

Tumor suppressive functions of WNT5A in rhabdomyosarcoma

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Abstract. Rhabdomyosarcoma (RMS) is a highly aggressive soft tissue malignancy that predominantly affects children. The main subtypes are alveolar RMS (ARMS) and embryonal RMS (ERMS) and the two show an impaired muscle differentiation phenotype. One pathway involved in muscle differentiation is WNT signaling. However, the role of this pathway in RMS is far from clear. Our recent data showed that the canonical WNT/ β -Catenin pathway serves a subordinate role in RMS, whereas non-canonical WNT signaling probably is more important for this tumor entity. The present study investigated the role of WNT5A, which is the major ligand of non-canonical WNT signaling, in ERMS and ARMS. Gene expression analysis showed that WNT5A was expressed in human RMS samples and that its expression is more pronounced in ERMS. When stably overexpressed in RMS cell lines, WNT5A decreased proliferation and migration of the cells as demonstrated by BrdU incorporation and Transwell migration or scratch assay, respectively. WNT5A also decreased the self-renewal capacity and the expression of stem cell markers and modulates the levels of muscle differentiation markers as shown by sphere assay and western blot analysis, respectively. Finally, overexpression of WNT5A can destabilize active β -Catenin of RMS cells. A WNT5A knockdown has opposite effects. Together, the results suggest that WNT5A has tumor suppressive functions in RMS, which accompanies downregulation of β -Catenin.

Introduction

Rhabdomyosarcoma (RMS) is the commonest soft tissue sarcoma in children and young adults (1). The two major subtypes are alveolar RMS (ARMS) and embryonal RMS (ERMS) that have emerged based on specific light microscopic

features. Whereas ARMS cells are distributed around an open central space thus resembling the alveoli of a lung, ERMS cells resemble immature skeletal myoblasts (2). Indeed, ARMS is molecularly different from ERMS and is characterized by genetic translocations of PAX3 or PAX7 and FOXO1. These fusion-positive ARMS are very aggressive. By contrast, fusion-negative ARMS clinically and molecularly resemble the less aggressive ERMS subtype (3). Although these subtypes are distinguishable by genetics and prognosis they share an impaired muscle differentiation phenotype and are probably derived from a muscle or a mesenchymal progenitor cell (2,4).

The development of skeletal muscle from immature precursors is partially driven by WNT/ β -Catenin (canonical) and β -Catenin independent (non-canonical) WNT signaling pathways. Indeed, a number of WNT ligands and receptors take part not only in embryonic myogenesis and skeletal muscle formation, but also in muscle differentiation and expression of myogenic regulatory factors [for a review see (5)].

WNT signaling is regulated by 19 secreted WNT proteins that bind to more than 15 receptors or co-receptors. When synthesized, WNT ligands are modified at the endoplasmic reticulum and then transferred to the plasma membrane, where they are secreted. How these secreted WNT ligands reach neighboring cells is still a matter of debate. One model proposes a transport of the WNT ligands by extracellular vesicles to the membrane receptor of other cells, whereas the other model favors the direct contact between cell-membrane-tethered WNT ligands and the receptor cells (6,7).

Of the WNT signaling pathways the canonical WNT/ β -Catenin pathway is the best characterized. In the inactive stage β -Catenin is phosphorylated and ubiquitinated by a destruction complex, which targets it for degradation by the proteasome. Activation of the WNT/ β -catenin pathway is induced by binding of extracellular WNT ligands to Frizzled (FZD) and their LRP5/6 co-receptors. This causes inhibition of the β -Catenin destruction complex. Consequently, β -Catenin is stabilized, accumulates in the cytoplasm and translocates to the nucleus, where it triggers the expression of WNT target genes such as *AXIN2* and *cMYC* (6,8).

The non-canonical β -Catenin-independent WNT/ Ca^{2+} or WNT/planar cell polarity (PCP) pathways are activated mainly by WNT5A. Binding of WNT5A to FZD 2, 3, 4, 5 or 6 and their co-receptors ROR1 and ROR2 results in WNT/ Ca^{2+} signaling (9). For induction of PCP signaling, WNT5A must interact with ROR2 (10). Depending on the receptor context,

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WNT5A also can either activate or inhibit canonical WNT/ β -Catenin signaling. Indeed, inhibition of β -Catenin accumulation by WNT5A is observed in several cell lines (11).

Although WNT signaling is involved in muscle development, its role in RMS remains to be elucidated. With respect to canonical WNT/ β -Catenin signaling, only a few studies have been published. This may be due to the fact, that RMS does not show mutations in relevant components of the pathway and only rarely shows nuclear β -Catenin (12). However, it has been reported that stimulation of RMS cell lines with recombinant WNT3A leads to nuclear translocation of β -Catenin and blocks cellular proliferation (13,14). In addition, downregulation of the main inhibitor of WNT/ β -Catenin signaling Dickkopf1 leads to expression of active β -Catenin and inhibition of proliferation and invasion of RMS tumor cells (15). These results imply that activation of WNT/ β -Catenin signaling has antitumoral effects in RMS. By contrast, data from our group show that activation of WNT/ β -Catenin or a β -Catenin knockdown barely affects proliferation, apoptosis or myodifferentiation of RMS tumor cells. In addition, RMS incidence, multiplicity or latency time are not altered by a hypomorphic *Wnt3a* allele or a conditional knockout of β -Catenin in genetically engineered mice (16,17). Furthermore, genetic data show decreased RMS multiplicity on a *Wif1*-deficient background (16). The latter experiment suggests that WNTs normally bound by WIF1 inhibit RMS formation or growth, either by blocking RMS initiation or by preventing the progression of already initiated tumors. WIF1 has high affinity to WNT3A, WNT4, WNT5A, WNT7A, WNT9A and WNT11 (18), of which WNT5A, WNT7A and WNT11 activate non-canonical WNT signaling pathways (19). Together with the fact that canonical WNT signaling only serves a subordinate role in RMS aggressiveness recent data suggest that non-canonical WNTs may have antitumoral functions (16).

The present study investigated the basic functions of WNT5A, the major ligand of non-canonical WNT signaling, in RMS. Until now, alterations of WNT5A expression levels were reported as a secondary phenomenon in RMS. Thus, in ERMS cells from p53/c-fos double mutant mice WNT5A is downregulated in comparison with normal myoblasts (13), whereas another study shows higher WNT5A expression in ERMS compared with ARMS cell lines (20), which is somewhat contradictory. Moreover, *WNT5A* sequence variants have been found in ARMS, but their effect on WNT5A function is unclear (21).

To elucidate the role of WNT5A in RMS in more detail, the present study investigated *WNT5A* expression in human *PAX3/FOXO1* positive ARMS and in ERMS. It also stably overexpressed or knocked-down WNT5A in ERMS and fusion-positive ARMS cell lines and investigated the growth behavior, migration and differentiation of the cells. The present study also analyzed the effect of WNT5A on β -Catenin stability.

Materials and methods

Biopsy specimens. For reverse transcription-quantitative (RT-q) PCR analyses 20 RNA (10 ARMS and 10 ERMS) samples from the Cooperative Weichteilsarkom Studiengruppe (CWS) tissue bank (Stuttgart, Germany) were analyzed (for

patient characteristics see Table SI). Muscle tissue from five (anonymous) separate patients served as controls. For analysis of WNT5A protein expression a tumor microarray (TMA) with 125 RMS biopsies from the Pediatric Tumor Register, Kiel, Germany was used. The histopathological features of all cases were centrally reviewed by the late Professor I. Leuschner (Member of the CWS and Director of the Pediatric Tumor Registry, Kiel, Germany). All patients were treated according to CWS protocols (CWS-96 or CWS-2002P). TMA studies and the use of normal muscle were authorized by the approval 158/2009/b02; University of Tübingen, Tübingen, Germany; April 2, 2009 within the framework of the CWS and that for the RT-qPCR studies additionally by the approval 2017-802R-MA (University of Heidelberg, University Medical Centre Mannheim). Written informed consent, according to the Declaration of Helsinki, was obtained from all patients or their legal guardians, depending on the age of the patients.

In addition, two publicly available RMS datasets with 103 (37 ARMS and 66 ERMS; dataset 1) (22) and 70 (34 ARMS and 36 ERMS; dataset 2) patients with RMS (3) were analyzed. Note that samples with unclear diagnosis were omitted and that only *PAX3-FOXO1* translocation positive ARMS and translocation negative ERMS were considered for analysis. As described previously, a Custom CDF Version 20 with ENTREZ-based gene definitions was used to annotate the arrays (23). The raw fluorescence intensity values were normalized applying quantile normalization and RMS background correction. All probes belonging to one gene (probeset) were summarized to one intensity value. It was not necessary to exclude or average the probesets for a pathway or gene ontology analysis. Gene analysis was performed by SAS JMP10 Genomics, version 6, from SAS (SAS Institute).

Cell lines and antibodies. The translocation positive ARMS cell lines CRL2061 (identical to Cellosaurus cell line SJCRH30) and RH30 and the ERMS cell lines RD and TE671 were obtained from the American Type Culture Collection (CRL2061 and RD) or the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (RH30 and TE671). The cell lines CRL2061 and RH30 were verified for the *PAX3-FOXO1* fusion gene and all cell lines routinely for mycoplasma contamination. ARMS cell lines were cultured in RPMI1640 and ERMS cell lines in DMEM, both supplemented with 10% (v/v) FCS (MilliporeSigma). Culturing conditions were 37°C and 5% CO₂. For the experiments, cells were used during the exponential growth phase. In addition, the cell lines RH30, RD and TE671 were authenticated by STR profiling (Eurofins Genomics Germany GmbH).

For protein detection, anti-human antibodies diluted in TBS were applied. Source and dilutions of the antibodies are listed in Table SII. HRP-conjugated anti-mouse or anti-rabbit IgGs (Cell Signaling Technology, Inc.) were used as secondary antibodies for western blotting as detailed below.

Plasmids and cloning. The pcDNA3-WNT5a plasmid for transient expression was from the Addgene Vector Database [Addgene plasmid cat. no. 35911 (24)]. For stable overexpression, WNT5A from the pcDNA3-WNT5a plasmid was cloned in the retroviral pBABE vector [Addgene plasmid cat. no. 1764 (25)]. WNT5A short hairpin (sh)RNA (mature

WNT5A targeting sequence 5'-GGACGTTAAGAGATA TTCAAA-3') cloned into the retroviral pGIPZ vector was purchased from Horizon Discovery. Restriction enzymes (*Bam*HI, *Eco*RI) were purchased from New England BioLabs, Inc. Purification of cloning products was performed with the QIAquick Gel Extraction kit (Qiagen GmbH). Gene products were ligated with T4 ligase from New England BioLabs, Inc. All plasmids were isolated with the EndoFree Plasmid Maxi kit (Qiagen GmbH) according to the manufacturer's instructions.

Stable WNT5A overexpression (WNT5AOE) or knockdown (WNT5AKD). The pBABE-WNT5A plasmid and the packaging plasmids pUMVC and VSV-G [Addgene plasmids cat. no. 8449 and cat. no. 8454 (26)] were transfected into 293 cells (packaging cell line) using the calcium phosphate transfection kit (MilliporeSigma) according to the manufacturer's instructions. This procedure employs a sodium phosphate-containing HEPES-buffered solution that is mixed with a calcium chloride solution containing the DNA. This generates DNA-calcium phosphate precipitates that are attached on the cell membrane and are internalized into the cell. After sterile filtration, the supernatant was used to transduce the RMS cell lines.

For transduction of the WNT5A shRNA expression vector WNT5A-GIPZ, the vector was mixed with Trans-Lentiviral packaging plasmid mix from Horizon Technologies according to the manufacturer's protocol (Horizon Technologies) and transduced as described above.

In all settings and if not stated otherwise, RMS cells transduced with either the empty pBABE or the empty pGIPZ vectors served as respective controls [CTR; please note that it was decided to use an empty vector for the WNT5A shRNA validation experiments, because an empty vector was also used for the WNT5A overexpression experiments. In addition, empty vectors are frequently used in knockdown experiments (27,28)]. pBABE and GIPZ plasmids both contain a puromycin resistance cassette. Therefore, successfully transduced RMS cells were selected with puromycin (MilliporeSigma) at a concentration of 10 µg/ml. WNT5AOE or WNT5AKD was verified by RT-qPCR and western blotting.

Transient WNT5A expression. Transient transfection of the pcDNA3-WNT5a plasmid or the empty pcDNA3 vector was performed with the calcium phosphate transfection kit (MilliporeSigma) according to the manufacturer's instructions.

Cell viability and proliferation assays. Cell viability were monitored by WST-1 and MTT assay. For WST-1 or MTT assays, WNT5AKD, WNT5AOE and respective control cells (CTR; transduced with the respective empty vectors) were seeded in flat bottom 96-well plates at a density of 5×10^3 cells/well. After 48 h cell viability was verified by adding 100 µl of WST-1 solution (Roche Diagnostics; WST-1 reagent was diluted 1:25 in cell culture medium) or by adding 20 µl MTT/well (5 mg/ml in sterile PBS; Carl Roth GmbH). After an incubation for 3 h at 37°C, reduction of WST-1 by viable cells was determined using a SynergyMx microtiter plate reader and the Gen5 software (BioTek Instruments GmbH) at 450 nm and a reference wavelength of 655 nm. For the MTT assay, the supernatant was discarded and cells were lysed by adding 200 µl DMSO.

Reduction of MTT by viable cells was determined at 560 nm and a reference wavelength of 620 nm.

Proliferation was measured by BrdU assay using the Cell Proliferation ELISA BrdU kit from Roche Diagnostics. In this assay, RMS cells were seeded in black 96-well plates with a clear base at a density of 5×10^3 cells/well. Cells growing in the exponential phase were treated with 10 µl/ml BrdU for 24 h. BrdU incorporation was measured according to the manufacturer's instruction using a SynergyMx microtiter plate reader and Gen5 1.11 software (BioTek Instruments, Inc.). In both assays, and after a settling time of 3 h, the cells were also incubated for 24 h with 200 ng/ml rWNT5A (R & D Systems) at 37°C in a 5% CO₂ incubator.

Migration assays. The cell migration assay was performed in 24-well plates (BD Falcon; Becton, Dickinson and Company) using 0.8 µm pore size inserts (BD Falcon; Becton, Dickinson and Company). After 24 h of starvation, RMS cells were seeded into the upper part of the insert at a density of 1×10^5 in a total volume of 300 µl. The lower part of the wells was filled with 500 µl medium supplemented with 10% FCS (RPMI1604 medium for RH30 and DMEM medium for RD and TE671 cells). The cells were allowed to migrate for 8 h (RH30) or 24 h (RD and TE671) at 37°C in a 5% CO₂ incubator. Thereafter the media inside the insert was aspirated and cells that had not migrated were scraped off the upper side of the inserts using a cotton swab. The cells that had migrated to the lower side of the inserts were washed once with PBS and stained with 5 µM CellTracker Green CMFDA Dye (Thermo Fisher Scientific, Inc.) for 20 min at 37°C in a 5% CO₂ incubator followed by a washing step with PBS. Pictures of the migrated cells were taken by fluorescence microscopy at 100-fold magnification (Olympus BX 60 with cellSens Dimension software 1.6; Olympus Corporation). Migrated cells were quantified by counting the number of cells in at least four individual fields per insert. Data shown represent the mean of migrated cells of at least three independent experiments + SD.

Cell migration was additionally verified by scratch assays. For this purpose, RMS cells were seeded in 12-well plates at a density of 2×10^5 cells/well. After a settling time of 3 to 4 h, normal growth medium was replaced by starvation medium supplemented with 1% FCS (without FCS the cells would have died) and cells were cultured for another 24 h. After aspiration of the medium, a cross-shaped scratch was made with a 200 µl pipette tip. To remove any detached cells, each well was washed twice with PBS and fresh starvation medium was added. Images were captured with an inverse microscope (Leica Microsystems GmbH) after 0, 6, 12 and 24 h and analyzed with ImageJ (version 1.48t, National Institutes of Health) and each scratch was measured at three different fixed positions.

Sphere assay. Sphere assay was performed as previously described (29). In brief, RMS cells were seeded at a density of 1 cell/µl in ultra-low attachment plates (MilliporeSigma) and cultured in neurobasal medium (Thermo Fisher Scientific, Inc.) freshly supplemented with 2X B27 (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.), 10 ng/ml EGF (cat. no. 236-EG; R&D Systems) and 20 ng/ml b-FGF (cat. no. 233-FB; R&D Systems). Spheres were counted under the microscope and pictures were taken

using an inverse microscope (Leica Microsystems GmbH) with phase contrast at x10 magnification.

RT-qPCR. Total RNA from 90% confluent cells was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was generated from 0.5 µg total RNA using the PrimeScript RT Reagent kit (Takara Bio, Inc.). The relative mRNA levels were detected by the Step one plus system (Thermo Fisher Scientific, Inc.) using TB Green Premix Ex Taq II (Takara Bio, Inc.). These steps were performed according to the respective manufacturer's instructions. All primers were designed with PRIMER3 and are listed in Table SIII. The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The quantification was performed by the $2^{-\Delta\Delta C_q}$ method (30). *GAPDH* was used as a reference gene for relative quantification (please note that the reference genes *B2M*, *RPL13A* and *TBP* for RMS were also tested and it was found that all housekeeping genes yielded the same results; data not shown). Respective primers are given in Table SIII).

Western blotting. RIPA buffer (Thermo Fisher Scientific, Inc.) complemented with proteinase inhibitor complete Tablets (EDTA-free; Roche Diagnostics GmbH) was used for cell lysis and protein isolation. For western blotting, 2×10^5 cells were seeded in each well of a 6-well plate. After 24 h cells were harvested in 200 µl RIPA buffer. 20 µl of this lysate were mixed with 5 µl loading buffer and loaded on the gel. Nuclear and cytoplasmic proteins were separated by the nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Isolation of supernatant proteins was performed from 1 ml medium with trichloroacetic acid 20% (Merck KGaA) as previously described (31). Equivalent protein amounts (determined by quantification of the respective loading controls on the blots) were mixed with protein loading dye (Bio-Rad Laboratories, Inc.), separated on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature with 5% milk powder (w/v) in TBST [TBS supplemented with 0.05% Tween20 (v/v)] for 1 h, washed three times for 10 min with TBS-T and incubated overnight at 4°C with the primary antibody diluted in PBS supplemented with 0.02% (w/v) sodium azide, 2% (w/v) BSA and 0.001% Tween20 (v/v). After washing three times with TBS-T, membranes were incubated for 1 h at room temperature with the respective secondary antibody diluted in 5% milk powder in TBST. After washing three times with TBST, protein bands were visualized with ECL Plus Substrate (Thermo Fisher Scientific, Inc.) and the Fusion SL Imaging system (Peqlab Biotechnologie GmbH). Data were quantified with ImageJ (version 1.48t, National Institutes of Health). Re-blot stripping solution (MilliporeSigma) was used to strip the membrane. For details of the antibodies used see Table SII.

Statistical analysis. All analyses of transduced cell lines summarize the results obtained from ≥ 3 independent experiments (each measured in duplicates for RT-qPCR, BrdU, MTT and scratch assays) and are represented as mean \pm SD. If not indicated otherwise, the data were analyzed by an unpaired Student's t-test (to determine if the difference between two

data sets is significant) or with one-way ANOVA followed by Tukey's multiple comparison (to determine if the differences between more than two data sets are significant) using GraphPad Prism 6.0 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

WNT5A is expressed in RMS. Initially, the present study assessed the WNT5A expression profile in two publicly available ERMS/ARMS datasets [dataset 1 and 2 published by Davicioni *et al* (22) and Williamson *et al* (3), respectively] and in 10 ARMS and 10 ERMS RMS samples (validation dataset) from the CWS study group (32). Note that only *PAX3-FOXO1* positive ARMS were used for the analysis. The results of the validation cohort were additionally compared with WNT5A expression in normal skeletal muscle.

Analysis of WNT5A in dataset 1 and 2 revealed a significantly higher expression level of WNT5A in ERMS compared with ARMS (Fig. 1A). This was similar in the validation dataset (Fig. 1B). In addition, the analysis of the validation set shows that the WNT5A expression level of ARMS resemble that of normal skeletal muscle (Fig. 1B). This suggests that WNT5A mRNA expression is rather associated with the ERMS subtype.

The present study also examined a TMA with 125 RMS for WNT5A expression by immunohistochemical analysis. After quality control, 41 ERMS and 7 fusion-positive ARMS samples were evaluable (all tumors, which showed mitoses but were Ki67 negative were excluded). The analyses revealed that all RMS samples were positive for WNT5A although to a variable intensity. Unfortunately, tumor- and stromal cells could not be clearly distinguished so that a scoring (which would have been multiplication of the percentage of WNT5A positive cells by staining intensity) was not possible. Nevertheless, it was found that 4, 23 and 14 ERMS express WNT5A at low, intermediate and high intensity, respectively and that 2 ARMS showed intermediate and 5 ARMS showed high WNT5A intensity (Fig. 1C shows a tumor with low and one with high WNT5A intensity).

The present study also analyzed the expression of some WNT5A-associated receptors (i.e., *FZD2*, *FZD4*, *FZD5*, *ROR1* and *ROR2*) in datasets 1 and 2. In both sets, ERMS show higher expression of *FZD2* and *FZD4* compared with ARMS. In addition, *ROR1* and probably also *ROR2* seem to be elevated in ERMS (Table SIV).

Similarly, the *PAX3/FOXO1* positive ARMS cell lines CRL2061 and RH30 and the ERMS cell lines RD and TE671 expressed WNT5A mRNA (Fig. 1D). In addition, the ARMS cell line RH30 and the ERMS cell lines RD and TE671 expressed and secreted WNT5A (Fig. 1E; top left shows WNT5A protein in the supernatant of the cells; bottom left shows cellular WNT5A protein expression and the respective loading control). Furthermore, the mRNA levels in general correlate with the respective WNT5A protein levels (compare Fig. 1D and E bottom right). Note that the WNT5A antibody detects a protein of the correct size (i.e., 45 kDa) in the supernatant of WNT5A producing fibroblasts, in media supplemented with 200 ng/ml rWNT5A (R&D Systems, Inc.) and in supernatants of RMS cells transiently transfected with WNT5A (Fig. S1A and B).

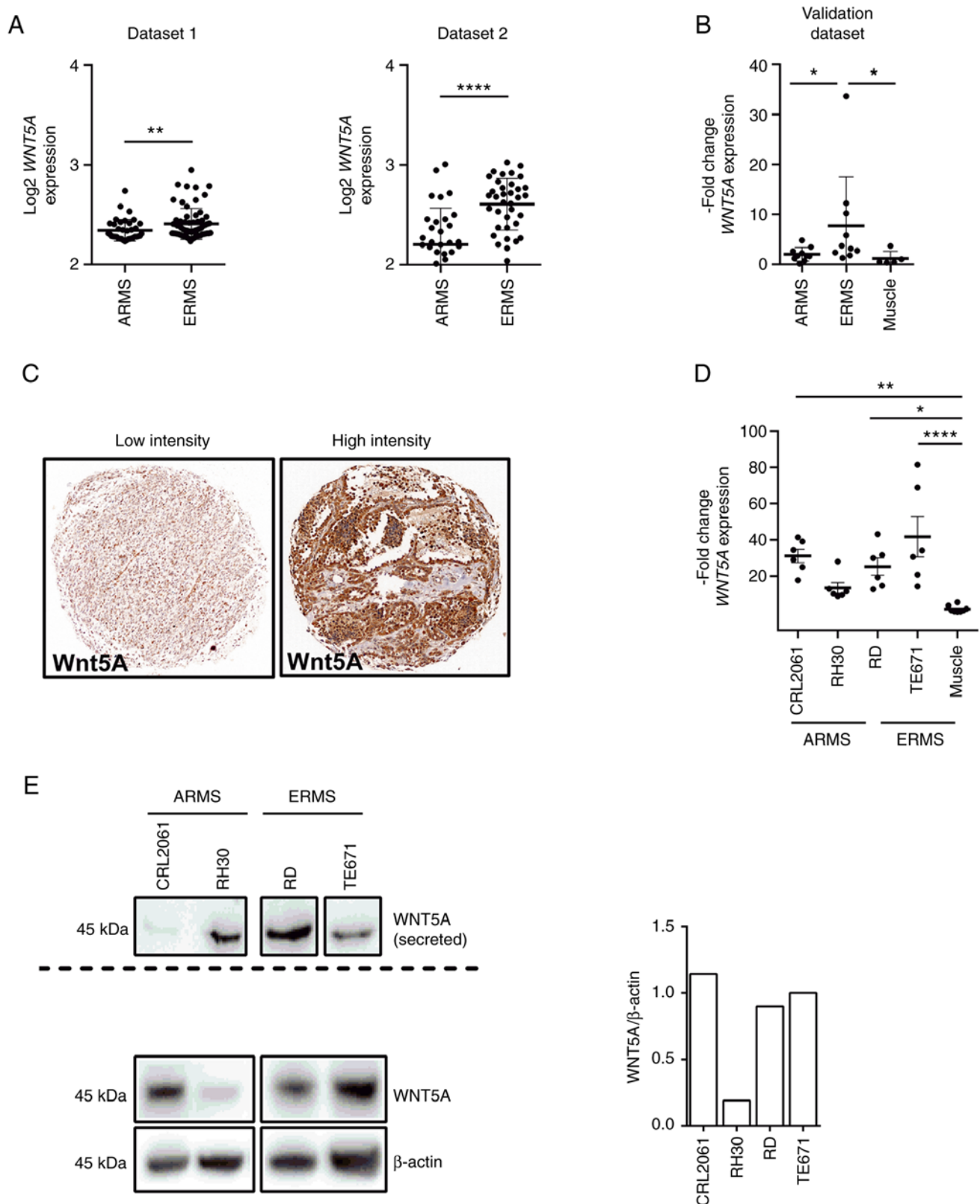


Figure 1. WNT5A expression in human RMS. WNT5A expression level (A) of ARMS and ERMS from two publicly available microarray datasets [see (22) and (3) for datasets 1 and 2, respectively] and (B) of 10 ARMS, 10 ERMS and five normal skeletal muscle samples in the validation data set from the CWS study group measured by reverse transcription-quantitative PCR. (C) Immunohistochemical analysis of human RMS showing low or high expression of WNT5A when using the WNT5A monoclonal antibody (3D10) from Thermo Fisher Scientific, Inc (magnification, 50x). (D) WNT5A mRNA expression (three independent experiments each measured in duplicates) of the human ARMS cell lines CRL2061 and RH30 and ERMS cell lines RD and TE671 in comparison with normal skeletal muscle, which was set=1. (E) Western blot analysis of WNT5A protein levels in the supernatant (top left; please note the quantification of secreted WNT5A using HSC70, which is occasionally secreted by tumor cells (58), was not possible because RMS cell lines apparently do not secrete HSC70) and on another gel in the respective whole cell lysates (bottom left) of RMS tumor cell lines. β -Actin detection served as loading control for whole cell lysates (bottom) and the right panel shows the respective quantification. Horizontal lines in (A-B) are mean \pm SD; bars in (D) are also \pm SD. Statistical analyses in (A-B) were performed by non-parametric Mann-Whitney tests and that in (D) by one-way ANOVA. * P <0.05, ** P <0.01, **** P <0.0001. RMS, rhabdomyosarcoma; ARMS, alveolar RMS; ERMS, embryonal RMS.

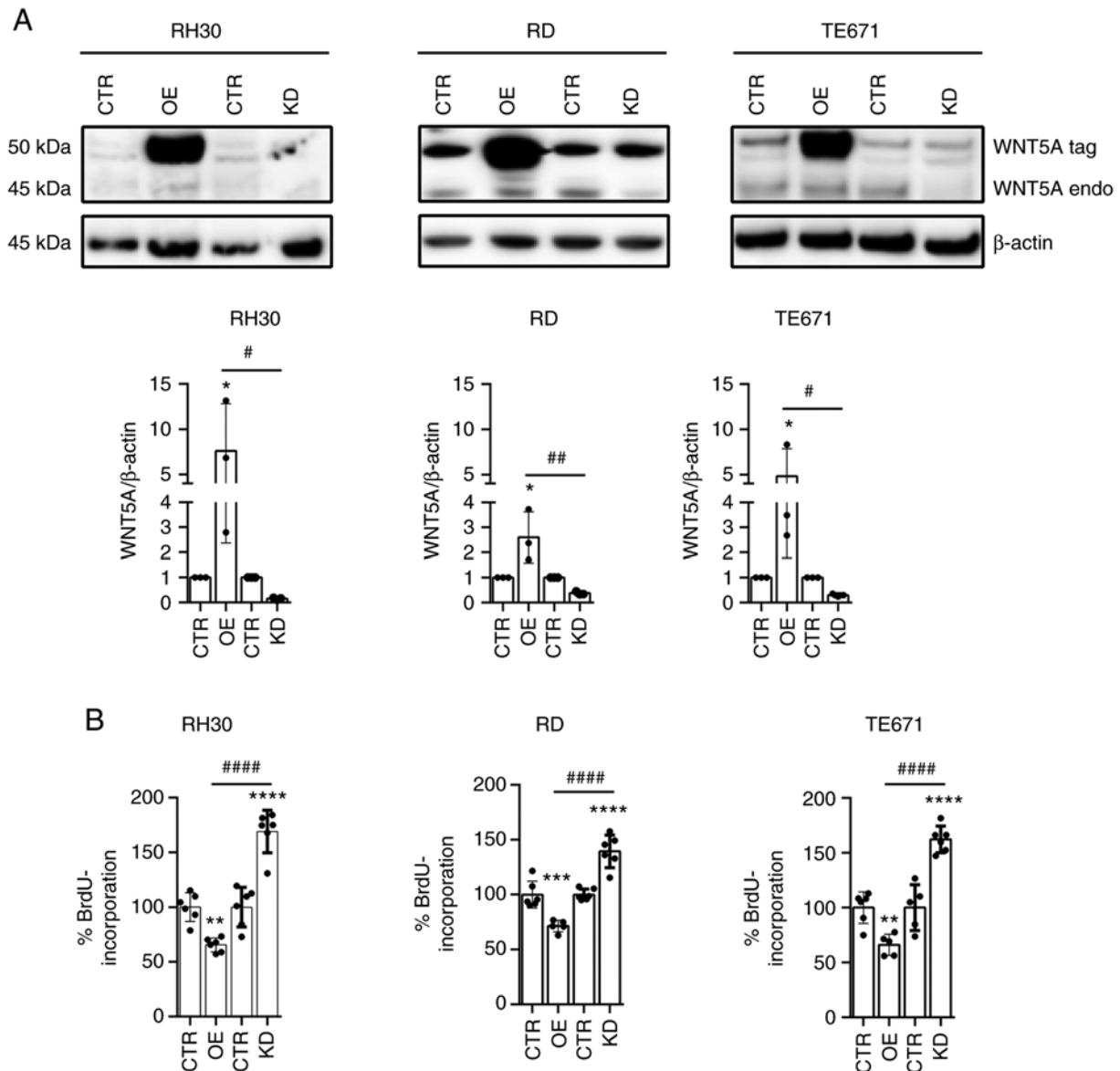


Figure 2. Protein expression levels of WNT5A and proliferation of WNT5AOE and WNT5AKD RMS cell lines. (A) Representative western blot analyses for WNT5A protein detection. Endogenous (WNT5A endo) and V5-tagged overexpressed (WNT5A tag) WNT5A proteins were detected using the mAb rabbit anti-Wnt5a cat. no. 2530 from Cell Signaling Technology, Inc. β -Actin served as the loading control. Note the overexpressed WNT5A tag protein is larger than the WNT5A endo protein due to the V5-tag. Furthermore, the WNT5A antibody detects an unspecific band of ~55 kDa that overlaps with the tagged WNT5A protein. Lower panel shows protein quantification from 3 independent experiments (values of WNT5A endo were used for quantification of controls and WNT5AKD and values of WNT5A tag for quantification of WNT5AOE). Values of CTR were set to 100%. Bars are mean \pm SD; statistical analysis was one-way ANOVA. *indicates significance vs. CTR; #indicates significance between OE and KD. (B) BrdU-incorporation assays (3 independent experiments each measured in duplicates) of the human ARMS cell line RH30 and ERMS cell lines RD and TE671 stably overexpressing WNT5A or stably expressing a WNT5A shRNA to induce a WNT5A knockdown. Cell lines transduced with the respective empty vectors (pBABE or pGIPZ) served as controls. * $^{\circ}$ P<0.05, ** $^{\circ}$ ##P<0.01, *** $^{\circ}$ P<0.001, **** $^{\circ}$ ####P<0.0001. CTR, control; OE, overexpressing; KD, knockdown.

However, it does not detect a protein in the supernatants of L-cells or WNT3A producing L-cells (Fig. S1A). Therefore, the anti-WNT5A antibody can be considered specific.

Finally, all cell lines produce β -Catenin and FZD5 protein and most of them FZD2 and ROR2, whereas ROR1 is rather expressed by ERMS cell lines (Fig. S1C). This indicated that ERMS cell lines express all WNT5A receptors analyzed (i.e., FZD2, FZD5, ROR1 and ROR2), whereas ARMS cell lines occasionally lack expression of these receptors.

WNT5A inhibits cellular proliferation and migration and may decrease expression of MYOD, DES and MYOG in RMS cells. To

investigate the effect of WNT5A on RMS growth behavior and differentiation, the present study stably overexpressed or knocked down WNT5A in the ARMS cell line RH30 and in the ERMS cell lines RD and TE671 (WNT5AOE or WNT5AKD, respectively; Fig. 2A). Note that WNT5A is V5-tagged (24) and therefore is larger than the endogenous WNT5A protein. Also note that the used monoclonal anti-Wnt5A antibody gave an unspecific band of around 55 kDa that overlapped with the tagged WNT5A protein. The present study was not able to stably overexpress WNT5A in the ARMS cell line CRL2061. Therefore, all subsequent experiments were performed with RH30, RD and TE671 cells, which all express and secrete WNT5A (Fig. 1D and E).

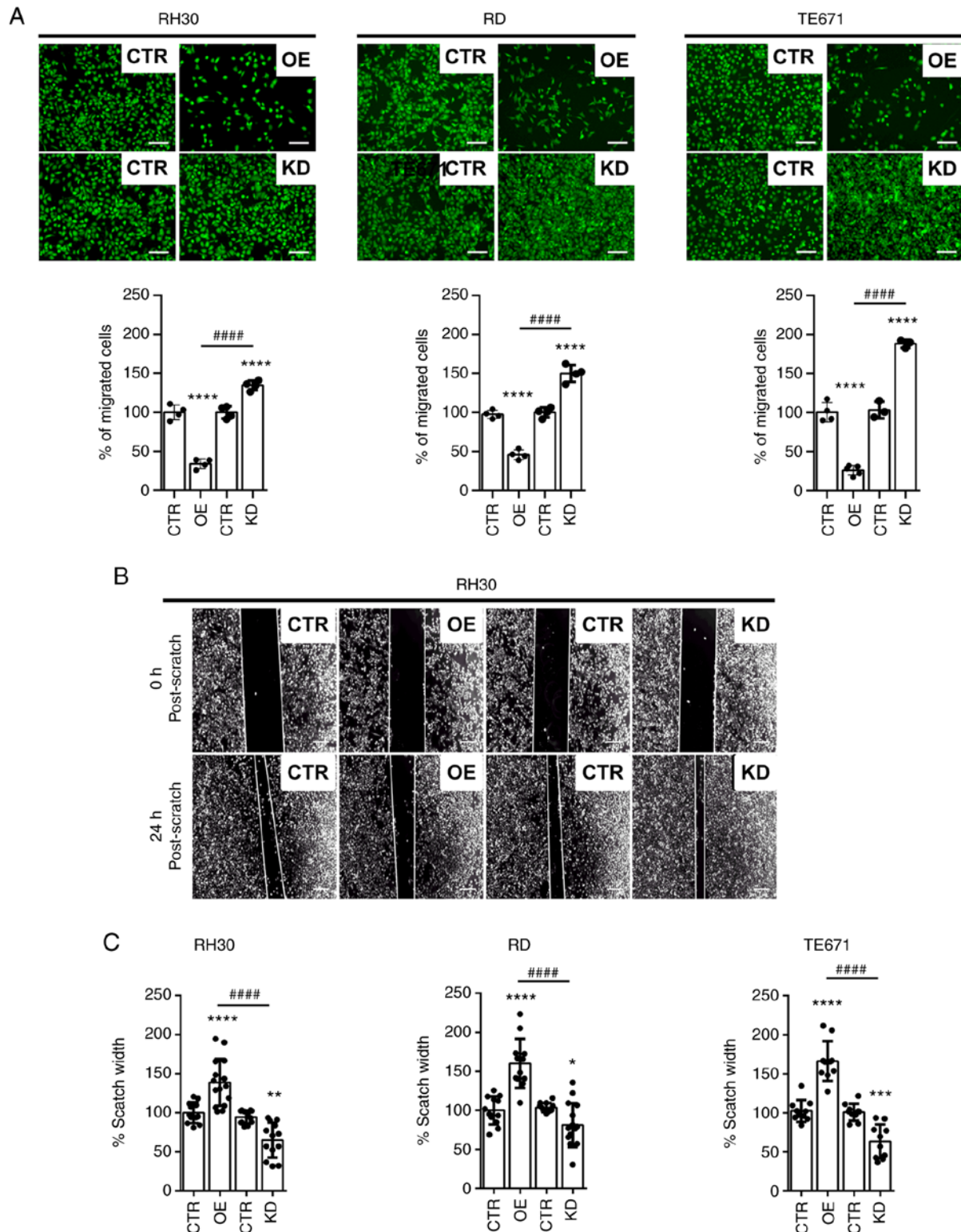


Figure 3. Migration of WNT5AOE and WNT5AKD RMS cell lines. (A) Representative photographs (top; scale bars=100 μ m) and percentage (from ≥ 3 independent experiments; bottom) of migrated cells measured by Transwell migration assays and (B) representative images (magnification, x40) of scratch assays using RH30 cells with the edges of the scratch wounds indicated as lines and (C) respective quantification of the scratch width 24 h post-scratching of all cell lines. Cells were starved for 24 h before making the scratch. Scratch width was measured at three different positions 24 h post-scratching in ≥ 3 independent experiments. Cell lines transduced with the respective empty vectors (pBABE or pGIPZ) served as controls. Values of CTR were set to 100%. Bars are mean \pm SD; statistical analysis was performed by one-way ANOVA. *indicates significance compared with CTR; #indicates significance between OE and KD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****/ $P < 0.0001$. RMS, rhabdomyosarcoma; CTR, control; OE, overexpressing; KD, knockdown.

At least two different cell batches, i.e., cells that have been transduced at different time points, were collected. Each batch was analyzed for *WNT5A* expression and for cell viability by

MTT assay. As shown in Fig. S2A and B, all batches showed an almost identical *WNT5A* mRNA level or proliferative behavior and thus the first batches were chosen for further experiments.

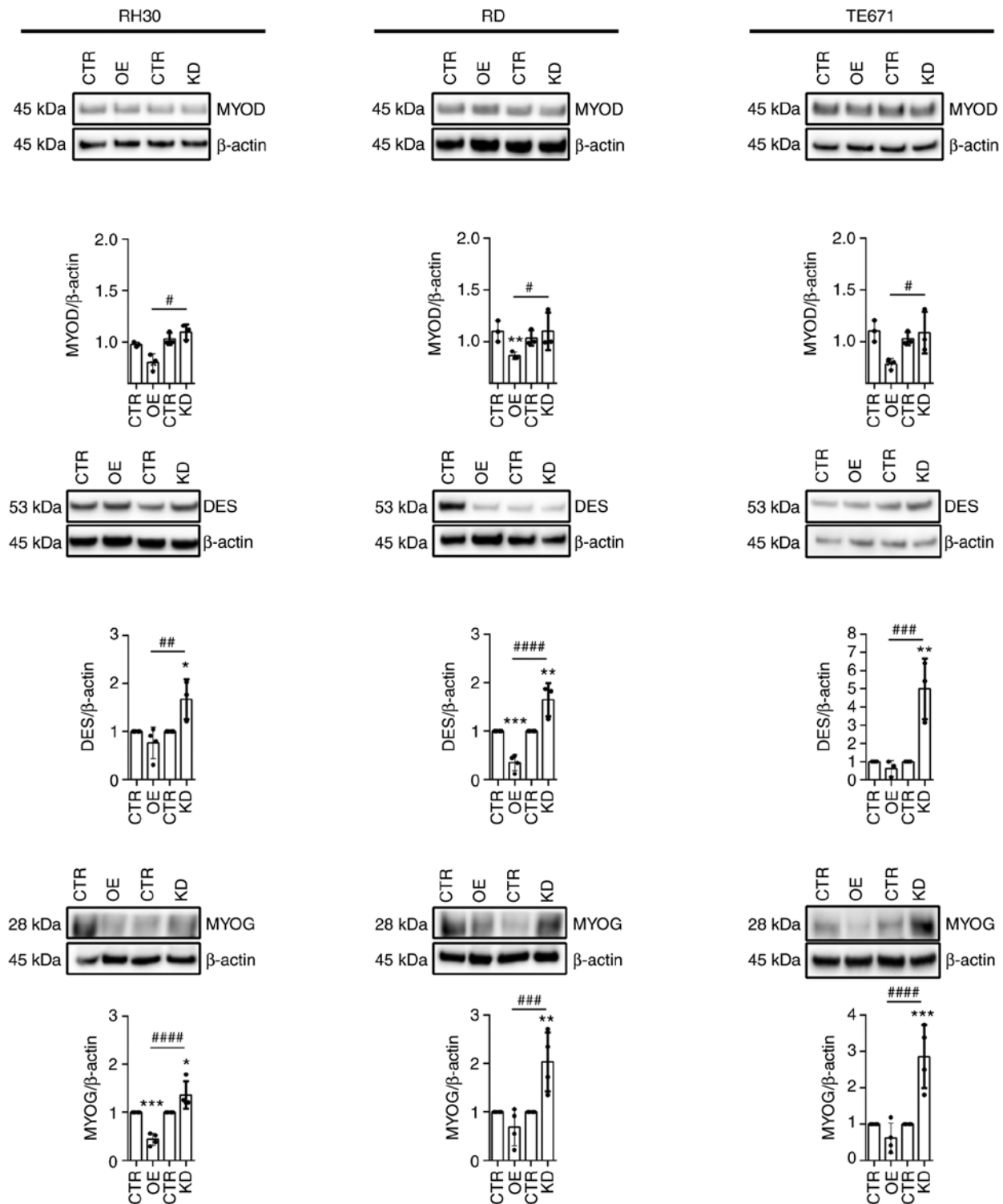


Figure 4. Expression of muscle markers in WNT5AOE and WNT5AKD RMS cell lines. Western blot analyses (top) along with the respective protein quantification (bottom) of the muscle markers MYOD, DES and MYOG of the human ARMS cell line RH30 and ERMS cell lines RD and TE671 stably overexpressing WNT5A or stably expressing a WNT5A shRNA to induce a WNT5A knockdown. Cell lines transduced with the respective empty vectors (pBABE or pGIPZ) served as controls. Detection of β -Actin served as loading control. For protein quantification from ≥ 3 independent experiments, values of CTR were set to 1. Bars are mean \pm SD; statistical analysis was performed by one-way ANOVA. * indicates significance vs. CTR; # indicates significance between OE and KD. */P<0.05, **/P<0.01, ***/P<0.001, ****/P<0.0001. RMS, rhabdomyosarcoma; ARMS, alveolar RMS; ERMS, embryonal RMS; CTR, control; OE, overexpressing; KD, knockdown.

Notably, when compared with cells transduced with the respective vector control, WNT5AOE significantly inhibited cellular proliferation of all RMS cell lines (Fig. 2B; for cell viability see Fig. S3A). By contrast, the WNT5AKD enhances

the respective parameters (Fig. 2B; for cell viability see Fig. S3A). Similarly, when respective parental cells were incubated with rWNT5A, proliferative capacity and viability of the cells were inhibited (Fig. S3B and C). However, this

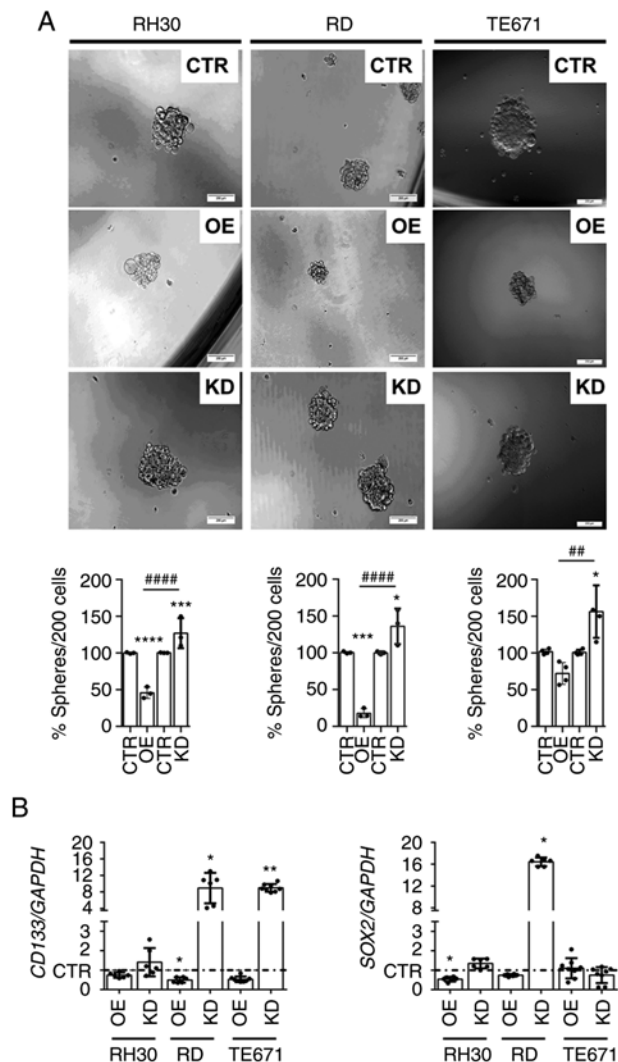


Figure 5. Sphere formation capacity and stem cell marker expression of WNT5AOE and WNT5AKD RMS cell lines. (A) Representative phase contrast pictures (top) and quantification (bottom) of sphere cultures and (B) Reverse transcription-quantitative PCR-based quantification of *CD133* and *SOX2* expression levels of 2D-cultured human ARMS cell line RH30 and ERMS cell lines RD and TE671 stably overexpressing WNT5A or stably expressing a WNT5A shRNA to induce a WNT5A knockdown in relation to cell lines transduced with the respective empty vectors (pBABE or pGIPZ) served as controls (CTR). (A) Pictures were taken after 1-week sphere culture of RH30, RD or TE671 cells, respectively (bottom) of 5 independent experiments. Scale bars=200 μ m. (B) Results represent data from ≥ 3 independent experiments each measured in duplicates. For sphere quantification, values of CTR were set to 100% in (A). CTR values of the gene expression analyses were set to 1 and are presented as a dashed line to ensure greater clarity (B). Bars are mean \pm SD; statistical analysis in (A) was performed by one-way ANOVA and that in (B) by Student's t-test. *indicates significance vs. CTR; #indicates significance between OE and KD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. OE, overexpressing; KD, knockdown. RMS, rhabdomyosarcoma; ARMS, alveolar RMS; ERMS, embryonal RMS; sh, short hairpin; CTR, control.

was only seen in RH30 and RD cells, but not in TE671 cells. This suggests that both membrane-tethered and extracellular WNT5A is responsible for these effects.

Next, the influence of WNT5A on migration of RMS cells was investigated by Transwell assay. The data showed that the migratory capacity of RH30, RD and TE671 cells with a WNT5AOE is significantly lower compared with the vector

controls, whereas that of WNT5AKD is significantly higher (Fig. 3A). This was confirmed by a scratch assay (Fig. 3B shows representative pictures of RH30 cells and Fig. 3C shows quantification of the scratch widths of all cell lines; note that the scratch width of WNT5AOE cells is higher and that of WNT5AKD cells is smaller). This demonstrated that WNT5A can inhibit the migratory capacity of RMS cell lines.

As WNT signaling serves an important role in myogenesis, the influence of WNT5A on the expression of myogenic markers was also analyzed. These were myoblast determination protein 1 (MYOD), myogenin (MYOG) and desmin (DES), which are correlated with a higher growth rate of RMS in zebrafish (33). Indeed, MYOD has an unappreciated and dominant oncogenic role to regulate human RMS growth (33). The results of the present study showed that WNT5AOE significantly decreased MYOD in all cell lines. WNT5AOE also decreased DES and MYOG protein, which was significant for RD and RH30 cells, respectively (Fig. 4). By contrast, the WNT5AKD significantly increased MYOG and DES protein levels in all analyzed cell lines (Fig. 4). Together, these data indicated that WNT5A may decrease the expression of MYOD, MYOG and DES in RMS cells, which goes along with concomitant inhibition of proliferation and migration. However, differences in muscle marker expression between the two control cell lines (CTR) was also detected, which were transduced with the backbones pBABE and pGIPZ for WNT5AO and WNT5AKD cells, respectively (Fig. S4). Therefore, these data need to be looked at with care, although other parameters such as WNT5A expression and cellular proliferation were identical to each other and in most cases also to the untransduced parental cell lines (see Fig. S4 for more details).

WNT5A inhibits sphere formation of RMS cells. It has been demonstrated that ERMS cell lines RD and TE671 and RH30 cells form rhabdospheres. Since rhabdospheres show a much higher tumorigenicity than adherent cells (29,34), the rhabdosphere colony formation assay is a powerful assay to analyze the self-renewal potential of RMS and to identify signaling pathways, which are essential for growth and maintenance of these stem cell-like and tumor-propagating RMS cells. After 7 days in culture, all cell lines had robustly formed rhabdospheres, independently of whether they were genetically manipulated or not. However, WNT5AOE spheres of all cell lines were smaller compared with control or WNT5AKD spheres. This phenomenon was already observed after 1 week in culture (Fig. 5A, shown by images in the upper panel). Notably, rhabdosphere numbers of WNT5AOE cells were also significantly lower compared with the control, whereas the numbers of WNT5AKD cells were significantly higher (Fig. 5A, lower panels). For RH30 cells, this characteristic was already observed after 1 week in culture (Fig. 5A, lower left panel) and for RD rhabdospheres after 2 weeks in culture (Fig. 5A, lower middle panel). To some extent this was also observed with TE671 cells after 8 weeks (Fig. 5A, lower right panel). Together, these data indicate that WNT5A inhibit self-renewal potential and maintenance of tumor-propagating RMS cells.

The present study also analyzed the expression of the stem cell markers *CD133* and *SOX2*, which both serve a role in RMS colony formation (29,35). WNT5AOE inhibited expression of *CD133* in all RMS cell lines, which was significant for

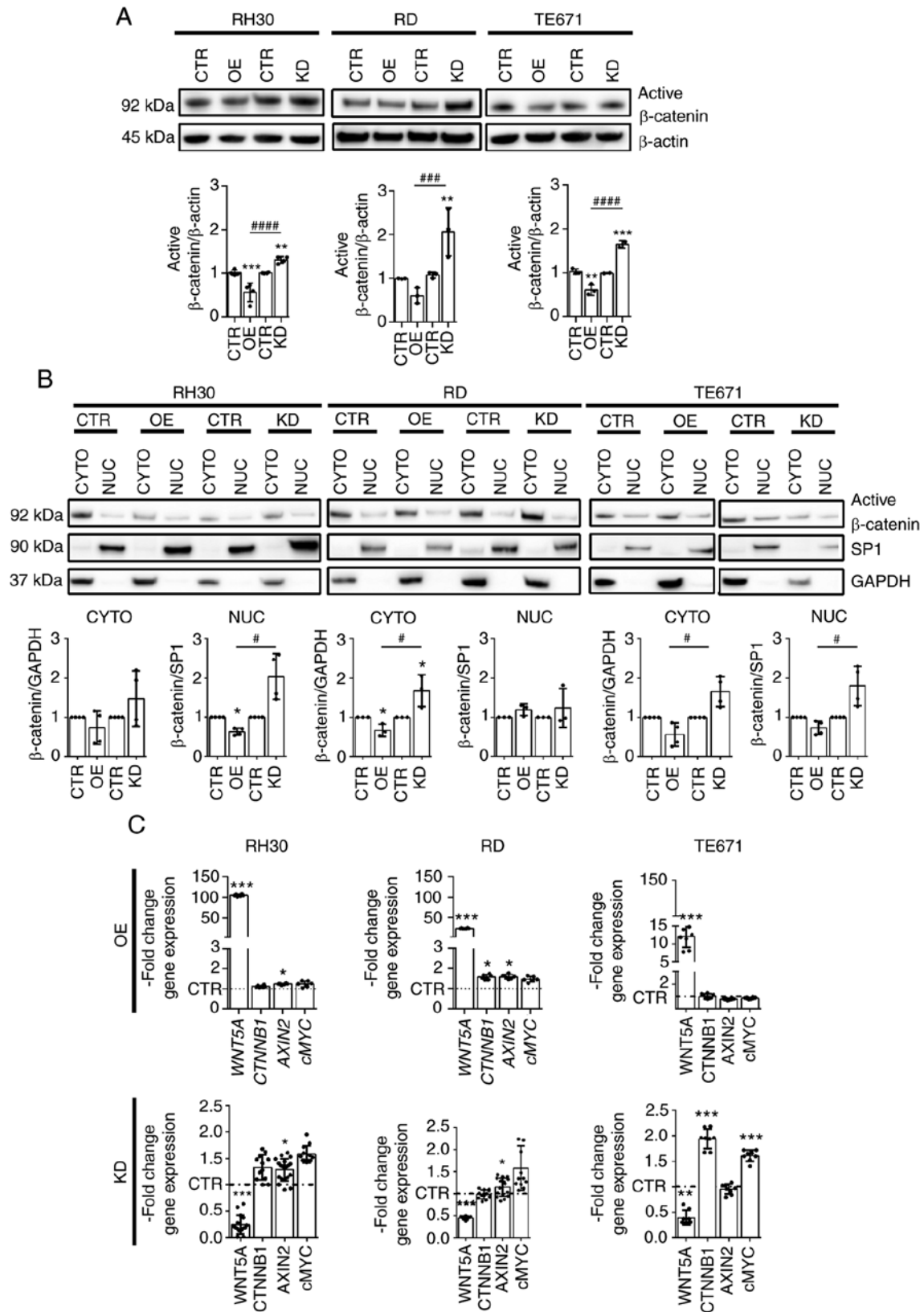


Figure 6. Stability of β-Catenin protein in WNT5AOE and WNT5AKD RMS cell lines. (A and B) Representative western blot analyses (top) along with the respective protein quantification (from ≥ 3 independent experiments; bottom) of active β-Catenin protein levels in (A) whole cell lysates and (B) cytoplasmic and nuclear fractions and (C) Reverse transcription-quantitative PCR analyses of *WNT5A*, *CTNNB1*, *AXIN2* and *cMYC* expression of the human ARMS cell line RH30 and ERMS cell lines RD and TE671 stably overexpressing WNT5A or stably expressing a WNT5A shRNA to induce a WNT5A knockdown in relation to cell lines transduced with the respective empty vectors (pBABE or pGIPZ), which served as controls. Detection of (A) β-Actin for whole lysates, (B) SP1 for nuclear and GAPDH for cytoplasmic fractions served as loading controls. (C) Gene expression levels were analyzed in ≥ 3 independent experiments each measured in duplicates and normalized to *GAPDH* expression levels. For protein and transcript quantification, values of CTR were set to 1 and in (C) the CTR values are presented as a dashed line to ensure greater clarity. Bars are mean \pm SD; statistical analysis in (A) and (B) was performed by one-way ANOVA and in (C) by Student's t-test. * indicates significance vs. CTR; # indicates significance between OE and KD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. OE, overexpressing; KD, knockdown; CYTO, cytoplasmic; NUC, nuclear; sh, short hairpin; CTR, control.

RD WNT5AOE cells. By contrast, *CD133* is overexpressed in WNT5AKD cells, which was significant for RD WNT5AKD and TE671 WNT5AKD cells (Fig. 5B, left panel). In addition, *SOX2* expression is significantly lower in RH30 WNT5AOE cells and is significantly higher in RD WNT5AKD cells (Fig. 5B). These data indicate that WNT5A can modulate the expression of stem cell markers in RMS cells.

WNT5A destabilizes or downregulates β -Catenin. As WNT5A can destabilize β -Catenin (11,36,37), the present study next analyzed the quantity and distribution of active non-phosphorylated β -Catenin protein in WNT5AOE and WNT5AKD cells. As shown in Fig. 6A the β -Catenin protein level was slightly decreased in WNT5AOE cells, whereas it was unequivocally increased in WNT5AKD cells (Fig. 6A). When the distribution of β -Catenin was investigated it appeared that both the cytoplasmic level of active β -Catenin and the nuclear level (at least in RH30 and TE671 cells) were decreased in WNT5AOE cells, whereas they were elevated in WNT5AKD cells compared with the control (Fig. 6B, upper and lower panel). This is different at the mRNA level. As shown in Fig. 6B, WNT5AOE did not block transcription of β -Catenin mRNA *CTNNB1* in any of the cell lines (Fig. 6B; indeed, in RD WNT5AOE cells *CTNNB1* is rather induced). By contrast, WNT5AKD did not induce *CTNNB1* in RH30 and RD cells. The exception was TE671 cells, in which the WNT5AKD resulted in upregulation of *CTNNB1* mRNA. This, by contrast to all other cell lines, accompanies significant induction of the β -Catenin downstream target *cMYC*. By contrast, the expression of *AXIN2* was not much altered by WNT5AOE or WNT5AKD (Fig. 6B) and if so, then *AXIN2* expression was upregulated.

In summary, these data demonstrate that WNT5AOE decreased the β -Catenin protein level in all RMS cell lines, whereas the WNT5AKD increased it. In almost all settings, this β -Catenin regulation is not correlated with alterations of the respective *CTNNB1* mRNA levels, indicating that WNT5A can destabilize β -Catenin protein. The exception is TE671 WNT5AKD cells, in which the increase in β -Catenin protein is associated with increased *CTNNB1* transcription. This shows that, depending on the cellular context, WNT5A can also modulate *CTNNB1* transcription.

Discussion

WNT signaling pathways control a number of developmental processes and contribute to a variety of human cancers. Numerous studies on the WNT/ β -Catenin pathway have been published and also encompass those in childhood tumors (17,38–40). However, knowledge about the role of WNT5A and non-canonical WNT signaling and the interaction with canonical WNT/ β -Catenin pathway in these tumors is sparse, particularly in RMS.

WNT5A can have either tumor suppressive or tumor promoting functions, which depends on the tumor entity. For example, WNT5A is a reliable marker for aggressive growth behavior and invasiveness of nasopharyngeal cancer (41), gastric cancer (42), oral squamous cell carcinoma (43) and melanoma (44,45), whereas it has tumor suppressive activity in thyroid carcinoma (46), prostate cancer (47), colorectal (48) and breast cancer (49). Indeed, it has been hypothesized that the function of

WNT5A as an oncogene or a tumor suppressor gene is dependent on the receptor context of the respective tumors (45,46,50).

The present study revealed that cell membrane-tethered and most likely also secreted WNT5A in RMS is rather tumor suppressive, since it inhibited proliferation, migration, the expression of stemness markers (e.g., *CD133*) and concomitantly sphere formation of RMS cells. These conclusions were based on *in vitro* experiments using RMS cell lines that overexpress WNT5A (WNT5AOE) or harbor a WNT5A knockdown (WNT5AKD). Although it is not currently known if cell membrane-tethered and secreted WNT5A acts through similar mechanisms, the WNT5A-mediated tumor suppressive effects are similar to those achieved in colon cancer (51) and hepatocellular carcinoma cells (52), in which WNT5AOE effectively inhibits cell proliferation. Furthermore, also in accordance with the present study, Cheng *et al* (51) observed reduced migration of colon cancer cells after WNT5AOE and increased migration after WNT5AKD. In addition, the authors showed an antagonizing effect of WNT5A on the canonical WNT/ β -Catenin pathway with decreased expression of cytoplasmic/nuclear β -Catenin after WNT5AOE (51). Therefore, at first glance, the WNT5A-induced inhibition of the WNT/ β -Catenin pathway seemed to be associated with a decrease in cell proliferation and migration in both colon cancer and RMS cells. However, this is rather not the case, since a recent study showed decreased proliferation with concomitant activation of WNT/ β -Catenin signaling in RMS cell lines deficient for DKK1 (15) and since we recently showed that canonical WNT signaling and β -Catenin do not affect aggressiveness of RMS cell lines or in a murine RMS model (17).

WNT5A may also inhibit the expression of muscle differentiation markers, such as MYOD, DES and MYOG. Although not all results were significant and muscle marker expression was apparently influenced by transduction of the empty vectors, the present study found that WNT5AOE in general downregulated these factors, whereas WNT5AKD increased their expression at least on protein level (Fig. 3B). These data supported a tumor suppressive role of WNT5A, because at least MYOD has an unexpected oncogenic function in RMS (33).

As already mentioned, WNT5A-mediated inhibition of the canonical WNT signaling pathway depends on the expression of specific receptors and is highly complex. Until now, we did not analyze the non-canonical signaling pathways that are activated in RMS cells by WNT5A. However, it has been shown that WNT5A-mediated β -Catenin degradation can involve the induction of the E3 ubiquitin-protein ligase SIAH2 (53). Furthermore, an interaction of WNT5A with FZD2 can activate WNT/ Ca^{2+} signaling and can concomitantly block binding of WNT3A by internalization of FZD2, which results in inhibition of β -Catenin accumulation and thus inhibition of canonical WNT Signaling (11). Moreover, binding of WNT5A to FZD4, which normally induces β -Catenin-dependent signaling, can be blocked in the presence of ROR2 (54) that is also involved in activation of WNT5A/JNK signaling (55). Notably, RH30, RD and TE671 cell lines express ROR2 and FZD2 (Fig. S1D). Because WNT5A-mediated activation of these receptors may involve destabilization of β -Catenin (11,36,37), it will be useful to investigate whether these receptors and their downstream signaling pathways are indeed involved in WNT5A-mediated inhibition of canonical WNT/ β -Catenin signaling in the RMS

cell lines RD and RH30. In TE671 cells, on the other hand, WNT5A also appeared to regulate β -Catenin on transcriptional level, because *CTNNB1* mRNA (and its target *cMYC*) was significantly upregulated in TE671 WNT5AKD cells.

In the present study, WNT5A also counteracted RMS stemness because it is negatively associated with RMS sphere formation and *CD133* expression. This is also seen in colon cancer cells, in which WNT5A is frequently silenced. In this tumor entity, reactivation of WNT5A leads to inhibition of tumor cell clonogenicity, which is due to antagonizing the WNT/ β -Catenin pathway (56,57). However, in RMS this mechanism is rather unlikely, because WNT3A does not alter RMS sphere formation (17).

Together, the present study revealed WNT5A expression in RMS *in vivo* and a tumor suppressive function of WNT5A in one ARMS and two ERMS cell lines *in vitro*, where WNT5A also inhibited migration and stem cell properties. This accompanied a decrease in MYOD, MYOG and DES expression and of active β -Catenin. How these processes are regulated and whether canonical and/or non-canonical WNT pathways are involved is currently unclear and more mechanistic data are needed for an improved understanding of the role of WNT5A in RMS. Thus, it remains to be analyzed if secreted WNT5A affects tumor proliferation via other mechanisms compared with cell membrane-tethered WNT5A. It also will be useful to investigate whether the WNT5A-induced changes in muscle marker and β -Catenin expression are directly involved in the anti-tumoral activities of WNT5A.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

NR, HH and KK conceived the present study. NR, JB and KK were responsible for the methodology and investigation. NR, JB and KK. HH and KK wrote the manuscript, which was reviewed and edited by AU and AM. HH and KK were responsible for project administration and funding acquisition. NR, KK and HH confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

TMA studies and the use of normal muscle were authorized by the approval 158/2009/b02; University of Tübingen, Tübingen,

Germany; April 2, 2009 within the framework of the CWS and that for the RT-qPCR studies additionally by the approval 2017-802R-MA (University of Heidelberg, University Medical Centre Mannheim). Written informed consent, according to the Declaration of Helsinki, was obtained from all patients or their legal guardians, depending on the age of the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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