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The tumour suppressor hSNF5/INI1 controls the differentiation potential of malignant rhabdoid cells

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ABSTRACT

Malignant rhabdoid tumours (MRT) are highly aggressive cancers of early childhood that arise in different organs or tissues. The unifying criterion for these tumours is the presence of inactivating mutations of the hSNF5/INI1 tumour suppressor gene which encodes a core subunit of the chromatin remodelling SWI/SNF complex. Using a variety of markers we analysed the phenotypic traits of MON and DEV cell lines derived respectively from an undifferentiated abdominal MRT and from a brain MRT. DEV cells express spontaneously a wide range of neural and glial markers. It can be induced to differentiate into the neural lineage following hSNF5/INI1 expression with appearance of neurite processes, strong increase of neural markers and decrease of glial markers. A less pronounced neural differentiation is also observed with MON cells, which possess more primitive polyphenotypic features with positivity for markers from the three embryonic layers. Finally, we show that the neural differentiation of rat PC12 cells in the presence of nerve growth factor (NGF) is strongly impaired when hSNF5/INI1 expression is inhibited by RNA interference. Altogether these results indicate that hSNF5/INI1 is an essential subunit for SWI/SNF-dependant induction of neural differentiation programs. Further experiments should enable documentation of whether it provides instructive or permissive signals for differentiation.

1. Introduction

Malignant rhabdoïd tumour (MRT) was originally described as a rare and highly aggressive renal malignant neoplasm of infancy and early childhood.[1] Subsequently, extrarenal MRTs have been reported in a variety of organs including the central nervous system (CNS), liver, skin, and soft tissues. Histologically, MRT is composed of sheets of round or polygonal cells with vesicular nuclei and prominent nucleoli. Rhabdoid cells frequently contain eosinophilic hyaline intracytoplasmic inclusions corresponding to whorls of intermediate filament.[2] Apart from constant positivity for vimentin, numerous studies have reported variable immunostaining reactivity for phenotypic markers including mesodermic,[3] myogenic,[4] epithelial[5] and neuro-ectodermal.[6] Variable differentiation areas can be observed within the same tumour. According to this phenotypic diversity of MRT, various cellular origins have been proposed for the original rhabdoid progenitor, which is still unknown.

This phenotypic variability contrasts with a strong genetic homogeneity. Indeed, MRTs are characterised by biallelic inactivation of the hSNF5/INI1 tumour suppressor gene localised at the 22q11.2 chromosome region.[7] Constitutional mutation of this gene defines a hereditary condition, which predisposes

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to renal and extra-renal rhabdoid tumours.[8–10] The tumour suppressor role of hSNF5/INI1 was confirmed in mice since 15–30% of mice heterozygous for hSNF5/INI1 mutation develop poorly differentiated tumours resembling MRT.[11–13] Moreover the conditional somatic inactivation of hSNF5/INI1 results in the extremely rapid development of aggressive lymphoma.[14]

hSNF5/INI1 encodes a 47 Kda subunit of the SWI/SNF multiprotein complex, which remodels nucleosomes *in vitro* in an ATP-dependant manner. The SWI/SNF complex plays a critical role in the chromatin remodelling associated with transcription regulation. More specifically, it plays a co-activator role with various transcription factors involved in the induction of differentiation processes.[15,16] It also interacts physically and functionally with pRB and HDAC1 to repress E2F transcription factor activity and inhibit S-phase entry.[17,18] In agreement with hSNF5/INI1 playing an essential role within the SWI/SNF complex, recent studies have shown that expression of hSNF5/INI1 in rhabdoid cell lines is able to inhibit S-phase entry and promotes G1-arrest in a Rb-dependant manner.[19–23] The observation that the undifferentiated phenotype of MRT is associated with a loss of hSNF5/INI1 function suggests that hSNF5/INI1 may be involved in differentiation processes. However, hSNF5/INI1 function in SWI/SNF-dependant transcriptional regulation of differentiation programs remains to be determined.

In this study we have investigated phenotypic markers diversity of two rhabdoid cell lines and analysed differentiation potential following hSNF5/INI1 reexpression as compared to cyclic adenosine monophosphate (cAMP) treatment. Our study indicates that hSNF5/INI1 expression in rhabdoid cells induces a neuronal differentiation phenotype as documented by increased expression for neural markers and neurite formation. In agreement with the hypothesis of hSNF5/INI1 playing a crucial role in neuronal differentiation we show that hSNF5/INI1 knockdown can inhibit the NGF-induced differentiation of PC12 cells.

2. Materials and methods

2.1. Cell lines and differentiation media

MON[7] and DEV[24] cell lines were grown in RPMI supplemented with 10% fetal calf serum (FCS) and 10 μg/ml Penicillin/Streptomycin (Invitrogen, Life Technologies). For induction experiments, dibutyl cAMP (0.5 mM, Sigma Aldrich) was added to the culture medium. The PC12 cell line was grown on collagen coated dishes (10 ng/ml, Sigma) in DMEM supplemented with 10% heat inactivated horse serum, 5% FCS and Penicillin/Streptomycin. The differentiation of PC12 cells was induced by 50 ng/ml 7S-NGF (Chemicon International) in DMEM supplemented with 0.5% iHS.

2.2. Antibodies

Monoclonal mouse anti-human antibodies (Mab) against alpha-smooth muscle actin (ASMA) (clone 1A4), CK8 (35βH11), CK18 (DC10), CD31 (JC70A), CD34 (QBEnd 10), CD83, CD117 (104D2), epithelial membrane antigen (EMA) (E29), isotype control IgG (A57H), Neurofilament protein (NFP) (2F11), $p75^{NGFR}$ (NGFR5), vimentin (V9), and polyclonal rabbit anti-cow glial fibrillary acid protein (GFAP) and pS100 were purchased from DAKO. Mab CD68 (Y1/82A), CD146 (P1H12), CD44 (G44-26) were from Pharmingen BD Biosciences, microtubule associated protein 2 (MAP2) (HM-2) from Sigma, Bone Sialo Protein (BSP) from BioDesign International; polyclonal goat anti human HNF-3β (M-20) and polyclonal rabbit anti human VEGFR (C20 sc-315) from SantaCruz.

2.3. Fluorescent-activated cell sorter (FACS) analysis

Membrane antigen expressions were analysed on fresh cells cultivated until 50–70% of confluence. Cells were stripped from the culture dish using PBS-EDTA 5 mM, washed twice in PBS-0.5% BSA and incubated with primary antibody (1/100^e) for 15 min at 4 °C. After washing, cells were incubated with 1/100^e FITC-conjugated donkey anti-mouse, anti-rabbit or anti-goat IgG secondary antibody (Jackson ImmunoResearch) according to the same procedure. Intracellular antigen expressions were analysed on fixed and permeabilised cells. Cells were stripped, washed twice, then fixed in 3.7% paraformaldehyde (Sigma) for 15 min at 4 °C. Cells were washed twice in PBS and treated with PBS-0.1% Triton for 5 min at 4 °C, except for HNF-3β antigen expression which was analysed on methanol permeabilised cells (5 min at 4 °C). Labelling was performed as on fresh cells. FACS analysis was performed on a FAC–Scalibur analyser (Beckton Dickinson). For each marker, means of at least three independent experiments with at least $n > 6$ values were analysed using *t*-test.

2.4. Plasmids, siRNA and transfections

Cells were seeded 24 h prior to transfection at a density of 3×10^5 cells/well in 6-well plates. At day 0, cells were rinsed two times with non-supplemented RPMI medium, and transfected with plasmid DNA or siRNA, as described further. The pcDNA3-INI1 expression vector was previously described.[19] Empty pcDNA3 vector was used as control (Clontech BD Biosciences). Plasmidic DNA were transfected using Effecten reagent (Qiagen) according to modifications of the manufacturer protocol: 1 μg of DNA was transfected with 6 μl of enhancer and 18 μl of Effecten for a 30 mm dish of MON cells (two times more for a 30 mm dish of DEV cells), in fresh medium supplemented for induction of differentiation as indicated in each experiment. The control and the SMARTpool™ hSNF5/INI-specific siRNA (accession number AB017343) were purchased from Dharmacon, MWG Genomic Company. Twelve micro grams of siRNA were transfected on PC12 cells in a 30 mm dish using 10 μl of Lipofectamine reagent (Invitrogen) according to manufacturer instruction, in serum free medium for 4 h at 37 °C. At this time, control or differentiation medium were added. Three days post-transfection, cells were transfected a second time to maintain the level of knockdown. Cells were harvested at day 5.

2.5. Immunofluorescence staining and analysis of process extension

DEV cells were grown onto 12 mm coverslips and transfected as described previously. At day 5, cells were fixed,

permeabilised and stained as described for FACS analysis. Cells were stained with rat anti-HA (Roche Diagnosis) or mouse anti-MAP2 primary antibodies and Alexa.Fluor-488-conjugated donkey anti-rat or Cy3-conjugated donkey anti-mouse secondary antibodies respectively. The coverslips were mounted in Vectashield medium containing Dapi (1.5 μg/ml) (Vector Laboratories). Immunofluorescent staining was observed using Zeiss Axioplan-2 microscope and Plan-NeoFluar opticals from Zeiss.

2.6. *Western blot analysis*

For the detection of hSNF5/INI1, 80 μg of protein extracts were subjected to SDS–PAGE[19] and transferred to nitrocellulose membrane (Amersham) using a semi-dry electroblotting system for 45 min, 3 mA/cm. Membranes were stained with Ponseau-S solution 0.1% (Sigma) to perform a loading and transfer control, and saturated for 1 h with 2% nonfat dry milk in TSBT (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). Blots were incubated overnight with polyclonal rabbit anti-hSNF5/INI1 (1/250[e]) antibody.[25] Membranes were rinsed three times for 5 min in TSBT and incubated for 1 h at RT with peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch). Bound antibody was detected using ECL kit (Pierce/Perbio, France).

3. Results

3.1. *MON and DEV rhabdoid cell lines present heterogeneous morphologies and antigen expression profiles*

MON and DEV cell lines display similar genetic alterations leading to the absence of hSNF5/INI1 expression.[7,9] In contrast, they were established from different tumour localisations and exhibit distinct morphological appearance in culture (Fig. 1A). The MON cell line was established from abdominal MRT and grows homogeneously as small polygonal and poorly adherent cells. The DEV cell line was established from a medulloblastoma[24] and was subsequently shown to exhibit a non-sense heterozygous mutation (W51X) of hSNF5/INI1 associated with a loss of heterozygosity (LOH) other allele therefore linking this tumour to MRT.[9] DEV cells grow heterogeneously as clusters of small hexagonal cells associated with more dispersed cells exhibiting membrane extensions.

In order to investigate and compare the phenotype of MON and DEV cell lines, particularly in relationship with differentiation states, we performed extensive FACS analysis of antigen expression summarised in Table 1. These antigens were chosen according to their mesenchymal, epithelial or stem cells specific expression. Among this last category, CD34 antigen, tested as a stem cell marker, was strongly expressed on MON cells but not on DEV cells. CD117 antigen, the c-KIT receptor for stem cell factor (SCF) involved in development and survival of a variety of primitive cells, was present on 10% of MON cells whereas DEV cells were negatives. Among more mature markers, subpopulation of these two cell lines were positives for CD68, a lysosomal associated membrane protein (LAMP) involved in endocytosis pathway and highly expressed in macrophages. CD83 which labels dendritic cells was positive in a subpopulation (14%) of MON cells, and CD44 antigen was highly expressed in 100% of MON cells. Contrarily these two markers were not expressed on DEV cells. ASMA isoform and BSP were expressed on these two cell lines. Hepatocyte Nuclear Factor 3β (HNF-3β), a mature marker of the hepatocyte lineage, was expressed in MON cells but not in DEV cell line.

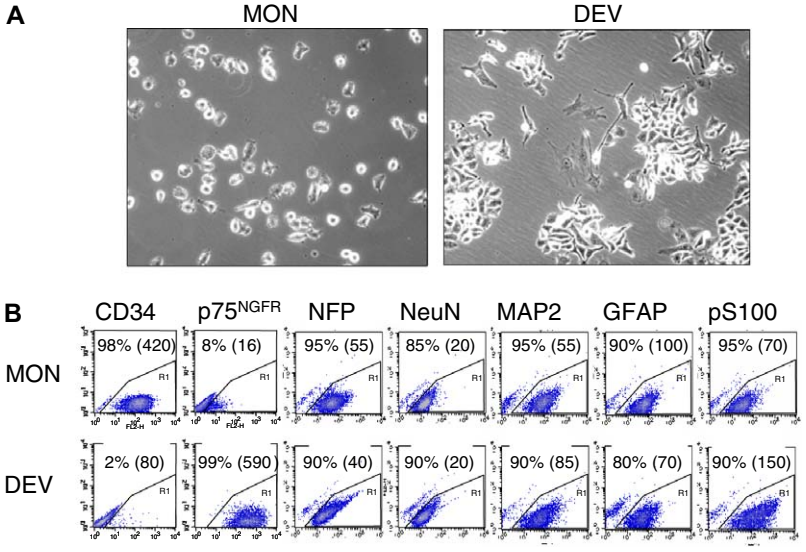

Fig. 1 – FACS analysis of antigen expression patterns on MON and DEV rhabdoid cell lines. (A) Light microscopy appearance (20×) of MON and DEV cell lines. (B) FACS analysis of markers expression. CD34 and p75^{NGFR} membrane antigen expressions were determined on non-permeabilised cells. NFP, NeuN, MAP2, GFAP and pS100 expressions were determined on triton permeabilised cells. FITC positive cells were gated (R1) as compared to isotypically matched control antibodies. The percentage (%) of positive cells and the mean of fluorescence intensity (MFI) are indicated. Each dot blot is representative of three independent experiments.

Interestingly, positivity for mesenchymal markers was associated with expression of EMA on sub-population of MON and DEV cells. Apart from vimentin which was highly expressed in these cells, cytokeratines 8 and 18, two cytoskeletal proteins essential for the development of epithelial cells, were expressed heterogeneously. Finally, the MON cell line exhibited positive staining for three endothelial markers (CD146 (S-endo), VEGFR2 (KDR), and CD31 (PECAM-1)) whereas DEV cells were clearly negatives.

Altogether these data showed that the expression of differentiation markers was highly heterogeneous from MON to DEV cell lines and also exhibited strong variations from one cell to the other within one cell line. Polyphenotypic traits with simultaneous positivity for both immature and differentiation markers were more pronounced for MON cells than for DEV cells.

3.2. MON and DEV cells co-express neuronal and glial markers

The nerve growth factor receptor $p75^{NGFR}$, a neural marker, was highly expressed in most DEV cells and more weakly in sup-populations of MON cells (Fig. 1B). In contrast to the differential expression of CD34 and $p75^{NGFR}$, other markers exhibited a remarkably similar level of expression between the two cell lines with significant expression of NFP, neuronal nuclei antigen (NeuN), MAP2 as well as GFAP and S100 protein, two antigens mainly expressed in cells of glial origin. Fig. 1 shows that more than 80% of MON and DEV cells co-expressed neuronal and glial markers, suggesting that these two cell lines may possess neuronal and/or glial differentiation potential. We therefore decided to investigate neuronal differentiation of MON and DEV cells.

3.3. Neuronal and glial differentiation potential of MON and DEV cells

In order to determine differentiation potential of MON and DEV rhabdoid cells in culture we performed differentiation-induction studies using different pharmacological agents such as All-*trans*-retinoic acid (ATRA), 12-O-tetradecanoyl phorbol-13-acetate (TPA), 1-β-D-Arabinofuranosylcytosine (Ara-C), and cAMP. Cells were incubated with various amounts of these inducers for different times. In these screening experiments we observed that the presence of 0.5 mM cAMP induced clear morphological differentiation of MON and DEV cells (Fig. 2A). These morphological changes were striking on MON cells, which produced numerous and long neurite-like processes in the presence of cAMP as compared to a round and polygonal morphology in control cultures. In the case of DEV cells, two different types of cells could be observed in control cultures: homogeneous colonies of polygonal cells without cytoplasmic extension, and more individual and dispersed cells with small neurite-like processes. In the presence of cAMP, all DEV cells harbored numerous and long neurite-like processes whereas polygonal cells were no longer observed.

These morphological observations were completed by FACS analysis of neuronal and glial markers, at day 5 of culture in the presence of cAMP as compared to control culture conditions (Fig. 2B). In MON cells, cAMP induced a strong increase of $p75^{NGFR}$ and MAP2 expression. The increase of NFP and NeuN was less pronounced. Glial markers were also slightly induced. Even more strikingly, DEV cells exhibited a strong induction of the four neuronal markers, and a significant decrease of glial antigens. These results indicated that the neuronal and glial phenotypes of rhabdoid cell could be modulated by cAMP and suggested that MON and DEV cells possess neuronal and/or glial potential.

3.4. hSNF5/INI1 expression leads to neuronal differentiation of DEV cells

We transfected expression vector encoding for tagged hSNF5/INI1 cDNA (HA-INI1) or corresponding empty vector (CT) into MON and DEV cells to determine the effect of hSNF5/INI1 expression on the differentiation potential of rhabdoid cells. We assayed the afore-indicated neuronal and glial markers expression patterns by FACS analysis after 5 days of culture. The transfection efficiency was determined by immunofluorescence staining using antibody against the HA tag, which reveal positive staining for HA-INI1 in 50% of the nuclei

Table 1 – FACS analysis of antigen expression on MON and DEV rhabdoid cell lines

	CD34	CD117	CD68	CD83	CD44	Asma[$]	BSP[$]	HNF3β[$]
MON	100% ++	10% +	5% +	14% +	100% ++	100% +	25% +	90% +
DEV	2% +	0	4% +	0	5% +	85% +	73% +	0

	EMA	Vim	CK8	CK18	CD146	VEGFr2	CD31
MON	23% +	100% ++	96% +	76% +	100% ++	24% ++	8% ++
DEV	85% ++	100% ++	50% +	28% +	0	0	0

For each marker, the percentage (%) of FITC positive cells and MFI (+ : MFI < 100; ++ : MFI > 100) of the positive population are indicated. ($) intracellular expression on permeabilised cells.

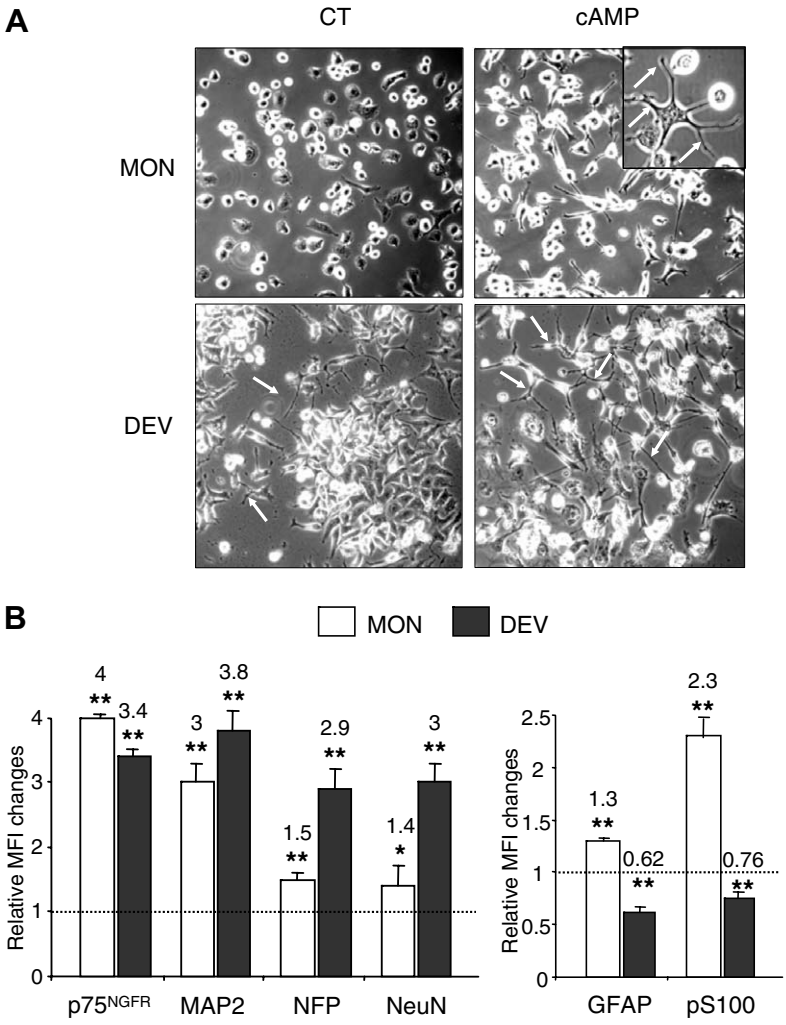

Fig. 2 – Effect of cAMP on neural and glial phenotype of MON and DEV cell lines. MON and DEV cells were grown for 5 days in control medium (CT) or in 0.5mM cAMP supplemented medium. (A) Light microscopy appearance (×40) of MON and DEV cells. Cytoplasmic extensions are indicated by white arrows. (B) FACS analysis of neuronal ($p75^{NGFR}$, MAP2, NFP, NeuN) and glial (GFAP and pS100) markers. Analyses were performed as described in Fig. 1. Values obtained for MON and DEV cells are represented by white and black bars respectively. Graphs represent the means of MFI fold changes of each marker in the presence of cAMP as compared to the MFI value of each marker in control medium (CT = 1, Relative MFI). Means of MFI fold changes and corresponding standard errors were obtained across three independent experiments and analysed using t-test. (*) p value <0.05. (**) p value <0.001.

(Fig. 3A). In DEV cells, hSNF5/INI1 expression increased the expression of neuronal markers such as MAP2, NFP and NeuN and clearly decreased GFAP and pS100 expression. In MON cells, the induction of neuronal markers and the decrease of glial markers by HA-INI1 were much less pronounced than in DEV cells (Fig. 3B).

The hypothesis of neural differentiation of DEV cells was further evaluated by the analysis of morphological changes upon hSNF5/INI1 expression (Fig. 4A). The numbers of cells with MAP2 positive cytoplasmic extensions together with the numbers of extensions per MAP2+ cell were strongly increased following hSNF5/INI1 expression (Fig. 4B). These morphological and quantitative changes, reminiscent of those observed in the presence of cAMP, were not observed with MON cells. These data clearly indicate that hSNF5/INI1 expression can induce an activation of the neurites formation process in DEV rhabdoid cells.

3.5. *hSNF5/INI1 knockdown inhibits neuronal differentiation of PC12 cells*

To evaluate the requirement of hSNF5/INI1 for neuronal differentiation, we assessed the effect of hSNF5/INI1 silencing upon differentiation of PC12 cells. The PC12 cell line, which was established from a rat pheochromocytome, constitutes a well-established cellular model of neuronal differentiation since the treatment of these cells with NGF in the presence of low serum induces neurites outgrowth and expression of neuronal markers. Differentiation experiments were conducted in the presence of either an hSNF5/INI1-specific siRNA

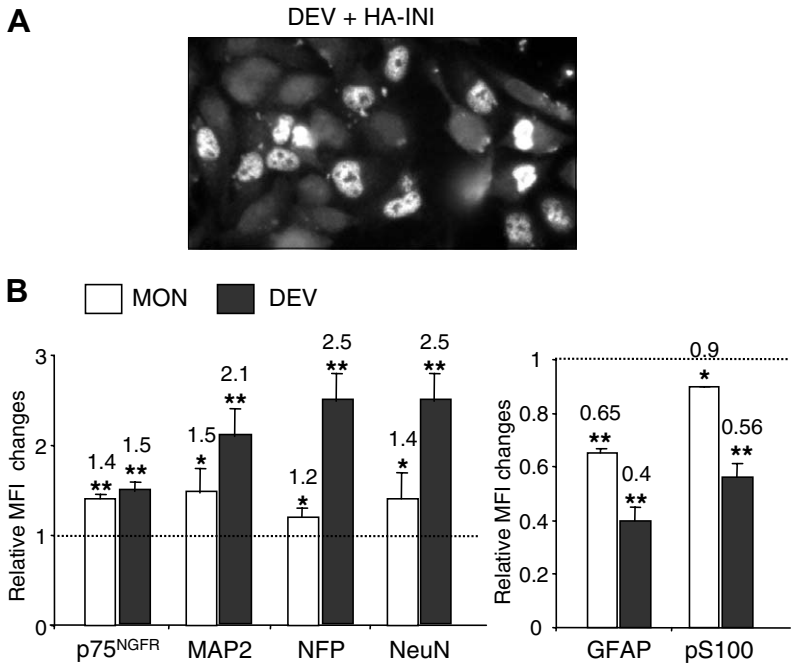

Fig. 3 – Effect of hSNF5/INI expression on neural and glial phenotype of MON and DEV cell lines. MON and DEV cells were transfected with empty vector (CT) or HA-tagged hSNF5/INI1 expression vector (HA-INI1) at day 0 and day 3, and induced to grow in control medium until day 5. (A) Immunofluorescence staining of HA-INI1 in transfected DEV cells at day 5. (B) FACS analysis of neuronal and glial markers. Analyses were performed as described in Fig. 1. Values obtained with MON and DEV cells are represented by white and black bars, respectively. Graphs represent the means of MFI fold changes of each markers in HA-INI1 transfected cells as compared to the MFI value of each markers in CT cells (CT = 1). Means of MFI fold changes and corresponding standard errors were obtained across three independent experiments and analysed using *t*-test. (*) *p* value <0.05. (**) *p* value <0.001.

(siR-INI) or a control scrambled siRNA (siR-CT). hSNF5/INI1 expression was not modified by control siR-CT throughout differentiation (Diff. D3 and D5, Fig. 5A). Contrarily, the specific siR-INI1 was able to knockdown hSNF5/INI1 expression with an almost complete disappearance of the hSNF5/INI1 protein (Fig. 5A). As expected, in the presence of siR-CT, NGF treatment of PC12 cells resulted in neurites formation and increased expression of MAP2 and NFP markers, as compared to undifferentiated cells (UD) (Fig. 5B and C). In contrast, the silencing of hSNF5/INI1 expression upon NGF treatment was associated with a decrease of the number and outgrowth of neurites and with a reduced induction of MAP2 and NFP expression (Fig. 5B and C). These results indicate that the knockdown of hSNF5/INI1 expression impairs the NGF-induced differentiation process of PC12 cells.

4. Discussion

We performed a morphological analysis and immunohistochemical characterisation of various MRT cell lines in culture and report here the results obtained for two of them, MON and DEV cell lines, which are demonstrative of the phenotypic heterogeneity of this group of tumours.

The expression of stem cells markers ($CD34^+$, $CD117^+$) suggests that MON cells possess stem cells properties. Expression of antigens specific to mature cells, such as CD68 and CD11c (macrophages), CD83 (follicular cells), ASMA (smooth muscle cells), BSP (osteocytes), CD44 and vimentin, strengthen the hypothesis of a mesodermic differentiation potential. The expression of endothelial markers raised the possibility that vasculogenic mimicry may play a role in malignant phenotype of MRT that remains to be tested.[26] Noteworthy, it would be of interest to study a possible correlation between CD146 and EMA expressions, two markers associated with tumour invasiveness, and MRT aggressiveness.[27,28] These results underline the polyphenotypic features of MON cells with positivity for markers from the three embryonic layers. This therefore suggests that the original cell from which derives this MRT has a very immature phenotype and that MON cells still retain some degree of differentiation potential along various lineages.

In contrast the phenotype of DEV cells appears much more restricted to the neural lineage since they do not express this variety of both mature and immature differentiation markers in different lineages. A number of reports have clearly documented the spontaneous or induced differentiation potential of the DEV cell line, which is derived from a medulloblastoma.[24,29,30] In particular, it was shown that this cell line, defined as a multipotent neural stem cell, could be induced to differentiate in the glial or neural lineage as a result of transfection with appropriate transcription factors.[30] We could recapitulate these experiments showing that cAMP treatment of DEV cells could trigger striking morphological changes and increase expression of $p75^{NGFR}$, MAP2, NFP and NeuN neuronal markers. In contrast, GFAP and pS100 astrocytic markers were decreased indicating a restriction of glial phenotype.

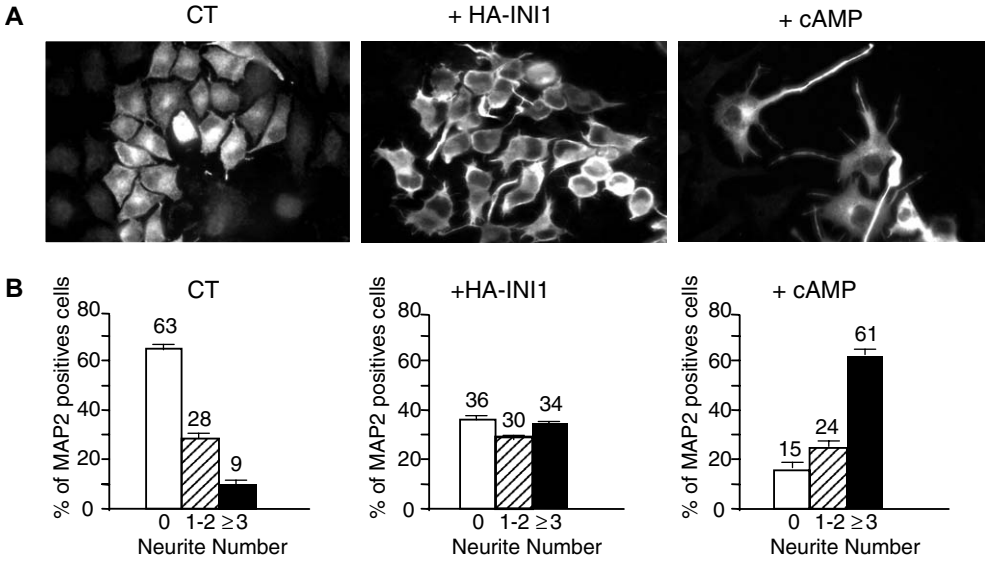

Fig. 4 – hSNF5/INI1 expression induces MAP2 positive neurites formation in DEV cells. (A) MAP2 immunostaining of DEV cells transfected with empty vector (CT) or HA-INI1 expression vector (+HA-INI1) and grown in control medium, or transfected with empty vector and grown in medium supplemented with cAMP (0.5 mM) until day 5. (B) Count of MAP2 positive neurite extension processes. DEV cells positive for MAP2 staining (MAP2+ cells) were counted on each coverslip and process extensions per MAP2+ cells were numerated. A neurite extension process was defined visually according to a length longer than that of cellular body. In each transfection and culture condition, the number of MAP2+ cells with 0 neurite, 1–2 (1 or 2) neurites, >3 (3 or more) neurites were numbered and are reported as percentage (%) of total MAP2+ cells in white, hatched and black bars respectively. Means of percentage values and corresponding standard errors across two independent experiments are shown.

The phenotype *of* hSNF5/INI1-transfected DEV cells indicates that hSNF5/INI1 expression is sufficient to increase neuronal markers expression and to decrease that of glial markers. It further suggests that, at least in DEV cells, hSNF5/INI1 acts as an instructive factor, at the branching point between neuronal or glial cell differentiation pathways.

MON cells only demonstrate a moderate increase of neural markers and decrease of glial markers upon treatment with AMPc or hSNF5/INI1 transfection. This difference between DEV and MON cells very probably reflects their different stage of differentiation. MON are more primitive cells and may require additional signals to achieve a neural differentiation.

To confirm the effect of hSNF5/INI1 in neural differentiation, we show that inhibition of hSNF5/INI1 expression, by specific siRNA in the rat PC12 cell line, is associated with the inhibition of neurites outgrowth and with a decreased expression of neuronal differentiation markers such as MAP2 and NFP.

Altogether, these results indicate that hSNF5/INI1 acts in the neural differentiation pathway. hSNF5/INI1 belongs to the SWI/SNF chromatin remodelling complexes, which have been shown to play a critical co-activator role in controlling gene expression, particularly during differentiation processes.[15,16] More particularly, recent findings involve BRG1, the catalytic subunit of the complex, in neural development and differentiation. Indeed, its expression is enriched in neural tissue during embryogenesis and Brg1 +/− heterozygous mice frequently display exencephaly, a neural tube abnormality.[31] Moreover, Brg1 mutant zebrafish have defects in terminal differentiation of retinal cells.[32,33] Very recently, Brg1 has been shown to be required for neuronal differentiation of mammalian P19 cells by mediating the transcriptional activities of proneural bHLH proteins.[34] In DEV or in hSNF5/INI1-inactivated PC12 cells, SWI/SNF-dependant regulation of neural transcription factors and, consequently, neural differentiation could be impaired by the absence of hSNF5/INI1. Contrarily, expression of hSNF5/INI1 would restore activity of these factors and trigger differentiation. In more primitive MON cells, the absence or reduced amount of these neural-specific transcription factors may limit the effects of hSNF5/INI1 expression. Further search for transcription factors with a differential expression between MON and DEV cells may enable this hypothesis to be tested. Besides these effects, hSNF5/INI1 role in differentiation may involve the remodelling of the cytoskeleton, possibly through the negative regulation of the activity of the RhoA protein.[25] Indeed, RhoA plays an important role in NGF-induced differentiation of PC12 cells as demonstrated by the ability of constitutively active forms of RhoA to impair neurite outgrowth in this system.[35]

More generally, the commitment of primitive multipotent stem cells to specific lineages is mediated by key transcription factors that activate downstream tissue specific subsets of genes in a temporally and spatially restricted manner. The dynamic assembly and disassembly of specialised chromatin structures determining the accessibility to transcription machinery is a key mechanism regulating this process. The immature and variable phenotype of rhabdoid cells together with the ability of hSNF5/INI1 to regulate differentiation suggest that, in addition to its roles in cell cycle activation and in

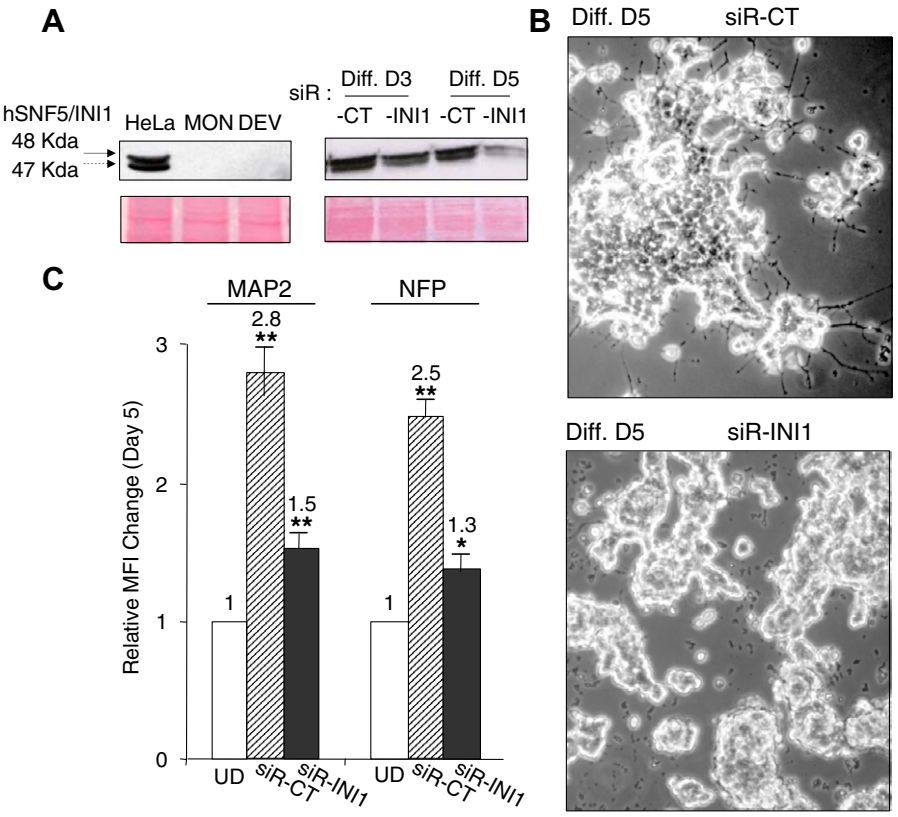

Fig. 5 – hSNF5/INI1 knockdown inhibits differentiation of PC12 cells. Differentiation of PC12 cells was induced from day 0 to day 5 in the presence of nerve growth factor (NGF) and low serum. Cells were transfected by control (siR-CT) or specific anti-hSNF5/INI1 (siR-INI1) siRNA at day 0 and day 3 of differentiation. (A) Western blot analysis of hSNF5/INI1 protein expression (both hSNF5/INI1 isoforms are detected). Staining of protein inputs is shown for each protein extract. Results from siR-CT and siR-INI1 treated cells at day 3 (Diff. D3) and day 5 (Diff. D5) of differentiation are shown. (B) Light microscopy appearance (×40) of siR-CT and siR-INI transfected PC12 cells at day 5 of differentiation (Diff. D5). (C) FACS analysis of MAP2 and NFP protein expression. Graph represents MFI fold changes for each marker. Values obtained for siR-CT (hatched bars) and siR-INI (black bars) transfected PC12 cells at day 5 of differentiation (Diff. D5) were compared to undifferentiated (UD) PC12 cells (white bars) used as control to define the baseline level of expression (relative MFI). Corresponding standard errors across three independent experiments are shown and analysed using *t*-test. (*) p value <0.05. (**) p value <0.001.

cytoskeleton reorganisation, hSNF5/INI1-deficiency plays a critical role in the inhibition of differentiation of rhabdoid cells. Further studies should document whether MRTs have a unique cellular origin but express variable phenotypic traits depending upon interaction with surrounding normal tissues or whether hSNF5/INI1 loss can transform various progenitor cells with distinct fates. This knowledge will be necessary to evaluate the potential of therapies based on the induction of differentiation in MRTs.

Conflict of interest statement

None declared.

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REFERENCES

1. Haas JE, Palmer NF, Weinberg AG, Beckwith JB. Ultrastructure of malignant rhabdoid tumor of the kidney. A distinctive renal tumor of children. *Hum Pathol* 1981;**12**(7):646–57.
2. Schmidt D, Harms D, Zieger G. Malignant rhabdoid tumor of the kidney. Histopathology, ultrastructure and comments on differential diagnosis. *Virchows Arch A Pathol Anat Histopathol* 1982;**398**(1):101–8.
3. Karnes PS, Tran TN, Cui MY, Bogenmann E, Shimada H, Ying KL. Establishment of a rhabdoid tumor cell line with a specific chromosomal abnormality, 46,XY,t(11;22)(p15.5;q11.23). *Cancer Genet Cytogenet* 1991;**56**(1):31–8.
4. Staehelin F, Bissig H, Hosli I, et al. Inv(11)(p13p15) and myf-3(MyoD1) in a malignant extrarenal rhabdoid tumor of a premature newborn. *Pediatr Res* 2000;**48**(4):463–7.

5. Parham DM, Peiper SC, Robicheaux G, Ribeiro RC, Douglass EC. Malignant rhabdoid tumor of the liver. Evidence for epithelial differentiation. *Arch Pathol Lab Med* 1988;**112**(1):61–4.
6. Fanburg-Smith JC, Hengge M, Hengge UR, Smith Jr JS, Miettinen M. Extrarenal rhabdoid tumors of soft tissue: a clinicopathologic and immunohistochemical study of 18 cases. *Ann Diagn Pathol* 1998;**2**(6):351–62.
7. Versteege I, Sevenet N, Lange J, et al. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* 1998;**394**(6689):203–6.
8. Sevenet N, Sheridan E, Amram D, Schneider P, Handgretinger O, Delattre O. Constitutional mutations of the hSNF5/INI1 gene predispose to a variety of cancers. *Am J Hum Genet* 1999;**65**(5):1342–8.
9. Sevenet N, Lellouch-Tubiana A, Schofield D, et al. Spectrum of hSNF5/INI1 somatic mutations in human cancer and genotype-phenotype correlations. *Hum Mol Genet* 1999;**8**(13):2359–68.
10. Biegel JA, Zhou JY, Rorke LB, Stenstrom C, Wainwright LM, Fogelgren B. Germ-line and acquired mutations of INI1 in atypical teratoid and rhabdoid tumors. *Cancer Res* 1999;**59**(1):74–9.
11. Guidi CJ, Sands AT, Zambrowicz BP, et al. Disruption of Ini1 leads to peri-implantation lethality and tumorigenesis in mice. *Mol Cell Biol* 2001;**21**(10):3598–603.
12. Roberts CW, Galusha SA, McMenamin ME, Fletcher CD, Orkin SH. Haploinsufficiency of Snf5 (integrase interactor 1) predisposes to malignant rhabdoid tumors in mice. *Proc Natl Acad Sci USA* 2000;**97**(25):13796–800.
13. Klochendler-Yeivin A, Fiette L, Barra J, Muchardt C, Babinet C, Yaniv M. The murine SNF5/INI1 chromatin remodeling factor is essential for embryonic development and tumor suppression. *EMBO Rep* 2000;**1**(6):500–6.
14. Roberts CW, Leroux MM, Fleming MD, Orkin SH. Highly penetrant, rapid tumorigenesis through conditional inversion of the tumor suppressor gene Snf5. *Cancer Cell* 2002;**2**(5):415–25.
15. Pedersen TA, Kowenz-Leutz E, Leutz A, Nerlov C. Cooperation between C/EBPalpha TBP/TFIIB and SWI/SNF recruiting domains is required for adipocyte differentiation. *Genes Dev* 2001;**15**(23):3208–16.
16. de la Serna IL, Carlson KA, Imbalzano AN. Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. *Nat Genet* 2001;**27**(2):187–90.
17. Strobeck MW, Knudsen KE, Fribourg AF, et al. BRG-1 is required for RB-mediated cell cycle arrest. *Proc Natl Acad Sci USA* 2000;**97**(14):7748–53.
18. Zhang HS, Gavin M, Dahiya A, et al. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* 2000;**101**(1):79–89.
19. Versteege I, Medjkane S, Rouillard D, Delattre O. A key role of the hSNF5/INI1 tumor suppressor in the control of the G1-S transition of the cell cycle. *Oncogene* 2002;**21**(42):6403–12.
20. Betz BL, Strobeck MW, Reisman DN, Knudsen ES, Weissman BE. Re-expression of hSNF5/INI1/BAF47 in pediatric tumor cells leads to G1 arrest associated with induction of p16ink4a and activation of RB. *Oncogene* 2002;**21**(34):5193–203.
21. Zhang ZK, Davies KP, Allen J, et al. Cell cycle arrest and repression of cyclin D1 transcription by INI1/hSNF5. *Mol Cell Biol* 2002;**22**(16):5975–88.
22. Ae K, Kobayashi N, Sakuma R, et al. Chromatin remodeling factor encoded by ini1 induces G1 arrest and apoptosis in ini1-deficient cells. *Oncogene* 2002;**21**(20):3112–20.
23. Reincke BS, Rosson GB, Oswald BW, Wright CF. INI1 expression induces cell cycle arrest and markers of senescence in malignant rhabdoid tumor cells. *J Cell Physiol* 2003;**194**(3):303–13.
24. Giraudon P, Dufay N, Hardin H, Reboul A, Tardy M, Belin MF. Differentiation of a medulloblastoma cell line towards an astrocytic lineage using the human T lymphotropic retrovirus-1. *Neuroscience* 1993;**52**(4):1069–79.
25. Medjkane S, Novikov E, Versteege I, Delattre O. The tumor suppressor hSNF5/INI1 modulates cell growth and actin cytoskeleton organization. *Cancer Res* 2004;**64**(10):3406–13.
26. Hendrix MJ, Seftor EA, Hess AR, Seftor RE. Vasculogenic mimicry and cell-cell plasticity: lessons from melanoma. *Nat Rev Cancer* 2003;**3**(6):411–21. Review.
27. Xie S, Luca M, Huang S, et al. Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. *Cancer Res* 1997;**57**(11):2295–303.
28. Yan X, Lin Y, Yang D, et al. A novel anti-CD146 monoclonal antibody, AA98, inhibits angiogenesis and tumor growth. *Blood* 2003;**102**(1):184–91.
29. Derrington EA, Dufay N, Rudkin BB, Belin MF. Human primitive neuroectodermal tumor cells behave as multipotent neural precursors in response to FGF2. *Oncogene* 1998;**17**(13):1663–72.
30. Buzanska L, Spassky N, Belin MF, et al. Human medulloblastoma cell line DEV is a potent tool to screen for factors influencing differentiation of neural stem cells. *J Neurosci Res* 2001;**65**(1):17–23.
31. Bultman S, Gebuhr T, Yee D, et al. A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. *Mol Cell* 2000;**6**(6):1287–95.
32. Gregg RG, Willer GB, Fadool JM, Dowling JE, Link BA. Positional cloning of the young mutation identifies an essential role for the Brahma chromatin remodeling complex in mediating retinal cell differentiation. *Proc Natl Acad Sci USA* 2003;**100**(11):6535–40.
33. Link BA, Fadool JM, Malicki J, Dowling JE. The zebrafish young mutation acts non-cell-autonomously to uncouple differentiation from specification for all retinal cells. *Development* 2000;**127**(10):2177–88.
34. Seo S, Richardson GA, Kroll KL. The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. *Development* 2005;**132**(1):105–15.
35. Nusser N, Gosmanova E, Zheng Y, Tigyi G. Nerve growth factor signals through TrkA, phosphatidylinositol 3-kinase, and Rac1 to inactivate RhoA during the initiation of neuronal differentiation of PC12 cells. *J Biol Chem* 2002;**277**(39):35840–6.