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Caveolin 1 is a marker of poor differentiation in Rhabdomyosarcoma

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This work is dedicated to Giorgio Fanzani

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ABSTRACT

Caveolins consist of three different membrane scaffolding proteins that play a variety of processes in different tissues. In skeletal muscle caveolins are differentially distributed, with Caveolin 1 (Cav-1) being uniquely expressed in satellite cells and Caveolin 3 (Cav-3) in mature myofibers. Rhabdomyosarcoma (RMS) represents the most common childhood soft-tissue sarcoma arising from mesenchimal precursors which fail to undergo proper commitment to muscle lineage. Cav-3 has been proposed as a marker of RMS with a high degree of differentiation, while biological significance of Cav-1 expression in RMS is still a matter of debate. In the present study we show that Cav-1 is predominantly expressed in the embryonal RMS histotype, as further confirmed by transcript and protein analysis in different *in vitro* human RMS cell lines. Immature cell phenotype of human embryonal RD line, carrying spontaneous activating RAS mutations, was significantly associated to ERK MAPK signalling pathway and featured by high Cav-1 levels, whereas pharmacological attenuation of the ERK pathway, improving cell differentiation, lead to Cav-1 down-regulation. Overall, these data place Cav-1 as a valuable marker of diagnosis for RMS characterised by low degree of differentiation.

1. Introduction

Caveolins comprise a group of three different scaffolding proteins,[1–3] named as Cav-1, Cav-2 and Cav-3 that display a unique hairpin conformation and play a pivotal role in the formation of *caveolae*, flask-shaped invaginations of the plasma membrane.[4–7] *Caveolae* and caveolins are expressed in a variety of tissues and are implicated in several processes, including vesicular transport, cholesterol homeostasis and regulation of signal transduction.[1,8–11] At the plasma membrane caveolins regulate the activity of several intracellular proteins by physical interactions, a property which is ensured by the presence of a characteristic caveolin scaffolding domain, which preferentially binds protein motifs characterised by aromatic residues.[12,13] In skeletal muscle, Cav-1 and Cav-3 are both expressed. In particular, Cav-1 expression is

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinases; MEK, MAP/ERK kinase kinases; MMLV-RT, moloney murine leukemia virus reverse transcriptase; MyHC, myosin heavy chain; PBS, phosphate buffer solution; TBS, Tris buffer solution.

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restricted to satellite cells,[14] which represent a pool of quiescent reserve elements,[15,16] whereas Cav-3 is expressed in myoblasts undergoing differentiation and in mature myofibers,[17,18] suggesting that a timely coordinated expression of Cav-1 and Cav-3 contributes to skeletal muscle homeostasis. Rhabdomyosarcoma (RMS) is the most common paediatric soft tissue sarcoma (MIM #268210) accounting for about 3% of childhood malignancies,[19] comprising of different histological subtypes (embryonal, alveolar and pleomorphic) that are all thought to arise from mesenchimal precursors that fail to undergo commitment towards myoblast lineage.[20] Translocations between chromosomes 2 and 13 have been associated to the alveolar RMS,[21,22] whereas embryonal RMS variants are frequently characterised by deregulation of imprinted genes in chromosome region 11p15.5[23,24] and activating mutations in N- or K-RAS genes.[25–27] In RMS Cav-3 represents a sensitive marker of advanced differentiation,[28] but, surprisingly, the expression of Cav-1 has never been investigated so far. Therefore, in the present study we evaluated Cav-1 levels in RMS biopsies as well as in different established human RMS cell lines. In addition, by employing the embryonal RD cells as RMS culture model line, we explored the subcellular distribution of Cav-1 and dissected the molecular pathways underlying its expression during the transition from cell proliferation to differentiation.

2. Materials and methods

All reagents were from Sigma-Aldrich, if not otherwise indicated.

2.1. Antibodies

The following primary antibodies were used: rabbit anti-Cav-1, rabbit anti-MyoD, mouse anti-phosphorylated (Tyr204) and anti-total ERK1/2 (Santa Cruz Biotechnology); mouse anti-Cav-3 and anti-GM130 (BD Transduction Laboratories); mouse anti-myogenin (Dako); mouse anti-MyHC (Hybridoma Bank, University of Iowa); mouse anti-phosphorylated p38 (Thr180/Tyr182) and anti-total p38 (Cell Signaling); mouse anti-alpha-tubulin (Sigma-Aldrich).

2.2. Immunohistochemistry

A total of 13 cases of RMS from all histological subtypes, including 8 embryonal, 4 alveolar and 1 pleomorphic, have been retrieved from the archive of the Department of Pathology in accordance with the Institutional Review Board of the Spedali Civili of Brescia. Sections have been taken from paraffin embedded blocks and subjected to immunohistochemical analysis. Briefly, sections were de-waxed, re-hydrated and endogenous peroxidase activity blocked by 0.3% H_2O_2/methanol for 20 min. Heat-induced antigen retrieval was performed using a microwave oven or a thermostatic bath in 1.0 mmol/L EDTA (pH 8.0) or 1.0 mM Citrate buffer (pH 6.0). Sections were then washed in TBS (pH 7.4) and incubated for 1 h or overnight in TBS/1% BSA with the specific primary antibody, as listed previously. Single immunostain has been revealed by ChemMATE EnVision HRP Labelled Polymer system (DAKO) or NovoLinkTM Polymer Detection System (NovocastraTM Laboratories Ltd) followed by diaminobenzydine as chromogen and Haematoxylin as counterstain. For double immunostains, after completing the first immune reaction, the second primary antibody has been applied and labelled using MACH 4TM Universal AP Polymer Kit (Biocare Medical); chromogen reaction was developed with Ferangi BlueTM Chromogen System (Biocare Medical) and nuclei were counterstained with Methyl Green. Images have been acquired by Olympus DP70 camera mounted on Olympus Bx60 microscope, using CellF imaging software (Soft Imaging System GmbH).

2.3. Cell cultures

Human embryonal RD cells[29] were purchased from European Collection of Cell Cultures (ECACC), whereas human RD/18 and RD/12 cells are clones originally derived from embryonal RMS subtypes.[30,31] Cells were routinely maintained under standard conditions (37 °C and 5% CO_2) in a humidified incubator, and cultured in growth medium (GM), consisting of high glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum and 100 μg/ml penicillin-streptomycin antibiotic. To induce differentiation, 80% confluent cells were switched to a low-serum medium (termed differentiating medium, DM), consisting of DMEM supplemented with 2% horse serum and antibiotic.

2.4. Drug treatments

PD98059 compound has been shown to inhibit *in vivo* MEK1 and therefore the activation of ERK MAPK cascade,[32] whereas SB203580 compound has been shown to inhibit the activity of p38 α and β kinases.[33] Each inhibitor was resuspended in DMSO vehicle and administered to RD cells at the final concentration of 5 μM.

2.5. Real-Time PCR analysis

Total RNA was isolated by Tri-reagent method and digested with DNAse (DNA-free, Ambion). Subsequently, 2 μg of RNA was reverse-transcribed in the presence of 400 Units of MMLV-RT (Promega) to obtain cDNA templates. Real-Time RT-PCR was performed using the ICycler iQ detection system (Bio-Rad Laboratories). For each quantification, a standard curve was created using a suitably appropriate quantity of cDNA, obtaining amplification efficiency values close to 2 for all primer combinations. The amplification profile used was: denaturation programme (95 °C for 3 min), 35 cycles of two steps amplification (95 °C for 15 s and 60 °C for 30 s) and melting curve (60–90 °C with a heating rate of 0.5 °C/10 s). Each reaction was performed in triplicate. Gene specific primers were designed with the Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), using published sequence data from the NCBI Reference Sequence (RefSeq) database (http://www.ncbi.nlm.nih.gov/RefSeq), and Oligo Analyzer software (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) to avoid potential hairpin formation and self dimerization. To evaluate differences in gene expression, we chose a relative quantification method

based on the standard curve approach.[34] Levels of expression obtained were normalised by geometric averaging of multiple internal control genes,[35] including ribosomal protein large P0 (RLP0), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tubulin, alpha 1 (TUBA1A).

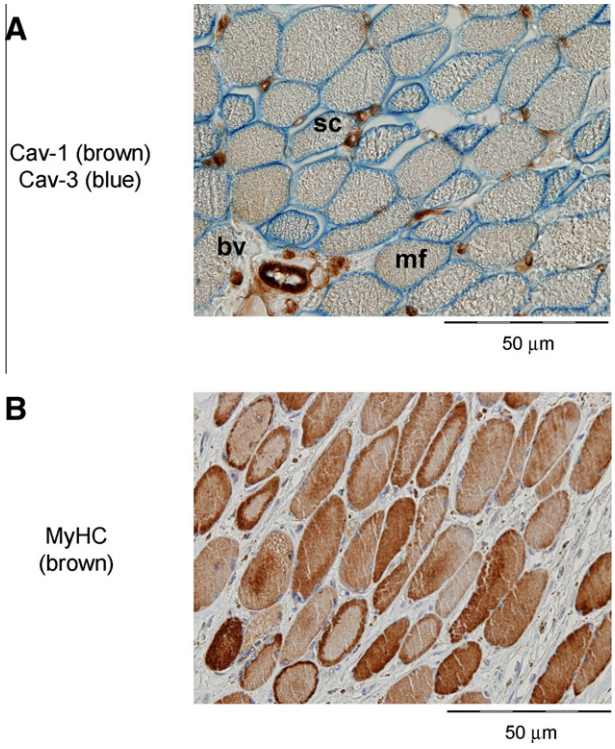

Fig. 1 – Cav-1 and Cav-3 mark different cell elements in skeletal muscle. (A) Immunostain of skeletal muscle tissue indicates that Cav-1 marks satellite cells (sc) and blood vessels (bv), while Cav-3 marks the plasmalemma of myofibers (mf). (B) Cross-sectional area of mature myofibers is evidenced by staining with MyHC antibody.

The following primer sequences have been used:

Gene	Primer	Sequence	Size
CAV-1	F	AACCGCGACCCTAAACACCT	103 bp
	R	CCTTCCAAATGCCGTCAAAA	
CAV-3	F	TGACCTGGTGAACCGAGACC	108 bp
	R	CACACGCCGTCAAAGCTGTA	
RPLP0	F	TGTGCCCTTCAACAGATTTTGG	147 bp
	R	GCAGCCTTTCATTCCTTCCATT	
GAPDH	F	CTCTCTGCTCCTCCTGTTCGAC	69 bp
	R	TGAGCGATGTGGCTCGGCT	
TUB1A	F	AAGCAGCAACCATGCGTGAG	98 bp
	R	GCCGTGTTCCAGGCAGTAGA	

2.6. *Western-blot analysis*

Protein concentration was calculated by bicinchoninic acid assay (Pierce). Equal amounts of protein samples were separated by SDS–PAGE under reducing conditions and transferred to PVDF membranes. Incubation with specific primary antibodies was followed by horseradish peroxidase-conjugated secondary antibodies (Chemicon, cat num AP132P, AP124P), and the resulting immunocomplexes were visualised using enhanced chemiluminescence reagent (Chemicon). To analyse caveolins expression, cells were harvested in a Triton buffer, composed by 10 mM Tris–HCl (pH 8.0), 1% Triton X-100, 5 mM EDTA, 150 mM NaCl, and a cocktail of proteases inhibitors (Roche). Subsequently, cell lysates were centrifuged (12,000*g* for 15 min at 4 °C) and the Triton-insoluble cell membranous fractions were further analysed. To analyse myogenin and MyHC expressions, protein lysates were obtained by harvesting myoblasts in cold RIPA lysis buffer, composed by 20 mM Tris–HCl (pH 7.6), 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaCl and a cocktail of proteases inhibitors. For immunodetection of phosphorylated ERK1/2 and p38 forms, cells were resuspended in a lysis buffer composed by 20 mM Tris–HCl (pH 7.4), 0.2% Triton X-100, 1 mM EDTA, 150 mM NaCl and a cocktail of proteases plus phosphatases inhibitors (0.5 mM NaF and Na_3VO_4).

Table 1 – Results of Immunohistochemical studies of RMS with MyoD, Cav-1 and Cav-3.

	Patients	Tumour body location	% MyoD	% Cav-1	% Cav-3	Ratio Cav-1/Cav-3	% Double positive Cav-1/Cav-3
ERMS	1	Occipital	100	80	20	4.0	30
ERMS	2	Dorsal	100	60	40	1.5	40
ERMS	3	Rhinopharynx	100	40	60	0.5	40
ERMS	4	Thigh	80	40	60	0.7	40
ERMS	5	Testis	100	50	70	0.7	50
ERMS	6	Rhinopharynx	80	20	50	0.4	40
ERMS	7	Pelvic	100	20	90	0.2	30
ERMS	8	Arm	90	90	90	1.0	80
	Mean		**93.7**	**50**	**60.0**	**1.1**	**43.8**
ARMS	9	Perineum	60	20	90	0.2	10
ARMS	10	Thigh	90	10	70	0.1	20
ARMS	11	Calf	70	20	80	0.3	10
ARMS	12	Elbow	100	10	90	0.1	10
	Mean		**80**	**15**	**82.5**	**0.2**	**12.5**
PRMS	13	Thigh	80	100	30	3.3	20

2.7. *Immunofluorescence microscopy*

Cells were cultured on 12 mm glass coverslips coated with 20 μg/ml laminin (Roche), fixed with paraformaldehyde for 10 min at 37 °C, washed with PBS/sucrose (2%) and treated with extraction buffer, composed by 20 mM Hepes (pH 7.4), 0.5% Triton X-100, 300 mM sucrose, 3 mM $MgCl_2$ and 50 mM NaCl. Then cells are treated with 3% BSA in PBS for 15 min, and incubated for 2 h in a humid atmosphere with the specific primary antibody, as listed previously. After PBS washing, samples were incubated for 1 h with a diluted 1:1000 anti-mouse or anti-rabbit CY3 conjugated secondary antibody (Jackson Immunoresearch). Cells were visualised using a Zeiss confocal microscope (Carl Zeiss S.p.A.), with the laser set on λ = 405–488–543 nm and the height of the scanning = 1 μm. Images (512 × 512 pixels) were then reconstructed using LSM Image Examiner software (Carl Zeiss S.p.A.). Alternatively, fluorescent staining was observed under an Axiovert S100

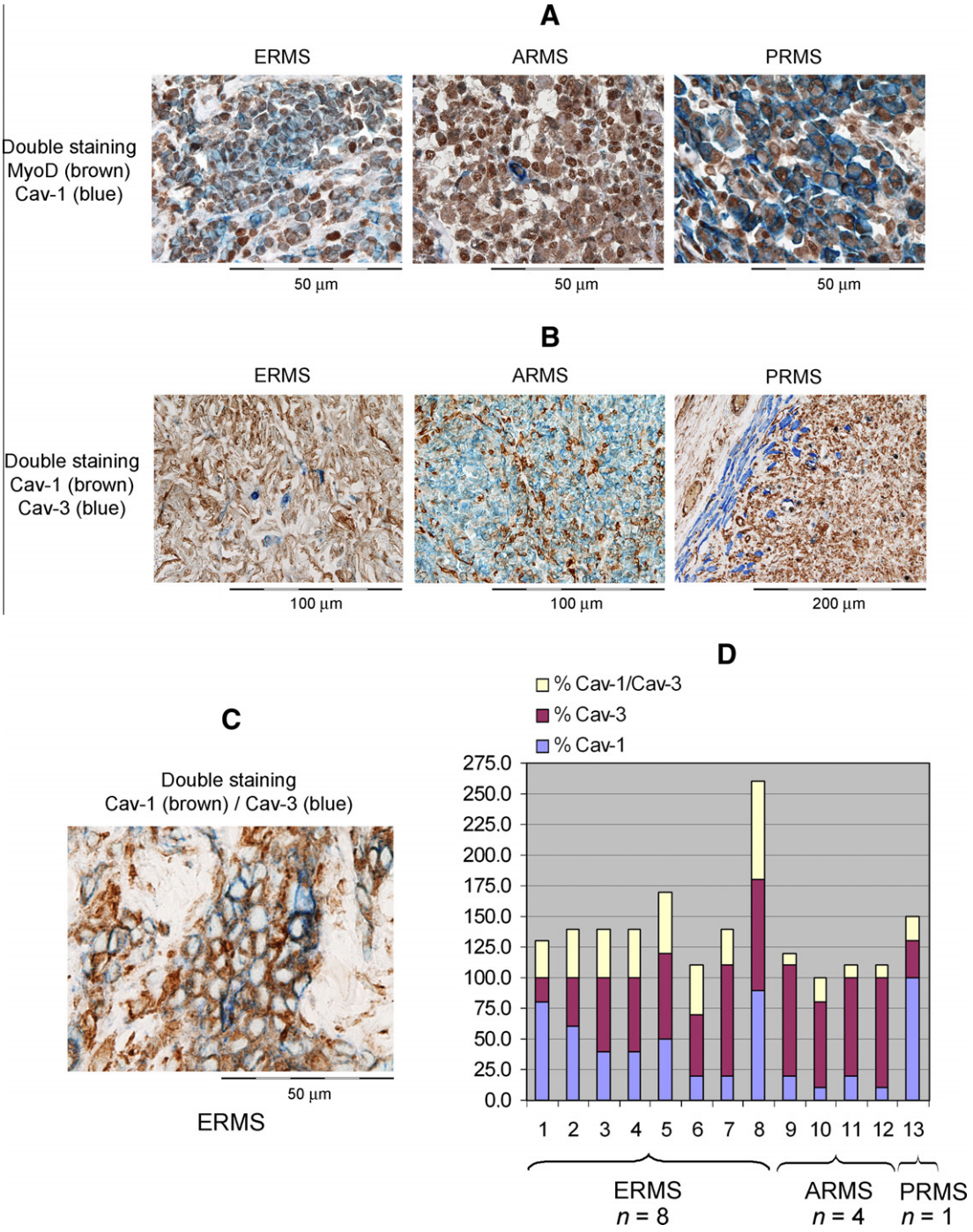

Fig. 2 – Cav-1 predominantly marks ERMS tumours. ERMS, ARMS and PRMS were co-stained with MyoD and Cav-1 (A) or with Cav-1 and Cav-3 (B), indicating that Cav-1 predominantly marks ERMS and PRMS. (C) In ERMS biopsy listed as number 8 in Table 1, the majority of cells exhibited staining for both Cav-1 and Cav-3. (D) The graph reports the percentage of cells positive for either Cav-1 or Cav-3 or for both Cav-1/Cav-3, as observed in each RMS tumour sample.

microscope (Zeiss), and pictures were taken with a digital camera (SensiCam) using the Image-Pro Plus software (version 6.2).

2.8. Statistics

All of the data are expressed as means ± S.E. Statistical significance was determined using *t*-Student analysis. A p value of <0.05 was considered significant.

3. Results

3.1. Cav-1 is predominantly detected in embryonal Rhabdomyosarcoma

Cav-1 and Cav-3 are markers of different cell elements in skeletal muscle. In particular, Cav-1 is a marker of satellite cells,[14] which represent the immature reserve elements of skeletal muscle,[15,16] whereas Cav-3 is expressed in mature myofibers.[17,18] Actually, as evidenced by immunostain of skeletal muscle tissue derived from thigh biopsy (Fig. 1A), Cav-1 expression was specifically recognised in the pool of cells interposed among the myofibers (e.g. satellite cells); in addition, Cav-1 marked blood vessels, being expressed in endothelial cells.[2] On the other side, Cav-3 was detected at the plasmalemma (Fig. 1A), which is the membrane surrounding the myofibers terminally differentiated and positive for the staining with MyHC (Fig. 1B). Subsequently, Cav-1 expression was investigated in Rhabdomyosarcoma (RMS), a soft-tissue tumour expressing a variety of markers typically observed in both embryonic and mature skeletal muscle,[19,20] such as MyoD[36,37] and Cav-3.[28] As summarised in Table 1, 13 different RMS biopsies, consisting of 8 embryonal (ERMS), 4 alveolar (ARMS) and 1 pleomorphic (PRMS), displayed high percentage of MyoD positive cells regardless of the occurrence in different body locations. Cav-1 was predominantly observed in ERMS and in the single PRMS analysed, while Cav-3 displayed a wide-ranging expression, reaching the peak in ARMS, as further indicated by the ratio between the percentage of Cav-1 and Cav-3 positive cells (Table 1). In addition, a significant percentage of cells exhibiting both Cav-1 and Cav-3 staining was especially observed in ERMS (Table 1). Representative images in Fig. 2A highlight that, beside the staining for MyoD was predominant in all the histotypes, Cav-1 was mainly recognised in ERMS and PRMS whereas Cav-3 was predominant in ARMS, as further evidenced by double staining with Cav-1 and Cav-3 antibodies (Fig. 2B). In Fig. 2C is shown an ERMS biopsy (listed as number 8 in Table 1), which exhibited a con-

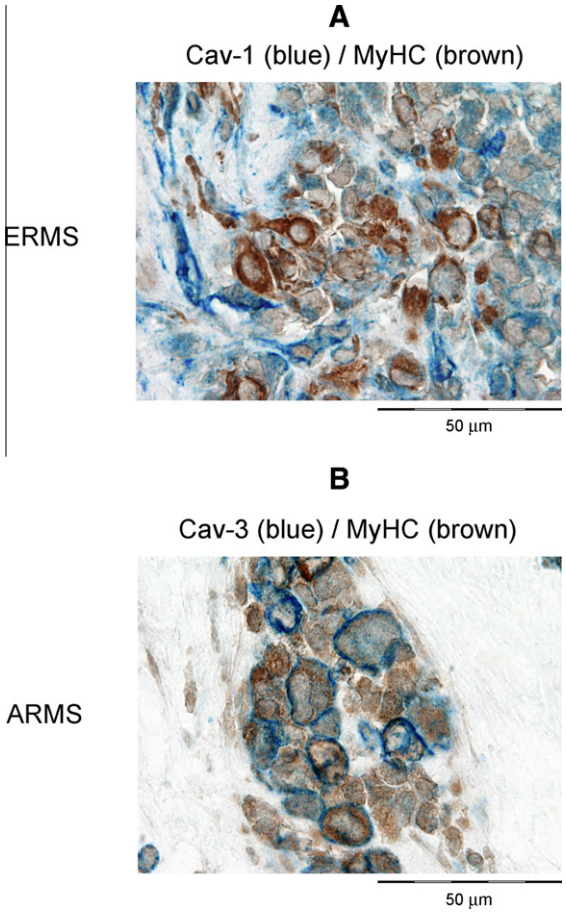

Fig. 3 – Cav-3, but not Cav-1, colocalises with MyHC in RMS cells. Double immunostains show that in ERMS Cav-1 and MyHC specifically stain different cell elements (A), while in ARMS Cav-3 and MyHC mark the same cells (B), suggesting that Cav-1 is a marker of immature cell elements.

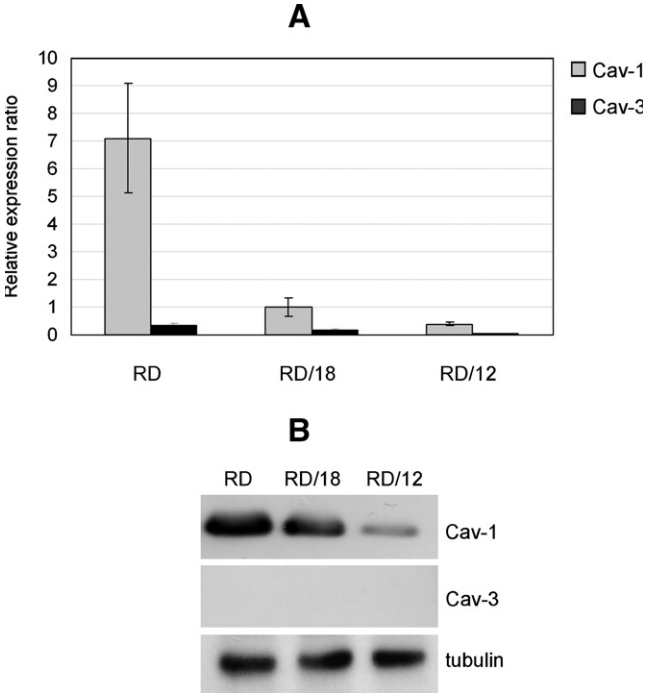

Fig. 4 – Cav-1 is significantly expressed in human ERMS cell lines. (A) Real-Time PCR analysis showing Cav-1 and Cav-3 transcript levels in ERMS cultures (RD, RD/12 and RD/18) maintained under proliferating conditions. Three independent experiments were performed. (B) Western-blot analysis showing Cav-1 and Cav-3 protein levels in ERMS cultures. Tubulin was used as loading control. Three independent experiments were performed.

sistent percentage of cells positive for both Cav-1 and Cav-3. By expressing the percentage of single or double Cav-1 and Cav-3 staining relative to each RMS analysed, graphical report in Fig. 2D clearly confirms that Cav-1 predominantly marked ERMS and PRMS, while Cav-3 configures more specifically as a marker for ARMS. Subsequently, to assess a correlation between Cav-1 expression and degree of RMS maturation, ERMS or ARMS tumour sections were subjected to immunostain for MyHC, a marker of terminal differentiation. In Fig. 3 are reported two ERMS and ARMS cases (listed as number 1 and 10 in Table 1), either exhibiting higher expression of Cav-1 or Cav-3, respectively. In ERMS Cav-1 positive cells resulted consistently negative for MyHC (Fig. 3A), while in ARMS Cav-3 and MyHC evidenced a merged staining in the same cells (Fig. 3B), confirming that Cav-3 is preferentially expressed in differentiated RMS cells, and indirectly suggesting that Cav-1 marks cell elements with lower degree of differentiation.

Taken together, these data suggest that despite both Cav-1 and Cav-3 being detectable in RMS, their specific expressions cannot be considered an exclusive hallmark of a certain histotype, because of the heterogeneity observed among the different RMS subtypes or within the same tumour. Unlikely Cav-3, which predominantly marks differentiated RMS cells, Cav-1 seems to be mainly associated to immature cell elements. In support of this evidence, Cav-1 is preferentially detected in ERMS, which can exhibit a cellular pattern particularly heterogeneous, from poorly to highly differentiated, bearing resemblance to the embryonic stages of the normal skeletal muscle.[20] Importantly, the co-expressions of Cav-1 and Cav-3, especially observed in ERMS tumours, may be indicative of a transition period between the immature and the mature cell phenotype.

3.2. *Cav-1 is expressed in human RMS cell lines from embryonal derivation*

To further characterise Cav-1 in RMS, different human ERMS cell lines were employed, consisting of RD, RD/18 and RD/12 cultures.[29–31] Two variants of Cav-1, playing non redundant roles, can be generated by alternative initiation or by transcription of a shorter splice variant,[38,39] with the Cav-1α isoform (residues 1–178) being the most commonly recognised.[40] By combining different primer sets, we verified the presence of Cav-1α isoform in RMS cultures (not shown). Subsequently, Real-Time PCR and Western-blot analyses were performed to quantify Cav-1 levels. Under proliferative conditions Cav-3 was undetectable, while Cav-1 was significantly expressed in ERMS cultures, as shown at transcript (Fig. 4A) and protein level (Fig. 4B). Taken together, these data suggest that Cav-1 is expressed in ERMS cells, as previously observed in RMS tumours.

3.3. *In RMS embryonal RD cells Cav-1 properly localises at plasma membrane*

Upon exit from Endoplasmic Reticulum/Golgi compartments, wild-type Cav-1 oligomers localise at the plasma membrane.[1,2] In addition, Cav-1 can recycle back to intracellular compartments, and therefore can be found in the Endoplasmic Reticulum, caveosomes,[9,41,42] exocytotic/secretory vesicles,[43,44] cytosolic lipid particles[45] or lipid-droplet structures.[46] In contrast, mutated Cav-1 forms accumulate in the Golgi apparatus and trigger loss of Cav-1 at the plasma membrane through a dominant-negative fashion, as occurs in some cancer types.[47–49] Indeed, confocal microscopy experiments were undertaken to investigate Cav-1 localisation in RMS embryonal RD cells. As shown in Fig. 5, Cav-1 was predominantly localised at the plasma membrane (Panel A) or distributed in intracellular vesicles at or near the plasma membrane (Panel B). Importantly, Cav-1 did not improperly accumulate in the Golgi apparatus, as evidenced by GM130

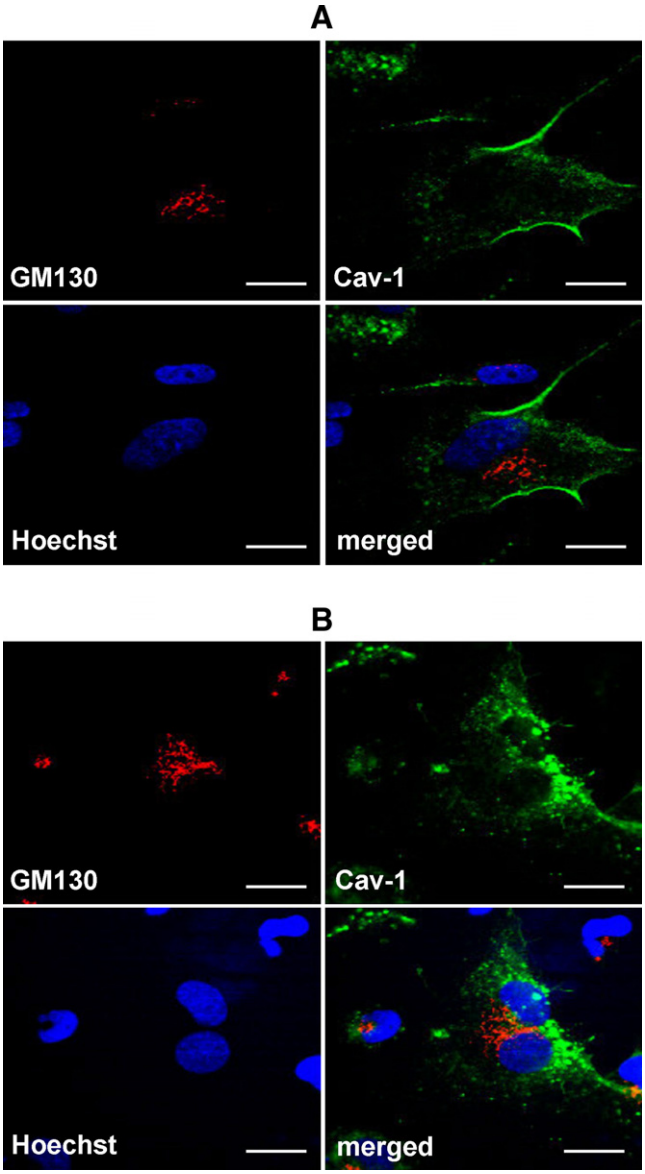

Fig. 5 – Cav-1 is correctly localised at plasma membrane of embryonal RD cells. Confocal microscopy analysis showing that Cav-1 localised at the plasma membrane (A) or in intracellular vesicles (B) of embryonal RD cells. GM130 staining was employed to mark the Golgi apparatus. Bars = 100 μm.

staining (Fig. 5A and B), indeed suggesting a wild-type distribution pattern, as confirmed by sequencing analysis that excluded the presence of inactivating mutations (data not shown). Altogether, these data clearly indicate that Cav-1 has a canonical subcellular distribution in human RD cells, supporting the idea that Cav-1 might exert a functional role in these cells.

3.4. *Cav-1 is predominantly expressed in immature RD cells*

Elevated ERK[25–27] and low p38 signalling[50] are molecular hallmarks contributing to impair differentiation in RMS cell lines, including human embryonal RD cells. As shown in Fig. 6A, after that RD cells were placed in a differentiating medium (DM), just a small subpopulation of them acquired a more mature phenotype, forming mono- or bi-nucleated thin myotubes that are positive for MyHC staining (Fig. 6A and B), suggesting that only a partial induction of myogenic differentiation can be obtained in RD cell line. Compared to proliferation, differentiation of RD cells was characterised by down-regulation of ERK phosphorylation and by a very modest increase of p38 phosphorylation, as detected by Western-blot analyses over a time-course for up to 72 h (Fig. 6C). In differentiating RD cells Cav-1 levels were decreased whereas Cav-3 and MyHC increased (Fig. 6C). Moreover, Real-Time PCR analyses showed that both caveolins were also transcriptionally regulated during differentiation of RD cells, as predicted by down-regulation of Cav-1 and increase of Cav-3 (Fig. 6D). By immunofluorescence analysis (IF), we evidenced that proliferating RD cells (GM) were positively labelled for Cav-1 but not for Cav-3 (Fig. 7A), while differentiating RD cells (DM)

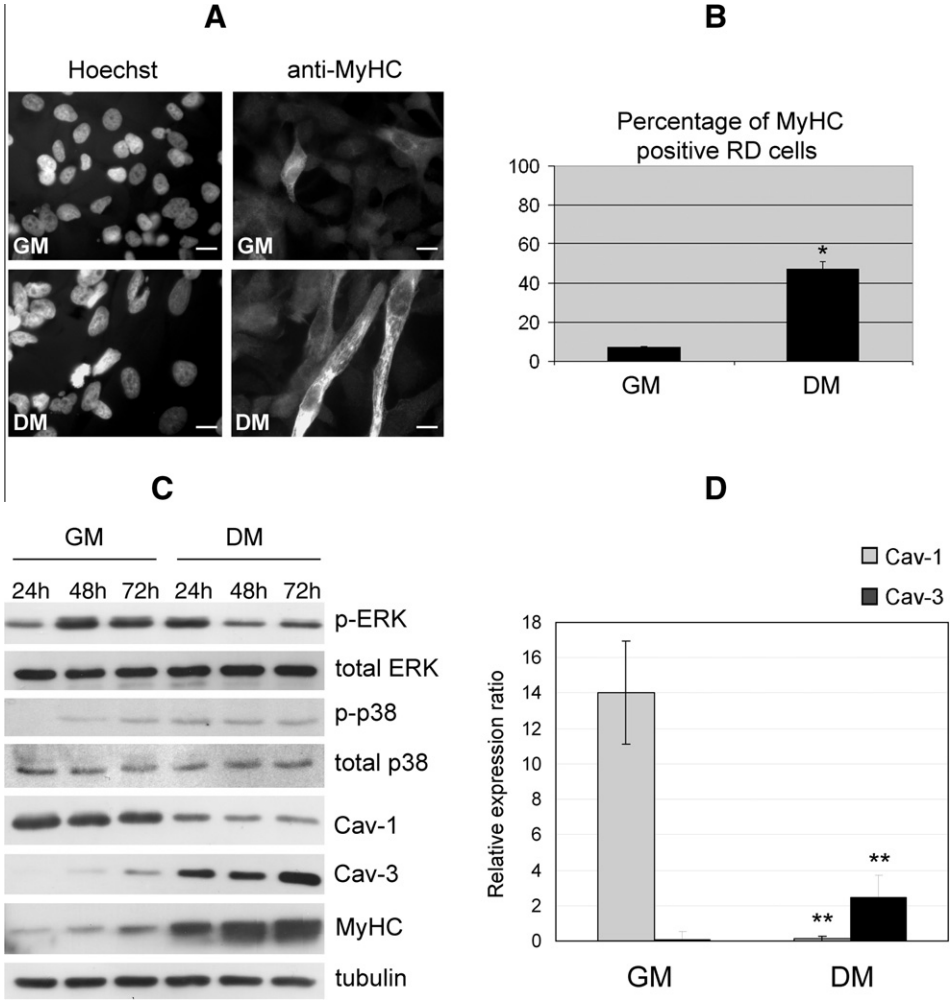

Fig. 6 – Cav-1 is down-regulated during myogenic differentiation of embryonal RD cells. Human RD cells (3.5×10^4 cells/6 cm plate) were maintained in a growth medium (GM) for up to 72 h, and then 90% confluent cells were switched to differentiating medium (DM) for an additional 72 h. (A) Subsequently, RD cells were subjected to immunofluorescence to visualise nuclei (Hoechst staining) and MyHC expression. Bars = 100 μm. (B) The percentage of MyHC-positive RD cells was increased during differentiation. Three independent experiments were performed. Significant at $^{*}p < 0.05$. (C) In RD cells cultured in GM or DM, Western-blot analyses was performed to compare the levels of phosphorylated ERK and p38 vs. total forms and the amount of Cav-1, Cav-3 and MyHC. Tubulin was used as loading control. (D) After 72 h of proliferation (GM) or differentiation (DM), Real-Time PCR has been performed to analyse Cav-1 and Cav-3 transcript levels in RD cells. Three independent experiments were performed. Significant at $^{**}p < 0.01$.

displayed increasing labelling for Cav-3 and loss of Cav-1 (Fig. 7B), as further graphically quantified (Fig. 7A and B, right panels). Taken together, these data suggest that myogenic RD differentiation, which requires attenuation of ERK signalling, enables loss of Cav-1 and increase of Cav-3, confirming that Cav-1 is a marker of poor differentiation in cultured RD cells.

3.5. *In RD cells Cav-1 expression correlates with the degree of ERK pathway activation*

Immature phenotype of RD cells is known to be widely dependent on elevated ERK signalling, because of the presence of N- and K-RAS activating mutations.[25,26] Accordingly, ERK pathway inhibition improves biochemical and phenotypical differentiation of RD cells, leading to a partial but significant restoration of p38 pathway.[51,52] To evidence a potential correlation between the degree of ERK pathway activation and Cav-1 expression, RD cells were cultured in presence of PD98059 (PD), a pharmacological MEK inhibitor.[32] Western-blot analyses detected the decreased ERK phosphorylation levels induced by PD treatment, that correlated with decrease of Cav-1 content and incremented amount of myogenin, MyHC and Cav-3 compared to DMSO-treated cells (Fig. 8A and relative graphical quantifications), leading to improved morphological differentiation (data not shown). On the other side, in presence of SB203580 compound (SB), a pharmacological inhibitor of p38 activity,[33] RD cells were retained in a completely immature morphological state (data not shown), featured by higher levels of ERK phosphorylation, increased Cav-1 amount and loss of myogenin, MyHC and Cav-3 expression compared to DMSO-treated cells (Fig. 8B and relative graphical quantifications). Overall, we might conclude that

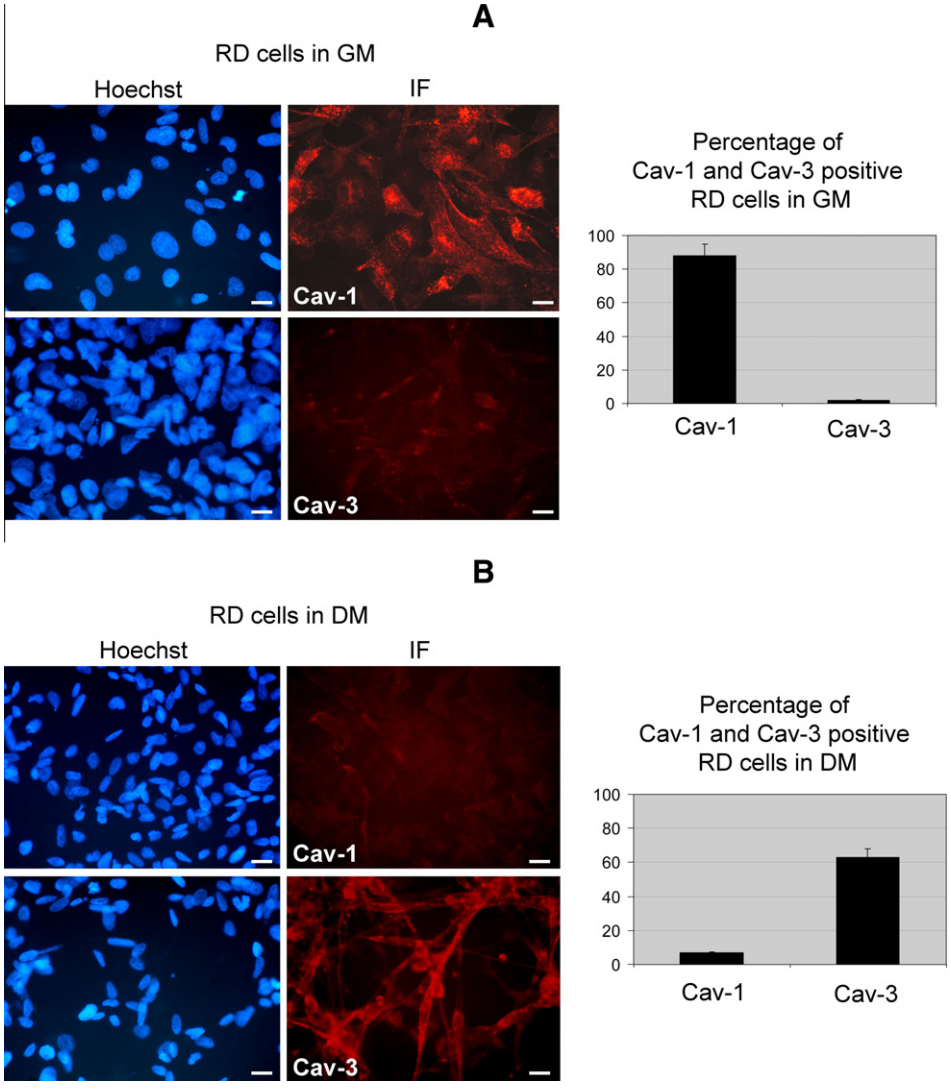

Fig. 7 – Cav-1 and Cav-3, respectively, mark mitotic or differentiating RD cells. (A) Proliferating RD cells displayed a round-shape morphology and were featured by high Cav-1 and low Cav-3 expressions. (B) In contrast, differentiating RD cells underwent elongated shape and displayed loss of Cav-1 and increased Cav-3 expression. Right diagrams show the percentage of Cav-1 and Cav-3 positive cells detected under GM or DM conditions, calculated by counting the cells positive for Cav-1 or Cav-3 staining in comparison with the total number of the Hoechst-positive nuclei. Bars = 200 μm.

Cav-1 expression levels are associated to the activity of ERK signalling pathway in human embryonal RD cells.

4. Discussion

In skeletal muscle Cav-1 is specifically expressed in reserve satellite cells,[14–16] in which it contributes to maintain quiescence by antagonizing RAS/ERK signalling,[14] while Cav-3 plays a crucial role in mature myofibers.[17,18,53,54] Data reported in this study propose Cav-1 as a valuable marker for the detection of the immature component within Rhabdomyosarcoma (RMS), a paediatric malignancy arising from mesenchimal precursors that fail to complete the commitment towards the myogenic lineage.[19–24] In particular, those

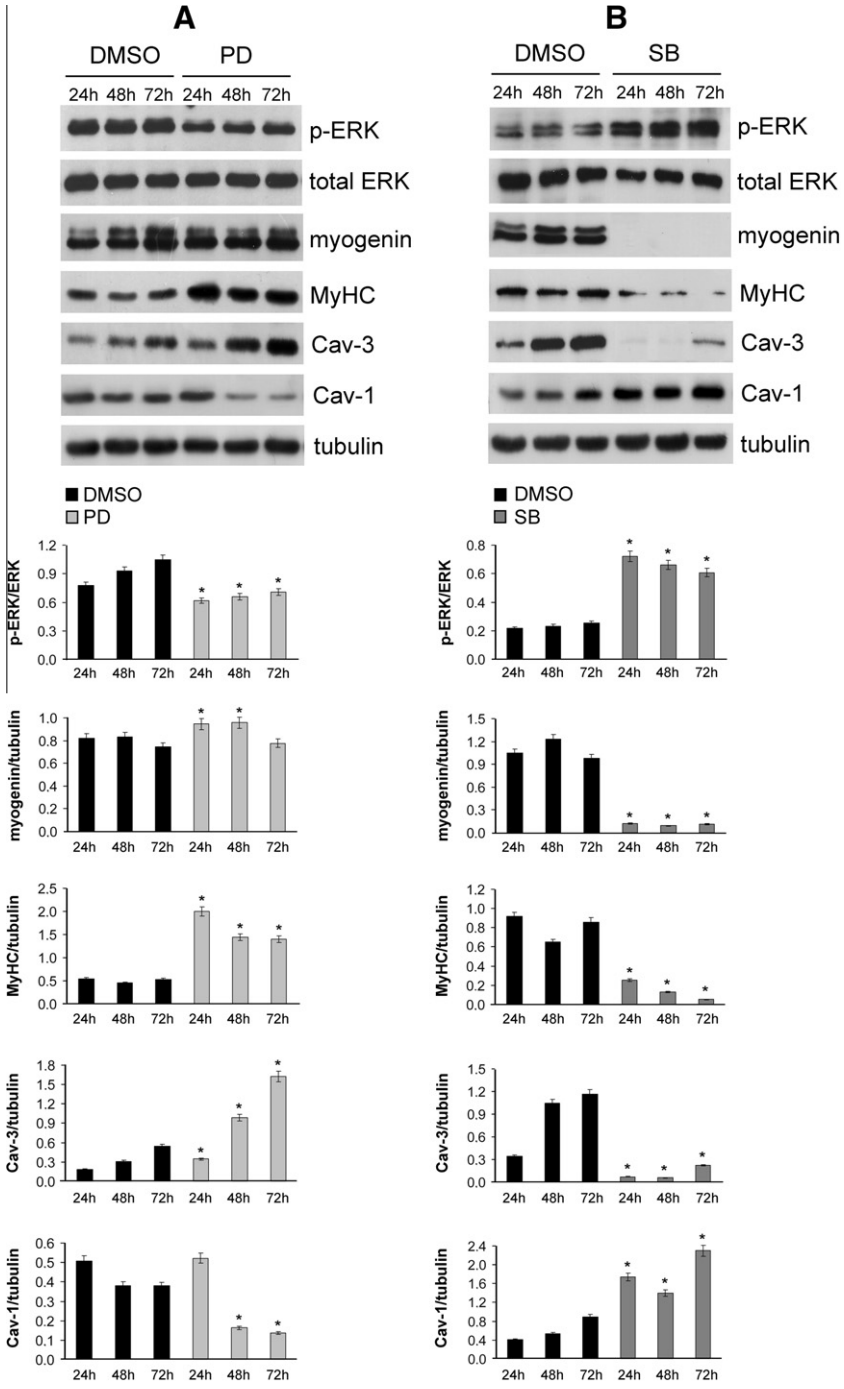

Fig. 8 – In embryonal RD cells Cav-1 levels are correlated to the activity of ERK MAPK pathway. RD cells were cultured in DM over a time-course for up to 72 h, left untreated (DMSO vehicle) or daily treated with PD98059 (PD) (A), an inhibitor of ERK phosphorylation, or with SB203580 (SB) (B), an inhibitor of p38 activity. Subsequently, Western-blot was performed to compare the levels of phosphorylated ERK vs. total forms **and the amount of myogenin, MyHC, Cav-3 and Cav-1. Tubulin was used as loading control. Densitometric analysis of protein levels are reported in the graphical diagrams. Results are the mean of three independent experiments. Significant at** $^{*}p < 0.05$.

tumours exhibiting advanced degree of maturation (e.g. ARMS) displayed preferential expression of Cav-3, therefore confirming previous findings.[28] On the other side, our analysis recognised Cav-1 predominantly expressed in ERMS tumours, which exhibit a cellular pattern particularly heterogeneous, from poorly to highly differentiated, bearing resemblance to the embryonic stages of normal skeletal muscle.[20] In support of this evidence, Cav-1 was also significantly detected in human ERMS cell lines, such as the commonly used RD cells,[29] and its expression was strictly correlated to the degree of ERK pathway activation. Activating RAS mutations in RD cells contribute to aberrant gain of function of ERK signalling[25–27] and also account for the half of ERMS tumours but not for the alveolar subtype,[55] contributing to malignancy.[51,52] In this scenario, Cav-1 seems to be aberrantly converted to a downstream target of the ERK pathway in human RD cells, giving rise to an ambiguous situation, in which immature RMS cells that display persistent Cav-1 expression are also mitotically active, in contrast to muscle satellite cells in which Cav-1 expression imposes quiescence.[14] Interestingly, detection of cell elements showing co-expression of Cav-1 and Cav-3, as predominantly observed in ERMS phenotype, suggests that aberrant ERK pathway activation, leading to deregulation between proliferation and differentiation, may give rise to phenotypes in which markers of specific and different cell states can be ambiguously co-expressed. In cancer progression, Cav-1 plays a dual role.[56–59] Targeted down-regulation of Cav-1 promotes cell transformation, anchorage-independent growth *in vitro* and tumour growth *in vivo*,[60] suggesting that Cav-1 acts as a tumour-suppressor mainly by limiting the ERK signalling pathway.[60,61] In support of this evidence, Cav-1 knock-out mice exhibit tissues characterised by cell hyper-proliferation.[62] For instance, Cav-1 is up-regulated in some cancers and contributes to increased malignancy,[63,64] confirming its ambiguous role in cancer. This complex behaviour can be considered an effect of multiple alterations in the cellular environment, that may promote a shift of Cav-1 activity from tumour-suppressor to oncogene, depending on the presence of a particular subset of caveolin partners in a certain stage of tumour progression, but also on the presence of Cav-1 inactivating mutations[47–49] or post-translational modifications.[65,66] In relation to RMS, it remains to be established whether Cav-1 might be associated to a favourable or poor prognosis. Given the contribution of ERK signalling in ERMS malignancy,[51,52] it is fascinating to speculate that the feedback control normally provided by Cav-1 on RAS/ERK signalling, as occurs in satellite cells,[14] might be lost in RMS tumours, changing the function of Cav-1 from antagonist to agonist of ERK pathway. In addition, persistent Cav-1 levels in myogenic precursor cells blocks the formation of myotubes,[14] and thereby can additionally contribute to retain RMS cells in an immature state. In this scenario, Cav-1 might provide a marker of unfavourable clinical outcome in ERMS tumours featured by elevated ERK signalling pathway, contributing to exacerbate immature phenotype and malignancy. Current and future investigations will be aimed to explain how the expression of Cav-1 might influence cell behaviour in RMS.

Statement of author contributions

SR, PLP, MC, AB and RV conceived and carried out experiments. AF conceived experiments and analysed data. AF, PLP and EM wrote the paper. All authors had final approval of the submitted and published versions.

Author's statement

The authors declare that the material includes an original research, which has not been previously published and has not been submitted for publication elsewhere while under consideration.

Conflict of interest statement

None declared.

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