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Characterization of Wnt/β-catenin signaling in rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and accounts for about 5% of all malignant paediatric tumours. β-Catenin, a multifunctional nuclear transcription factor in the canonical Wnt signaling pathway, is active in myogenesis and embryonal somite patterning. Dysregulation of Wnt signaling facilitates tumour invasion and metastasis. This study characterizes Wnt/β-catenin signaling and functional activity in paediatric embryonal and alveolar RMS. Immunohistochemical assessment of paraffin-embedded tissues from 44 RMS showed β-catenin expression in 26 cases with cytoplasmic/membranous expression in 9/14 cases of alveolar RMS, and 15/30 cases of embryonal RMS, whereas nuclear expression was only seen in 2 cases of embryonal RMS. The potential functional significance of β-catenin expression was tested in four RMS cell lines, two derived from embryonal (RD and RD18) RMS and two from alveolar (Rh4 and Rh30) RMS. Western blot analysis demonstrated the expression of Wnt-associated proteins including β-catenin, glycogen synthase kinase-3β, disheveled, axin-1, naked, LRP-6 and cadherins in all cell lines. Cell fractionation and immunofluorescence studies of the cell lines (after stimulation by human recombinant Wnt3a) showed reduced phosphorylation of β-catenin, stabilization of the active cytosolic form and nuclear translocation of β-catenin. Reporter gene assay demonstrated a T-cell factor/lymphoid-enhancing factor-mediated transactivation in these cells. In response to human recombinant Wnt3a, the alveolar RMS cells showed a significant decrease in proliferation rate and induction of myogenic differentiation (myogenin, MyoD1 and myf5). These data indicate that the central regulatory components of canonical Wnt/β-catenin signaling are expressed and that this pathway is functionally active in a significant subset of RMS tumours and might represent a novel therapeutic target.

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Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of adolescence and childhood, and accounts for 5% of malignant paediatric tumours.[1–3] Compared with embryonal RMS, alveolar RMS has a higher frequency in older children, is more aggressive, is more frequently associated with bone marrow metastases and has a significantly worse outcome.[2,4] Most alveolar RMSs have t(2;13) or t(1;13) translocations, involving the PAX3–FKHR and PAX7–FKHR fusion genes, respectively. The fusion gene product appears to deregulate the differentiation of muscle progenitor cells through mechanisms that are incompletely understood. The histological diagnosis for RMS is based on immunohistochemical expression of myogenin and MyoD1, both of which are known downstream products of Wnt signaling in muscle.[5]

Wnt signaling is an evolutionally conserved pathway that is operative in the skeletal development of invertebrates and vertebrates. The multifunctional nuclear transcription factor β-catenin is the major effector of the Wnt pathway and is involved in cell transformation.[6,7] Wnt signaling is also actively involved in myogenesis[5,8–11] and embryonic somite patterning.[12,13] The Wnt pathway is crucial in cell

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proliferation, cell migration, polarity and cell death.[14,15] Dysregulation of Wnt/β-catenin signaling facilitates cancer invasion and metastases in various tumours.[16,17]

Inactive Wnt signaling results in cytoplasmic β-catenin associating with a multimolecular complex containing casein kinase α, glycogen synthase kinase-3β (GSK-3β), axin and adenomatosis polyposis coli (APC) protein.[15,16] This complex promotes phosphorylation of β-catenin leading to its ubiquitination and subsequent degradation by the proteasome.[15,16,18] Wnt signaling is activated when Wnt ligands bind to Frizzled receptors and co-receptors on the cellular surface. Active Wnt signaling promotes the downstream signaling cascade and the activation of disheveled (Dsh) protein, which in turn inactivates GSK-3β.[14–18] This allows β-catenin to translocate into the nucleus where it binds to T-cell factor/lymphoid-enhancing factor (TCF/LEF), leading to activation of target genes including c-Myc,[19] cyclin D1,[20,21] c-Jun,[22] Slug[23,24] and Cox2.[25] In addition, TCF/LEF/β-catenin complex may cooperate with factors activated by other signaling pathways to modulate cellular remodeling. Indeed, many of these genes, such as cyclin D1 and c-Myc, have crucial roles in cell growth, proliferation and differentiation, and are deregulated in many types of cancer.[26–29]

Given the role of Wnt signaling in embryonal myogenesis and tumourigenesis, this study aimed to characterize the Wnt pathway in RMS using biopsy material and *in vitro* studies of the presence and activity of components of the pathway.

MATERIALS AND METHODS

Clinical Material

Fifty-four RMS samples were obtained from the histopathology archives of Alder Hey Children's NHS Foundation Trust ($n=40$, 1991–2009) and 'Policlinico Umberto I' Hospital, Sapienza University, Rome, Italy ($n=14$; 2005–2009). Approval from the ethical and research committees of Alder Hey Children's NHS Foundation Trust and 'Policlinico Umberto I' Hospital was obtained. All patients or their parents gave informed consent. The minimum follow-up period of the patients was 5 years. Forty-four cases out of 54 had sufficient tissue to be included. Patient data and tumour characteristics are presented in the supporting information (see Supplementary Information, Supplementary Table S1). The diagnosis of RMS was made by the characteristic morphological pattern (embryonal, alveolar, not otherwise specified) and immunohistochemical nuclear expression of myogenin and Myo-D1 (Figure 1). All alveolar RMS were assessed for Pax3/7–FKHR translocations using standard FISH analysis. Normal skeletal muscle ($n=8$), obtained at surgery for benign conditions, was used as the control.

Cell Culture

Human embryonal RMS (RD and RD18) and alveolar RMS (Rh4 and Rh30) cell lines were grown at 37 °C in 5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum, glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.

Tissue Microarray and Immunohistochemistry

Tissue microarrays (TMAs) containing a total of 136 samples from 34 RMS cases (4 samples per case containing viable tumour cells) and 16 samples from 8 controls was assembled in duplicate using eight 1 mm-deep cores. Ten cases were unsuitable for TMA because the blocks were very thin (<1 mm) ($n=10$); these cases were assessed on full-face sections. The primary antibody against β-catenin (1:200, monoclonal mouse anti-human β-catenin and clone β-catenin-1; DAKO, UK) was applied to 4 μm sections and biotinylated secondary antibodies used for the catalyzed signal amplification technique (Amersham, UK). β-Catenin expression was evaluated in a blind manner by two independent observers (TH and SA). Positive cases had at least four representative cores, each with at least 10% of neoplastic cells expressing β-catenin. The intensity of expression was scored as 0 = negative, 1 = weak, 2 = moderate, 3 = strong, and the pattern of expression defined as cytoplasmic/membranous or nuclear (Figure 1). Cases with tumour heterogeneity showing equal numbers of positive and negative cores were considered positive only if at least three cores showed 2+ expression. If the expression was weak (1+), the case was considered negative for β-catenin. Cases assessed on full-face sections ($n=10$) were regarded as positive only if at least 10% cells showed moderate or strong expression.

Protein Extraction and Western Blot Analysis

Protein extracts were obtained from cells using standard lysis buffer (for details, see Supplementary methods). For western blot analysis, equal amounts of proteins were resolved by SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) using standard procedures (for antibodies and other reagents, see Supplementary methods). All experiments were carried out at least three times.

Phosphorylation Status of β-Catenin in Response to Wnt3a Stimulation

Time course experiments

β-Catenin in its inactive state is phosphorylated by GSK-3β in cooperation with a degradation complex and is tagged for proteasomal degradation.[15,16] Inactivation of GSK-3β allows active β-catenin to accumulate within the cytoplasm and translocate to the nucleus to activate its downstream targets.[15–17] The activation status of the Wnt pathway was assessed in a time course experiment after treatment with Wnt3a. After overnight serum starvation, Rh30 and RD18 were stimulated with 200 ng/ml Wnt3a for 0′–10′–30′–60′–180′–300′–12 h–24 h, whereas untreated cells served as control. Protein extraction and western blotting was performed as described above. All experiments were carried out at least three times.

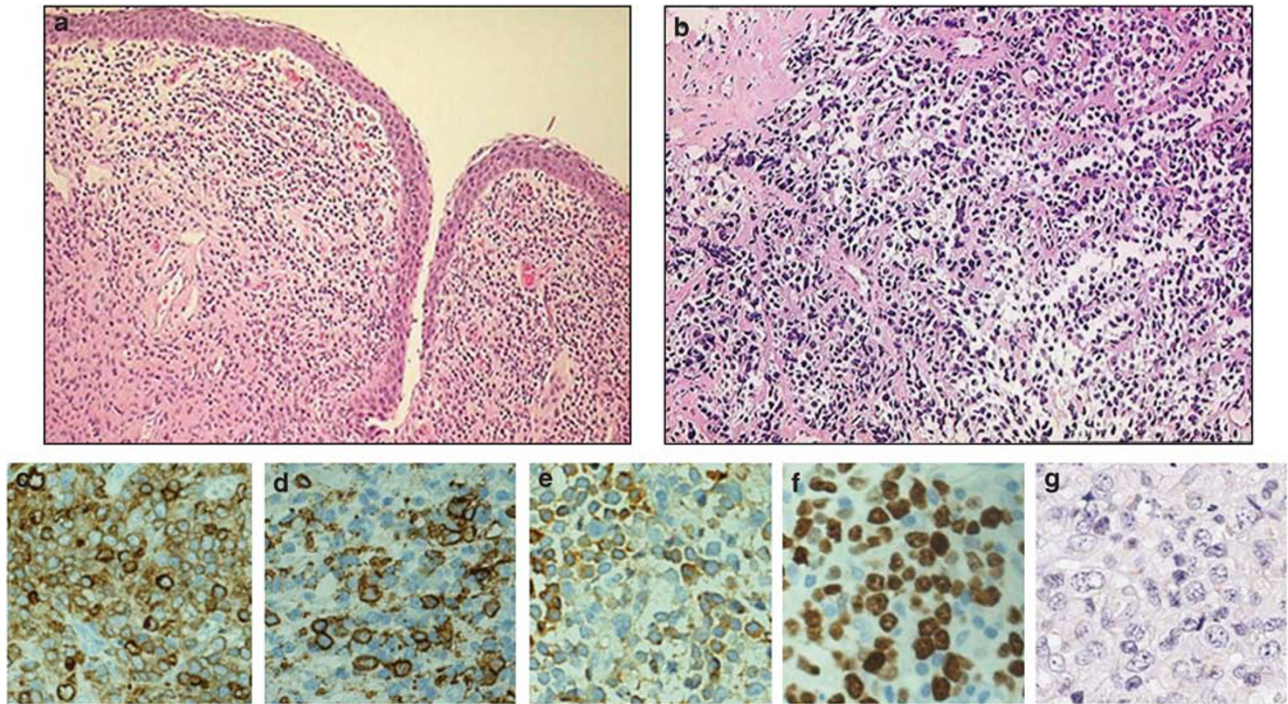

Figure 1 Typical morphological patterns of embryonal rhabdomyosarcoma (RMS)—botryoid type (**a**) and alveolar (**b**) rhabdomyosarcoma. (**c–g**) Membranous/cytoplasmic and nuclear immunohistochemical expression patterns and scoring for β-catenin.

Immunofluorescence

RD18 and Rh30 RMS cells were seeded on 12-mm diameter glass coverslips and used at a confluence of 70–90%. Untreated (serum-starved cells) and 200 ng/ml Wnt3a-treated cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Incubation with rabbit polyclonal anti-phospho-S33S37T41β-catenin, rabbit polyclonal anti-phospho-S552β-catenin, mouse monoclonal anti-β-catenin (Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal MyHC antibody (Millipore) or TRITC-conjugated phalloidin (Sigma-Aldrich) was carried out at room temperature for 1 h. After washing with PBS, cover slips were incubated with FITC or Texas red-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min in the dark. After final washing, nuclei were counterstained with DAPI (Sigma-Aldrich). The slides were then mounted in Moviol or Vecta Shield and examined by immunofluorescence microscopy. All experiments were carried out at least three times.

Cell Proliferation Assay

Cells were seeded in triplicate on 96-well plates and incubated with complete medium for 24 h and then starved with serum-free medium for 24 h. Quiescent cultures were treated with daily supplemented medium with or without 200 ng/ml Wnt3a for 24 and 48 h. The effect of Wnt3a on cell proliferation was determined by cleavage of tetrazolium salt WST-1 to formazan (Roche Applied Science, USA), by reading the absorbance of treated and untreated cells at 440 nm. The value for untreated cells was considered as 100% cell proliferation Each point represents a mean value and s.d. of three experiments with three replicates.

Transfection and Luciferase Reporter Gene Assay

For the detection of β-catenin-driven Wnt-transcriptional activity, Gaussia luciferase assay kit (New England Biosciences, UK) based on TOPflash/FOPflash reporter plasmid system was used (for details, see Supplementary Information, Supplementary methods 2). Gaussia luciferase has a secretory signal and upon expression is secreted into the cell medium. Medium from the treated and control cells were collected for Gaussia luciferase assay according to the manufacturer's instructions. Each point represents a mean value and s.d. of three experiments with three replicates.

Caspase-3/7 Apoptosis Assay

Apoptosis was measured by Apo-ONE homogeneous caspase-3/7 assay according to the manufacturer's protocol (Promega, UK; for details, see Supplementary methods).

Statistical Analysis

The experimental results are expressed as mean $\pm$ s.d. from at least three separate experiments performed in triplicate. Statistical analysis was done using Student's *t*-test and *P*-value <0.05 was considered as statistically significant.

RESULTS

β-Catenin Expression by Immunohistochemistry

In total, 44 RMS cases were assessed for β-catenin expression by immunohistochemistry—34 by TMA and 10 on full-face sections. As shown in Figure 1, RMS tumours exhibited a zonal expression pattern. In TMA, 17 of the 34 RMS samples expressed β-catenin in cytoplasm/membrane, whereas only 2 cases (both embryonal RMS) showed nuclear expression. Of the 10 cases studied using full-face sections (all embryonal RMS), 6 showed membranous/cytoplasmic β-catenin expression. In total, β-catenin expression was detected in 59% of all the RMS samples (26/44—64% (9/14) in alveolar RMSs and 56% (17/30) of embryonal RMSs (Figures 1c–g; Table 1). All alveolar RMS cases were evaluated for PAX–FKHR translocations by standard FISH analysis; β-catenin was expressed in five of the seven alveolar cases carrying PAX–FKHR translocations and in four of the seven alveolar cases that were translocation negative.

Wnt/β-Catenin Signaling Proteins are Expressed in RMS Cell Lines

In order to characterize Wnt/β-catenin pathway and identify its role in RMS, we conducted expression analysis using four cell lines derived from embryonal (RD and RD18) and alveolar (Rh4 and Rh30) RMS. Western blot experiments showed high levels of β-catenin and its inhibitor GSK-3β in all cell lines (Figure 2a). Also cadherin, a binding partner of β-catenin at adherens junctions, was present in the four cell lines although it was expressed in Rh30 alveolar cells at a lower concentration, which could only be detected after overexposure of the blot (Figure 2a). Other structural and functional Wnt pathway proteins, axin-1, naked-1, LPR6 and Dsh3, were also expressed in all RMS cell lines (Figure 2b). These results confirmed expression of central proteins of the Wnt pathway in RMS cells. The alveolar Rh30 and embryonal RD18 cell lines were therefore used for the rest of the studies.

Table 1 Immunohistochemical expression of β-catenin in alveolar and embryonal RMSs

	Alveolar RMS ($n=14$)	Embryonal RMS ($n=30$)
Total number of RMS cases (n = 44)		
TMA ($n=34$)	14	20
Full-face section cases ($n=10$)	0	10
Membranous/cytoplasmic expression		
TMA	9/14	9/20
Full section cases	0	6/10
Nuclear expression		
TMA	0	2/20
Full section cases	0	0
Total β-catenin expression (26/44)	9/14	17/30

Abbreviations: RMS, rhabdomyosarcoma; TMA, tissue microarray.

Phosphorylation Status of β-Catenin in Response to Wnt3a Stimulation

β-Catenin in its inactive state is phosphorylated on residues Ser 33, Ser 37 and Thr 41 by GSK-3β in cooperation with a degradation complex and is tagged for proteasomal degradation.[15–17] Inactivation of GSK-3β allows active β-catenin to accumulate within the cytoplasm and phosphorylate on residue Ser552 that induces its translocation into the nucleus and increases its transcriptional function activating its downstream targets.[30]

The activation status of the Wnt pathway was assessed in a time course experiment after treatment with Wnt3a. Rh30 control cells (0′) showed strong expression of phospho-S33-S37-T4-β-catenin that became faint between 10′ and 180′, with the lowest value at 60′ (Figure 2c). Unexpectedly, the inactive phosphorylated Ser9-GSK-3β levels decreased in response to Wnt3a treatment, whereas phospho-S552-β-catenin and total GSK-3β levels remained relatively constant (Figures 2c and d). Akt and phospho-Akt gradually decreased up to 60′, then returned back to basal level at 180’ and then decreased again. Similar results were obtained using RD18 cells but with different kinetics (Figure 2d). Phospho-S33–37-T4-β-catenin disappeared between 10′ and 60′, with the lowest level at 30′, together with a unexpected decrease in phospho-Ser9-GSK-3β. These results indicate that β-catenin shows dynamic changes in its phosphorylation status in response to Wnt3a stimulation in both alveolar and embryonal RMS cells.

Effect of Recombinant Wnt-3a on Subcellular Localization of β-Catenin

β-Catenin localized in different cellular compartments forms distinct complexes and executes differential cellular functions.[15–17] In order to assess whether Wnt-3a treatment affects β-catenin, in particular its intracellular redistribution, we performed immunofluorescence analysis. Wnt3a stimulation resulted in translocation of a fraction of β-catenin from cell–cell contact to mostly perinuclear/nuclear accumulation in Rh30 cells (Figure 3). These results were confirmed by immunoblot analysis with fractioned extracts (Figure 4a) and immunofluorescence using an antibody against β-catenin phosphorylated forms in RD18 and Rh30 cells (Figure 4b). Wnt3a stimulation concomitantly promoted nuclear accumulation of phospho-S552-β-catenin and a reduction of cytosolic phospho-S33S37T41-β-catenin in both alveolar and embryonal cell lines, whereas the amount of control nuclear protein lamin B remained

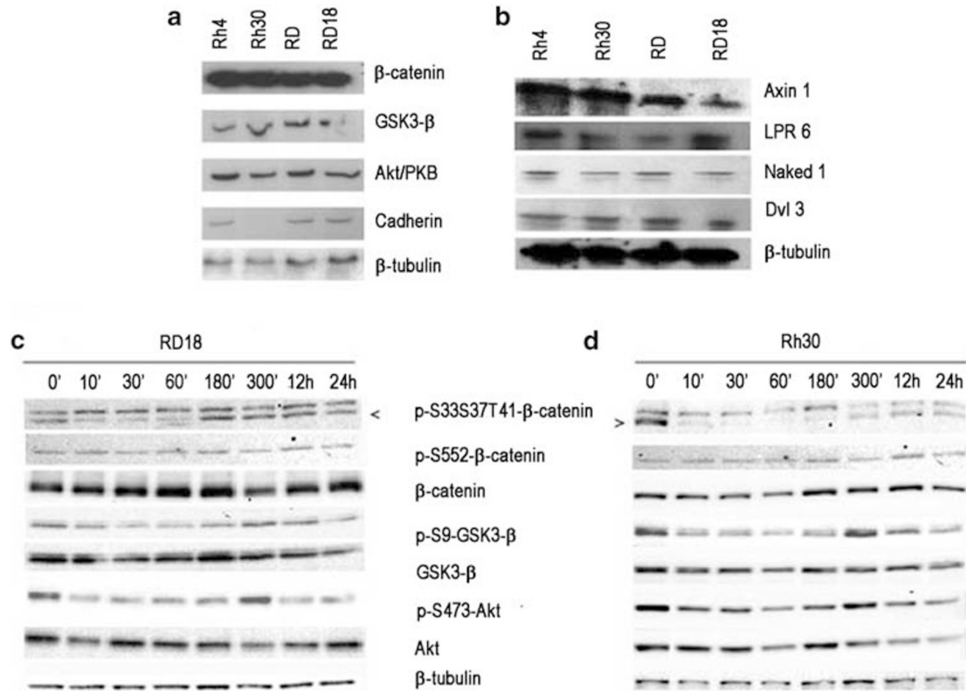

Figure 2 (**a**, **b**). Immunoblot analyses of Wnt proteins in ARMS and ERMS cell lines. Expression of phospho-β-catenin (S33S37T41), phospho β-cateninS552, total β-catenin, total glycogen synthase kinase-3β (GSK-3β), phospho-GSK-3β, total Akt and phospho-Akt in RD18 (**c**) and Rh30 (**d**) cells in response to Wnt3a (200 ng/ml) over 24-h time period.

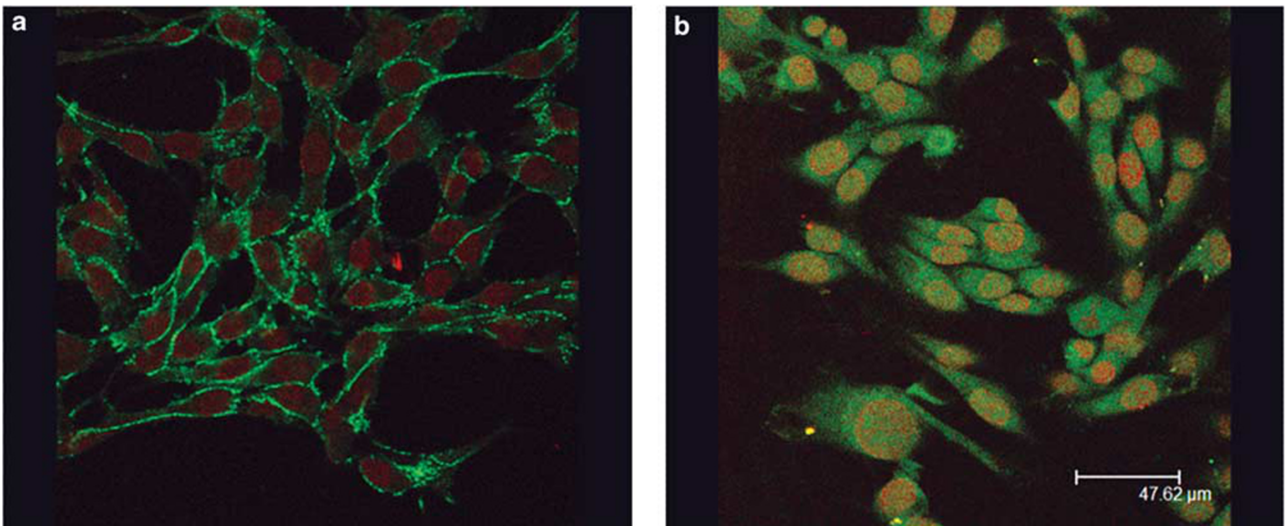

Figure 3 Immunofluorescence localization of β-catenin with (**a**) and without (**b**) Wnt3a (200 ng/ml) in Rh30 cells. The control cells show a predominantly membranous expression while the Wnt3a-stimulated cells show perinuclear localization.

unchanged (Figure 4a). In addition, immunofluorescence showed cytoplasmic localization of phospho-S552-β-catenin in control cells (0$'$), whereas a pronounced perinuclear/nuclear localization was evident in Wnt3a-stimulated RMS cells (RH30 at 120$'$ and RD18 at 60$'$; Figure 4b). Phospho-S33S37T41-β-catenin showed a cytoplasmic localization in control cells (0$'$) and a notable reduction in Wnt3a-stimulated RMS cells (RH30 at 120$'$ and RD18 at 60$'$; Figure 4b).

Regulation of TCF/LEF Transcriptional Activation

We evaluated the functional significance of the nuclear translocation of β-catenin in response to Wnt3a treatment in

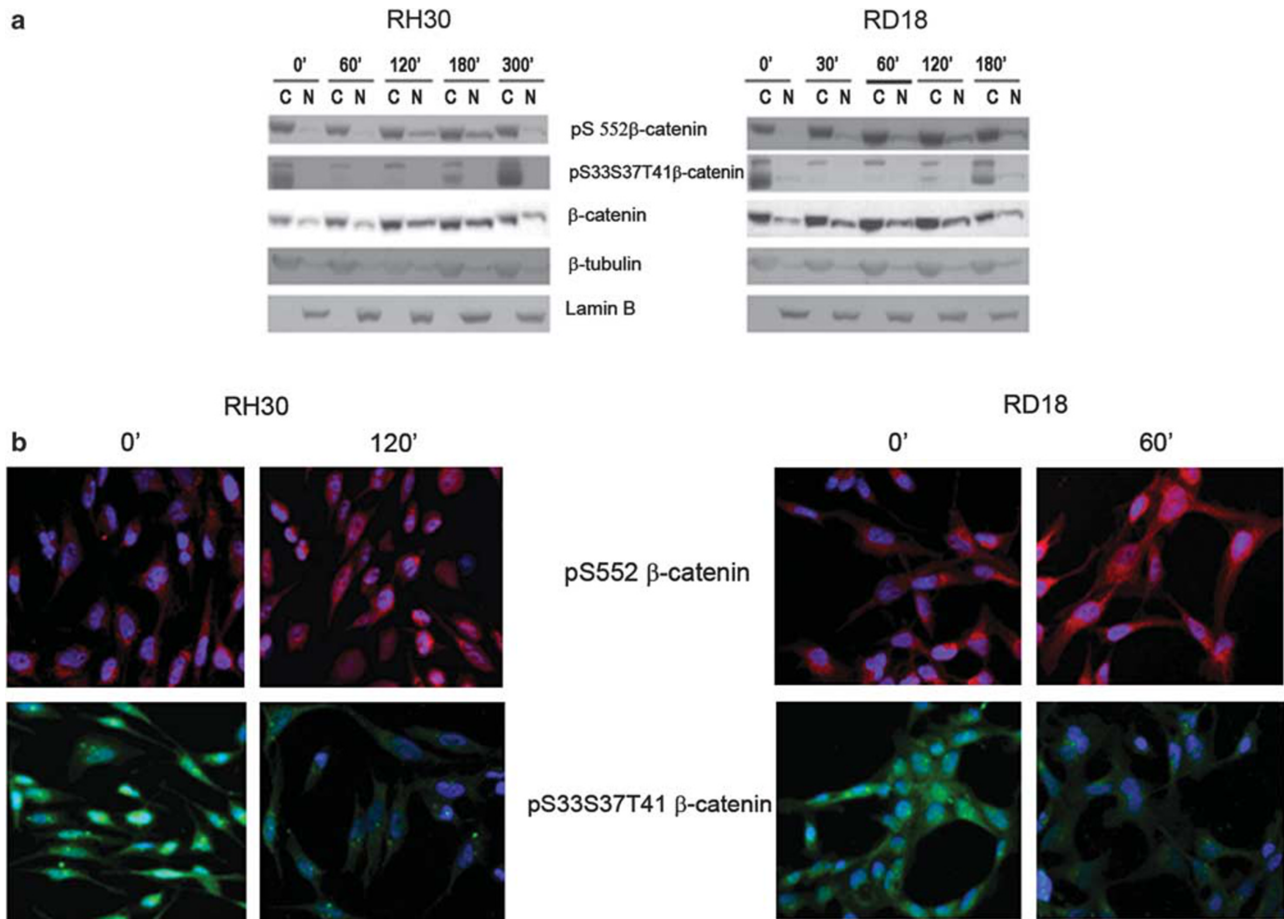

Figure 4 Expressional analysis of phospho-β-catenins (S552 and S33S37T41) in Rh30 and RD18 cells in response to Wnt3a (200 ng/ml) over time in the cytoplasmic (C) and nuclear (N) fractions by immunoblotting (**a**) and immunofluorescence (**b**). Both experiments confirm a concomitant nuclear increase in the phospho-S552-β-catenin fraction and a reduction of cytosolic phospho S33S37T41-β-catenin.

Rh30 and RD18 cells by the downstream transactivation of TCF/LEF complex using luciferase assay analysis. After Wnt3a stimulation, TOP flash-transfected Rh30 and RD18 cells, showed approximately sevenfold significant induction of β-catenin-driven TCF/LEF luciferase activity compared with untreated control cells; this effect appears to be specific because Wnt3a treatment does not affect luciferase activity of FOP flash control vector (Figure 5a).

Unexpectedly, immunoblot analysis for downstream Wnt targets in both Wnt3a-treated cell lines showed no significant changes in c-Jun, cyclin D1, slug and c-myc expression (Figure 5b). Conversely, expression of axin-2, another specific Wnt target, was increased in Rh30 and RD18 cell lines following Wnt3a stimulation (Figure 5c).

Effect of Recombinant Wnt-3a on Proliferation and Apoptosis in RMS Cell Lines

Given the cellular context dependency of Wnt signaling, we examined its role in mediating the proliferative capacity of RMS cell lines. After 48 h of Wnt3a treatment resulting in activation of canonical Wnt signal pathway, as confirmed by luciferase assay, we observed up to 20% decline in proliferation rates of both alveolar RMS cell lines, (Rh4 cells $P<0.02$ and Rh30 $P<0.05$; Figure 6a). There was no significant decrease in proliferation after Wnt3a treatment in the embryonal RMS cell lines (RD18 and RD; Figure 6a). To further delineate whether Wnt3a stimulation mediates its antiproliferative effect through apoptotic or cytostatic mechanisms, we assayed the activity of specific proteins directly involved in early apoptosis induction. Wnt3a stimulation did not produce a statistically significant increase in caspase-3/7 activity (see Supplementary Information, Supplementary Figure S1) suggesting that Wnt3a may act by promoting cell differentiation in alveolar RMS cell lines rather than inducing apoptosis or cytotoxic effects. Indeed, after 24–48 h Wnt3a treatment a definite induction of myogenic differentiation markers (MyoD1, myf5 and myogenin) occurred while desmin showed variable results in both cell lines (Figure 6b). Myosin and actin arrangement were assessed by immunofluorescence using MyHC antibody and phalloidin TRITC-conjugated after 96 h Wnt3a treatment. Treated cells were MyHC positive

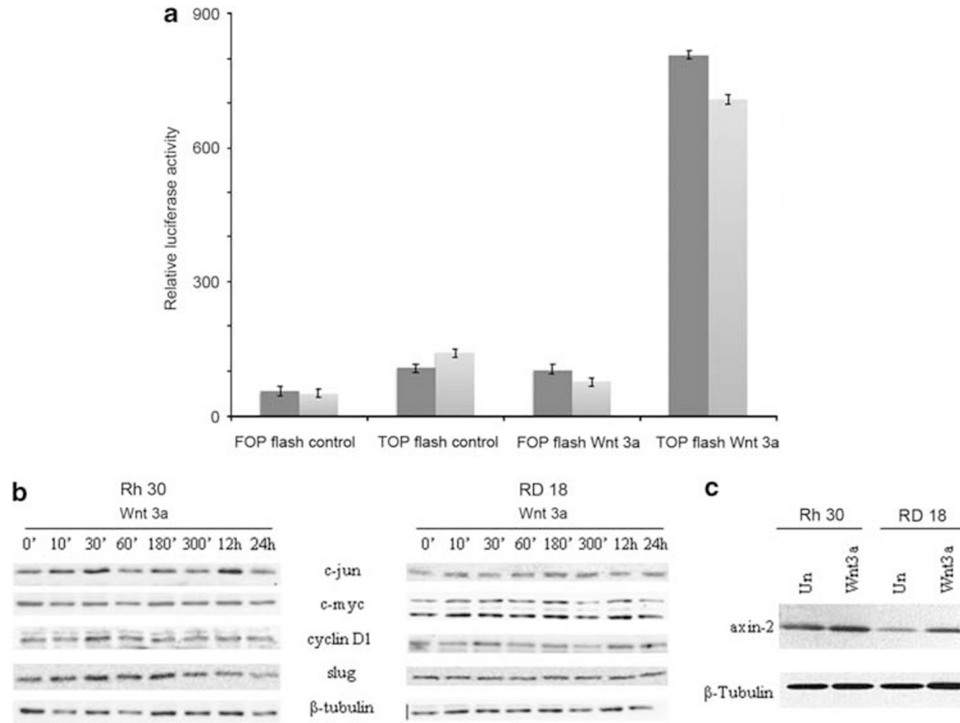

Figure 5 (**a**) Functional activation of Wnt signaling in RMS. Wnt-responsive Gaussia luciferase TOP/FOP reporter gene assay ($n = 3$) in untreated and Wnt3a (200 ng/ml) treated Rh30 Immunoblot analyses of Wnt target proteins in ARMS Rh30 and ERMS RD18 cell lines with and without Wnt3a (200 ng/ml) stimulation (**b**) Increased expression of Wnt-feedback inhibitor axin-2 in control and Wnt3a (200 ng/ml) stimulated Rh30 and RD18 cells (**c**). These results indicate functional activation of Wnt signaling in RMS.

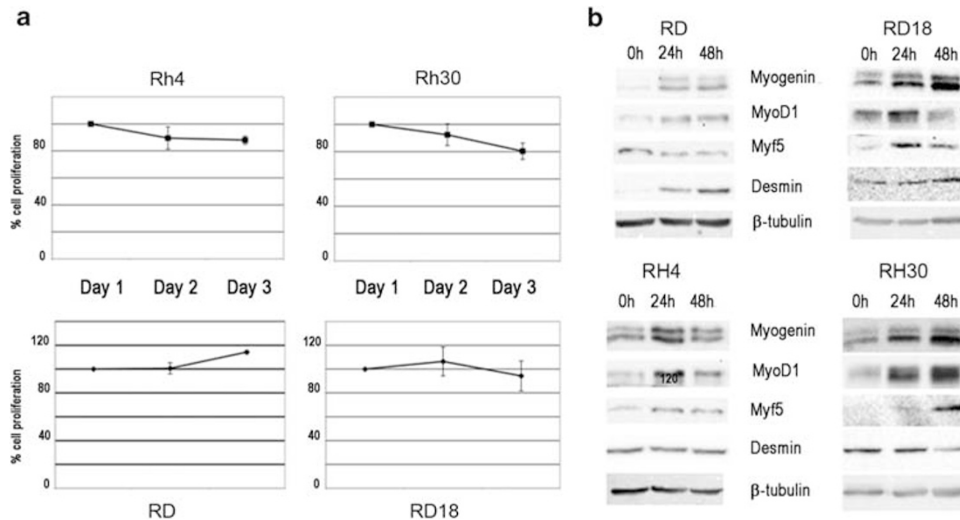

Figure 6 Effect of Wnt3a (200 g/ml) on the proliferation of alveolar (Rh4 and Rh30) and embryonal (RD and RD18) RMS cell lines expressed as % inhibition. (**a**) There was 15–20% decrease in proliferation rate of alveolar RMS cells (Rh4 $P < 0.02$ and Rh30 $P < 0.05$) and no significant change in embryonal RMS cells(RD $P > 0.05$ and RD18 $P > 0.05$; $n = 3$). (**b**) Increased expression of myogenic markers (myogenin, myf-5 and myoD1) after Wnt3a (200 ng/ml) stimulation in rhabdomyosarcoma (RMS) cells.

compared with untreated cells (data not shown) and, after phalloidin staining, areas characterized by a more concentrated assembly of actin bundles appeared (Figure 7a). Cells treated with Wnt3a revealed morphological features typical of differentiating muscle cells, progressively changing from fusiform or star shaped into elongated confluent cells as shown by confocal microscopy (Figure 7b). Therefore, increased differentiation appears to be a plausible

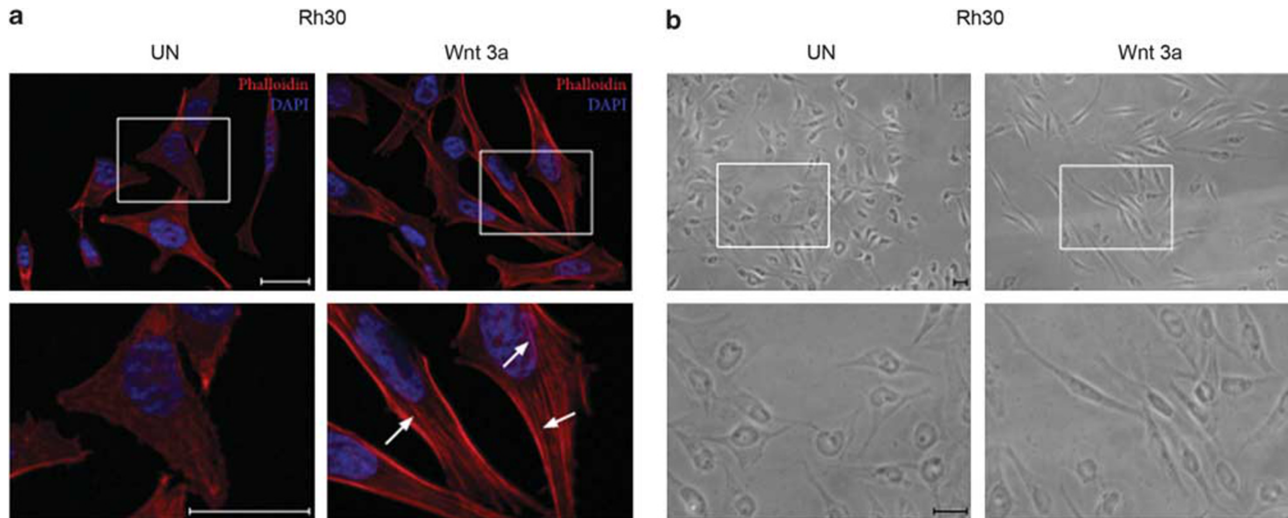

Figure 7 The effect of 96 h Wnt3a stimulation of Rh30 cells. (**a**) Actin rearrangement to form condensed filamentous bundles (arrows) is shown by immunofluorescence with TRITC-conjugated phalloidin. (**b**) Phase contrast microscopy shows a change in morphology from stellate to elongated cells. Representative images for each treatment group are shown with the areas in the upper panels enlarged in the lower bottom panels. Scale bars 20 μm.

explanation for the decline in proliferation in alveolar RMS cell lines.

DISCUSSION

Wnt signaling is involved in myogenic differentiation and proliferation[10,11] although recent studies have generated conflicting views of its relevance to the molecular pathways operative in RMS. Bouron-Dal Soglio *et al*[31] showed no mutations of β-catenin in their RMS cases so concluded that there was no evidence of activating mutations in the genesis of RMS. Singh *et al*[32] identified Wnt2 overexpression in embryonal RMS cells and described impaired Wnt signaling in embryonal RMS cells from p53/c-fos double mutant mice in comparison with normal myoblasts.

This study aimed to characterize in detail the Wnt/β-catenin signaling pathway in RMS and to explore its functional activity. Immunohistochemical assessment of biopsy material showed membranous/cytoplasmic β-catenin expression in 24/44 cases and nuclear expression in 2 cases, this being in agreement with expectations given that no activating mutations of β-catenin were found by others.[31] Wnt pathway proteins were identified in cell lines derived from alveolar and embryonal RMS. Activation of the Wnt pathway by its natural agonist, Wnt3a, stabilized the free form of the active β-catenin (phospho-S552) in a time-dependent manner with its subcellular redistribution by translocation from the membranes to the perinuclear/nuclear region.[30] As the levels of Akt and phospho-Akt remained unchanged, the Wnt3a-mediated β-catenin activation and GSK-3β inactivation appears to be independent of PI3K/Akt pathway. Usually, PI3K/Akt pathway activation by EGR, insulin, insulin-like growth factor, and so on, causes inactivation of GSK-3β by phosphorylation at Ser 9. The aberrant decrease rather than increase in phospho-GSK-3β levels in response to Wnt3a (Figures 2c and d) may be explained in part by an alternative mechanism for its inactivation following Wnt stimulation. Dvl-mediated physical sequestration of GSK-3β, rather than its inactivation by phosphorylation at Ser9 (as seen after PI3K/Akt pathway activation), may be one explanation for this observed decrease in the GSK-3β levels, as shown in rat skeletal muscle by Aschenbach *et al.*[33] Similar results were obtained by Rao *et al*[34] who proposed that APC and β-catenin interaction does not require phosphorylation by GSK-3β.

Indirect immunofluorescence and cell fractionation studies confirmed that free β-catenin (phospho-S552) translocates into the nucleus where it is transcriptionally active. Decrease in the level of phospho-S33S37T41-β-catenin in both cell lines after Wnt3a indicates a decrease in the inactive form of β-catenin, supporting the fact that β-catenin is rescued from degradation and is stabilized in the cytoplasm so that it can translocate to the nucleus to activate its downstream Wnt targets. In a reporter gene assay for β-catenin, Wnt3a specifically activated transcription and, on western blotting, stimulated expression of the downstream Wnt target axin-2. Axin-2 has been shown in previous studies[35,36] to be involved in a negative feedback loop to limit signaling pathways following transduction of Wnt. We believe that transient activation of Wnt/β-catenin signaling pathway may be sufficient to redistribute β-catenin from the membranes into the nuclei where, in the context of RMS cells, it may activate target genes.

The basic mechanisms involved in RMS growth and proliferation are incompletely characterized. We investigated the role of the Wnt signaling pathway in cell proliferation in RMS cell lines. Wnt3a stimulation resulted in about 20%

decrease over 3 days in the proliferation rate in both alveolar RMS cell lines, but there was no significant decline in that of embryonal RMS cell lines. Decrease in proliferation could be either because of increased apoptosis or increased cell differentiation. None of the cell lines showed increased apoptosis after Wnt3a stimulation but induction of myogenic differentiation markers (myogenin, myoD and myf5) indicating a possible role for Wnt signaling in promoting myodifferentiation in RMS, as suggested by *in vitro* studies.[11,31]

Why myogenic differentiation markers induced by Wnt3a failed to arrest proliferation in embryonal RMS cell lines remains unexplained, although it is possible that the Pax–FKHR fusion gene protein, which imparts a growth advantage to alveolar RMS cells, may be directly inhibited by Wnt signaling. Other cytogenetic events in embryonal RMS may uncouple cell proliferation and cell differentiation. More studies are needed to understand the mechanism underpinning this phenomenon. As activation of Wnt/β-catenin pathway decreases proliferation and increases myodifferentiation in RMS, activation of this pathway may be useful therapeutic tool in the management of aggressive alveolar RMS, for example, using lithium chloride to inhibit GSK-3β.[14] There appears to be a tumour-suppressive role for Wnt/ß-catenin signaling pathway in ARMS that needs to be investigated further.[28,29]

In summary, this study has shown that Wnt proteins are expressed in tissue samples from alveolar and embryonal RMS, and has provided *in vitro* data indicating that the pathway may be functionally active. To our knowledge, this is the first paper that comprehensively shows the expression and relevance of Wnt signaling in RMS. Our data support a possible tumour-suppressive role of Wnt/β-catenin signaling in ARMS that warrants further investigation. This pathway is a potential therapeutic target, in particular for patients with alveolar RMS who currently have a poor outcome with limited therapeutic options.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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AUTHOR CONTRIBUTIONS
SA designed the overall structure of the project including the experiments and was guided by HPM and CD. SA, HPM, CD and TH procured grants. SA and SCialfi carried out the experiments in Liverpool and Rome. SA, SCialfi, CD, HPM and TH analyzed data. The manuscript was written by SA and revised by SCialfi, CD, HPM and TH. All authors have approved the submitted version.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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