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The doppel (Dpl) protein influences *in vitro* migration capability in astrocytoma-derived cells

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Abstract. Doppel (Dpl) protein is the paralogue of the cellular prion (PrP^C) protein. In humans, Dpl is expressed almost exclusively in testis where it is involved in spermatogenesis. Recently, the protein has been described to be ectopically expressed in astrocytomas and its potential association to the brain tumor malignancy progression has been advanced. In this study, we aimed to investigate *in vitro* the potential involvement of Dpl in the tumor cell migration: to this purpose, Dpl expression was reduced in the IPDDC-A2 astrocytoma-derived cell line, by means of antisense and siRNA approaches; migration rates were then evaluated by means of a scratch wound healing assay. As a result, the cellular migration was sensibly reduced after Dpl silencing. Following a complementary approach, in HeLa cells, showing very low endogenous Dpl expression, the protein expression was induced by transfection and stabilization of an eukaryotic expression vector containing the doppel gene coding sequence. These stably Dpl-overexpressing cells revealed a significant increase in the migration rate, compared to untreated and control cells. In addition, Dpl-forced expression induced substantial changes in the cell morphology. Of note, in these cells, viability examination by means of tetrazolium-based assay did not reveal differences in the proliferation; on the contrary, a variation in density-dependent growth, leading to an increase of cell contact inhibition was highlighted. These results, in conclusion, might suggest a potential and functional role for Dpl in tumor cells migratory and morphological behaviours and address to future gene-targeted therapeutic interventions.

Keywords: Prion-like proteins, glial tumor, IPDDC-A2, HeLa, siRNA, antisense oligonucleotide, gene-silencing, migration scratch assay

Abbreviations

CNS:	Central nervous system,
D-MEM:	Dulbecco's modified eagle's medium,
GPI:	Glycosyl-phosphatidyl-inositol,
IRES:	Internal ribosome entry site,
MTS:	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphofenyl)-2H-tetrazolium,
NMR:	Nuclear magnetic resonance,
S-ODN:	Phosphothioate-oligonucleotide,
PBS:	Phosphate buffered saline,
SD:	Standard deviation,
SDS-PAGE:	Sodium dodecyl sulphate-polyacrilamide gel electrophoresis,
siRNA:	Short interfering RNA,
WHO:	World health organization.

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1. Introduction

The majority of malignant brain tumors is derived from glial cells and most of them display an astrocytic component, but the high degree of heterogeneity often makes it difficult to determine the cell of origin [23,24]. Four malignancy grades are accepted by the WHO, from grade I, the biologically least aggressive, to the grade IV tumors (glioblastoma), the most aggressive [30]. Despite advances in clinical and molecular fields, high grade astrocytomas remain fatal, particularly because of striking rates of *de novo* and acquired resistance to therapeutic intervention [7]. Up to date, no therapy has been able to give tangi-

ble improvements in the prognosis and the survival of patients with this pathology is often less than one year [44,46]. Malignant astrocytomas, like most aggressive cancers, present aberrant proliferation, diminished apoptosis, escaping from both growth control and immuno-regulation [23]. Moreover, these tumors exhibit a characteristic ability to infiltrate healthy brain tissue and form satellite tumors. To this regard, many genes have been identified to be associated with astrocytic tumors migration [15,40]. This migration capacity makes these tumors exceedingly difficult to treat and even after resection, invasive cells can give rise to tumors within centimeters of the resection site [15].

In the recent past, the first prion-like protein gene, doppel (*PRND*), was described in rodents, cattle, sheep, goat and human [28,36,38,51,53]. In 2004, we first reported an altered expression of this gene in brain tumors, and we hypothesized that doppel might be considered as an astrocytic tumors progression marker [10]. Doppel gene product, Dpl, is a paralogue of the cellular prion protein (PrP^C), the main causative agent of spongiform encephalopathies, but it seems not be involved in these diseases [52]. Human Dpl protein is composed of 126 aminoacids, GPI-anchored to the cytoplasmic membrane, highly glycosylated and NMR analysis confirmed a carboxy-terminal domain composed of three α-helices and two β-sheets [31]. Dpl, differently from PrP^C that is widely expressed in different tissues, is described only in adult testis where it is involved in spermatogenesis and fertilization capability [6,33,41]. As we recently documented, Dpl is ectopically expressed in brain tumors; additionally, *PRND* mRNA expression was associated with the glial tumor malignant transformation [11]. Furthermore, in these tumors, the transcript showed a nuclear retention and the protein product was subjected to an unusual glycosylation; finally, Dpl failed to be GPI-anchored at the astrocytic tumor cellular membrane and it was mainly localized in the cellular cytoplasm [8].

In this work, an *in vitro* functional analysis was performed to clarify the role of the doppel gene product in regulating cell migration in human brain tumors.

2. Materials and methods

2.1. Cell lines and culture reagents

Human astrocytoma-derived IPDDC-A2 and cervical carcinoma HeLa cell lines (ECACC, Salisbury, UK) were employed. Cells were maintained at 37°C, 5% CO_2 in D-MEM supplied with 10% fetal bovine serum, 1% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture media and supplements were provided by Invitrogen (Carlsbad, CA, USA). For transfectants selection, puromycin (Sigma-Aldrich, St. Louis, MO, USA) was employed.

2.2. siRNA, antisense DNA oligonucleotides and treatments

Three *PRND*-specific siRNA molecules were purchased from Ambion (Austin, TX, USA). Their sequences were: *Silencer*® siRNA ID# 133939-sense 5′-GCA AAU CCU GGC AAG UGA Ctt-3′ and 133939-antisense 5′-GUC ACU UGC CAG GAU UUG Ctc-3′, ID# 133940-sense 5′-GCC UAA UGA AGG CCA UCA Utt-3′ and 133940-antisense 5′-AUG AUG GCC UUC AUU AGG CTG-3′, ID# 133941-sense 5′-CCA CCU UAU UAG CUA AAA Att-3′ and 133941-antisense 5′-UUU UUA GCU AAU AAG GUG Gtg-3′. A *GAPDH*-specific *Silencer*® siRNA was employed as control (ID# 4624).

Two antisense DNA oligonucleotides (ODN), fully phosphorothioated and HPLC-purified, were provide by Sigma®-Genosys. *PRND*-specific antisense S-ODN sequence was 5′-ATG AGG AAG CAC CTG AGC TG-3′ and *GAPDH*-specific antisense S-ODN sequence was 5′-CCG ACC TTC ACC TTC CCC AT-3′, both complementary to the first twenty nucleotides of their respectively gene coding sequences.

Transfection reactions were carried out using Lipofectamine™ 2000 reagent (Invitrogen) on a cells monolayer of about 70% confluence previously plated on 30 mm Petri dishes (Corning, New York, NY, USA) and, after respectively 18 and 24 h, migration assay and western blot analysis were performed. Briefly, the *PRND*-specific siRNAs were co-transfected, each one at the final concentration of 0.10 μM, in presence of 5.0 μl of transfection reagent; antisense S-ODNs were transfected at the final concentration of 1.57 μM with 2.5 μl of liposome reagent. Antibiotics-free medium was employed.

2.3. Plasmids construction

For overexpression experiments, pIRESpuro3 plasmid (Clontech Laboratories Inc., Palo Alto, CA, USA) was employed. The human doppel protein coding sequence was amplified from genomic DNA (Clontech Laboratories Inc.) using the following primers: Dpl(EcoRI)-U, 5′-CCG GAA TTC ATG AGG AAG CAC CTG AGC TG-3′ and Dpl(NotI)-L, 5′-TGC GGC

CGC TTA TTT CAC CAT GAG CCA GAT CA-3′; the PCR product was then *Eco*RI/*Not*I-cloned into the plasmid, thus originating the pIRESpuro3-*PRND* construct. As control, the pIRESpuro3-*PRNP* construct was prepared as follow: the human prion protein coding sequence was amplified from genomic DNA (Clontech Laboratories Inc.) employing the following primers: PrP(*Eco*RI)-U, 5′-CCG GAA TTC ATG GCG AAC CTT GGC TGC-3′ and PrP(*Not*I)-L, 5′-ATT TGC GGC CGC TTA TCA TCC CAC TAT CAG GAA GAT GA-3′. The PCR product was then *Eco*RI/*Not*I-digested and cloned into the pIRESpuro3. PCR thermal profile was: 31 cycles at 94°C × 15 s, 62°C × 30 s and 72°C × 60 s.

2.4. Generation of stably transfected HeLa cells

HeLa cells were transfected using Lipofectamine™ 2000 reagent (Invitrogen), as described in the manufacturer's protocol, with, respectively, pIRESpuro3 empty vector, pIRESpuro3-*PRND*, pIRESpuro3-*PRNP* and both constructs pIRESpuro3-*PRND* and pIRESpuro3-*PRNP*. Briefly, 10^5 cells were plated in 24-wells plate; 24 h later, 1 μg of the construct of interest was transfected with 2 μl of Lipofectamine™ 2000 and, after a 24 h-incubation time, puromycin (Sigma-Aldrich) at a final concentration of 1 μg/ml was added. Transfection efficiency was evaluated by means of the pEGFP-N1 plasmid (Clontech Laboratories Inc.), transfected independently, and evaluating the originated green-fluorescent cells. The stable cell lines were isolated, by sub-culturing and selecting with 1 μg/ml of puromycin for 1 month, prior of performing the experiments.

2.5. Western blot analysis and antibodies employed

For immuno-blot analysis, cells monolayer was washed twice with 37°C pre-warmed phosphate-buffered saline (PBS), trypsinized and the pellet was lysed with ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5% sodium deoxycholate, 1% Nonidet P-40 (Roche, Basel, Switzerland), 0.1% sodium dodecyl sulphate) by a 30-min incubation on ice; cell debris was removed by centrifugation at 13,000 rpm for 12 min at 4°C and supernantant was recovered and supplemented with Complete™ Mini protease inhibitor cocktail (Roche). Protein samples were quantified by Qubit™ fluorometer (Invitrogen) following manufacturer's instructions. 100 μg of total protein extract was boiled in Laemmli sample buffer (2% SDS (w/v), 6% glycerol (v/v), 150 mM β-mercaptoethanol, 0.02% bromophenol blue (w/v) and 62.5 mM Tris-HCl pH 6.8). Proteins were blotted onto nitro-cellulose membrane Hybond™-C Extra (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Membranes were blocked with 2% non-fat milk in PBS containing 0.1% Tween® 20 (v/v). The following antibodies were employed for immuno-detection: rabbit polyclonal anti-human Dpl (kindly provided by Prof. Valentini), mouse monoclonal anti-human PrP^C (3F4, Sigma-Aldrich) and mouse monoclonal anti-*GAPDH* (Ambion), used at 1:10,000 dilution. The blots were then treated with species-specific peroxidase-coupled secondary antibodies (1:10,000, Amersham Biosciences) and protein signals were revealed by the "ECL Advance™ Western Blotting Detection Kit" (Amersham Biosciences).

2.6. In vitro scratch wound healing assay

The ability of cells to migrate in monolayer cultures was assessed by a scratch assay on 30-mm Petri dishes (Corning). For transient transfection experiments in IPDDC-A2 cells, eighteen hours after siRNA or S-ODN treatments, the assay was performed as follows. Cells, at a confluence of about 95%, were rinsed once with PBS and replaced with new complete medium. At the same time, three parallel lines were drawn with a permanent marker on the bottom of the dishes as reference and three parallel wounds were scraped, perpendicularly to these lines on the cells monolayer, by a sterile plastic micropipette tip: to measure the migratory capacity of the cells into the scrape wounds, nine points of reference were taken for each sample for each interval time (0–2–4–6–8 h). Microscopic photographs were taken where each wound crossed a marker line, first immediately after scrapings and then at the indicated time intervals. The migration distances were then deduced from the comparison of the t_0 (0 h) and the next intervals photographs and expressed in arbitrary units.

For the stably transfected cell lines, the day before, cells were plated in order to obtain approximately a 95% confluence at the start point of the assay. Cells were then rinsed once with PBS to remove cellular debris and replaced with new medium supplemented with 1 μg/ml puromycin and the scratch assay was performed as described above. For each plasmid construct, two different stable cell lines were tested.

For the transient transfection of S-ODN molecules in stably Dpl-expressing HeLa cells, seventeen hours after the treatment at a confluence of about 95% in

antibiotics-free medium (except 1 μg/ml of puromycin), the scratch assay was performed as previously described.

Morphology analysis and photographs were made using the inverted microscope Nikon Eclipse TS100 (Nikon, Tokyo, Japan).

2.7. Cell viability and cell growth assays

To test the cellular viability, an MTS-based assay, the "CellTiter 96® Aqueous One Solution Cell Proliferation Assay" (Promega, Madison, WI, USA) was employed. Cells were plated into 96-wells flat-bottom plate in triplicate at a density of 10^4 cells per well. First readouts were measured 24 h after plating and then at intervals of 24 h, following the manufacturer's instructions. Readouts were measures with a microplate reader (Titertek Multiskan, Huntsville, AL, USA) at 492 nm wavelength.

Growth curves were made employing a 96-wells plate: similarly, stably Dpl-expressing HeLa cells were plated at a density of 10^4 per well in triplicates. The cells were incubated up to 8 days: at intervals of 24 h, cells were trypsinized and counted by a hemocytometer.

2.8. Software employed and statistical analysis

The software employed for densitometric analysis was the GelixOne version 1.2.1 (Biostep GmbH, Jahnsdorf, Germany). Statistical analysis and graphs design were performed with Office Excel (Microsoft, Redmond, WA, USA); figures were processed with Illustrator® 10.0 (Adobe Systems Inc., San Jose, CA, USA). Data are expressed as mean ± SD of two independent experiments. The results were analyzed using the Student's t test; p value inferior to 0.05 was considered significant.

3. Results

Astrocytoma-derived cell line IPDDC-A2 (previously characterized for doppel expression in [8,14]) was transiently transfected with *PRND*-specific siRNA and antisense S-ODN molecules at a final concentration of 0.30 and 1.57 μM, respectively; immunoblotting analysis, carried out 24 h after transfection, showed a decreased Dpl protein expression of the previously described Dpl isoforms (Fig. 1(a)). Specifically, a more defined Dpl knockdown effect at this interval was obtained after S-ODN-treatment. A densitometrical analysis of the 30 kDa Dpl electrophoretic band, widely conserved in protein extracts from different tumor cells, in S-ODN- and siRNA-treated samples reported, respectively, an intensity of 35% and 49%, compared to the untreated sample (Fig. 1(a)).

To verify whether the doppel expression disruption was able to influence cell migration *in vitro*, we performed a scratch assay in IPDDC-A2 cells subjected or not to a *PRND* silencing. Since a strong reduction of Dpl was observed at 24 h after transfection with siRNA and S-ODN molecules, experiments addressing cellular migratory capacity were carried out accordingly. As shown in Fig. 1(b), both silencing techniques induced a sensitive decrease in the cell motility. As a negative control, validated *GAPDH*-specific siRNA and *GAPDH*-antisense S-ODN molecules were assayed: these interfering treatments did not influence cells migration ability. In particular, siRNA treatment showed the phenotipic effect earlier than S-ODN treatment, as shown in Fig. 1(c). The average migration rate varied from about 60 μm/h, in untreated and *GAPDH*-transfected cells, to approximately 40 μm/h, in Dpl-silenced cells. Finally, Fig. 1(b) shows some snapshots of the cells treated with the Dpl-silencing molecules respect to the not treated cells.

To extend the Dpl specific effect on the migrating phenotype, a different tumor cell line was assayed. HeLa cells, that showed a very low Dpl protein expression as reported in Fig. 2(a), have been stably transfected with the eukaryotic expression vector pIRESpuro3 (Clontech Laboratories Inc.) containing the *PRND* gene coding sequence. As shown in Fig. 2(a), Dpl protein displayed a heterodisperse intense band respects to the cell line stably selected for pIRESpuro3 plasmid alone and to the untreated cells. In addition, the *PRNP* gene coding sequence was cloned into the pIRESpuro3 plasmid and stably transfected HeLa cells overexpressing PrP^C protein, as shown in Fig. 2(a), were then assayed for migration capability. As reported in Fig. 2(c), the graph indicated an increase migration rate for Dpl-expressing cells (about 17 μm/h), while untreated cells, empty plasmid-selected cells and PrP^C-expressing cells exhibited a similar migration capability, significantly lower than Dpl-expressing cells (7 μm/h). Interestingly, stably expressing PrP^C and Dpl proteins cells, showed a migration rate similar to the Dpl transfectants (Fig. 2(c)). Finally, Fig. 2(b) showed photographs of Dpl-expressing and untreated cells, 24 h after performing the scratch.

Furthermore, stable Dpl transfectants were subjected to a treatment with the *PRND*-specific S-ODN

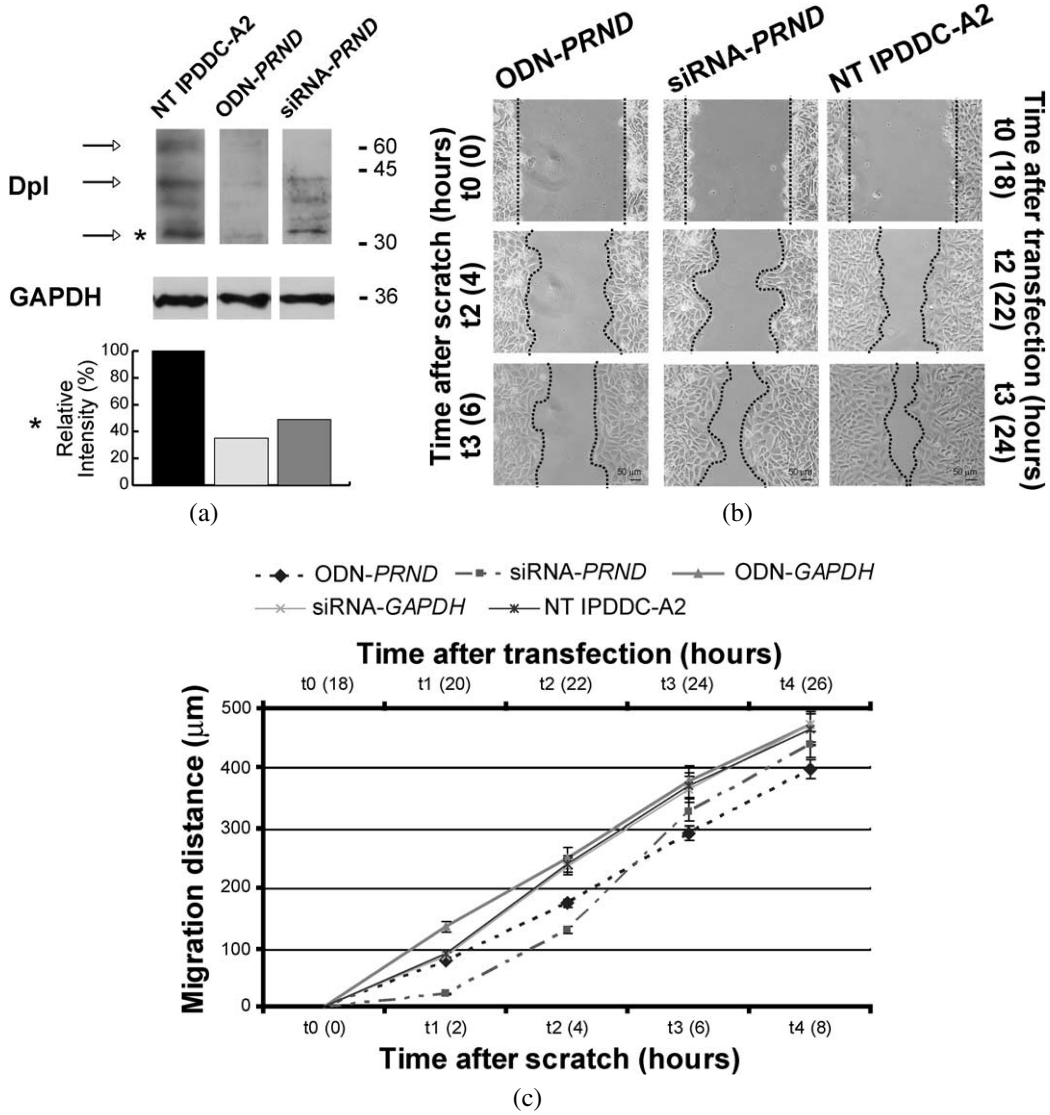

Fig. 1. IPDDC-A2 cells treatment with Dpl-silencing molecules and migration rate curves. (a) Doppel (Dpl) and GAPDH immuno-blot analysis of Dpl-silenced cell lysates, 24 h after transfection with *PRND*-specific S-ODN (ODN-*PRND*) and siRNA (siRNA-*PRND*), revealed an expression decrement of the three Dpl major isoforms (indicated by the arrows). The histogram reports the percentage relative intensities of the 30 kDa band (*), taken as representative band, normalized to the untreated sample (NT IPDDC-A2). Bands molecular weights are indicated in kDa. (b) The snapshots of the cell monolayer scratches indicate the higher migration rate of the untreated cells respects to the Dpl-silenced cells, respectively, at 4 h after scrape (i.e. 22 h after transfection) and at 6 h after scrape (i.e. 24 h after transfection). Scale bars are reported (50 μm). (c) The graphical representations of the migration curves show that both S-ODN (ODN-*PRND*) and siRNA (siRNA-*PRND*) treatment influences the migratory behaviour of IPDDC-A2 cells reducing the migration capability, while *GAPDH*-specific silencing treatment (ODN-*GAPDH* and siRNA-*GAPDH*) does not affect migration, being similar to the untreated control (NT IPDDC-A2). In particular, siRNA-*PRND* treatment shows the phenotypic effect earlier than ODN-*PRND* treatment, while an inverted trend is observed 5 h after scratch (i.e. 23 h after transfection). Statistical analysis was performed by Student's t test ($p < 0.05$).

molecules: a partial rescue of the migration rate of these cells was observed, respect to the untreated and to the *GAPDH*-specific S-ODN treated cells (Fig. 3).

Of note, average migration rate of untreated IPDDC-A2 cells on the Petri plastic support was much more higher than untreated HeLa, respectively 43 ± 0.1 μm/h and 7 ± 0.1 μm/h.

Dpl stably expressing HeLa cells were analyzed for cellular parameters such as viability, growth and morphology. As reported in Fig. 4(a), the viability assay

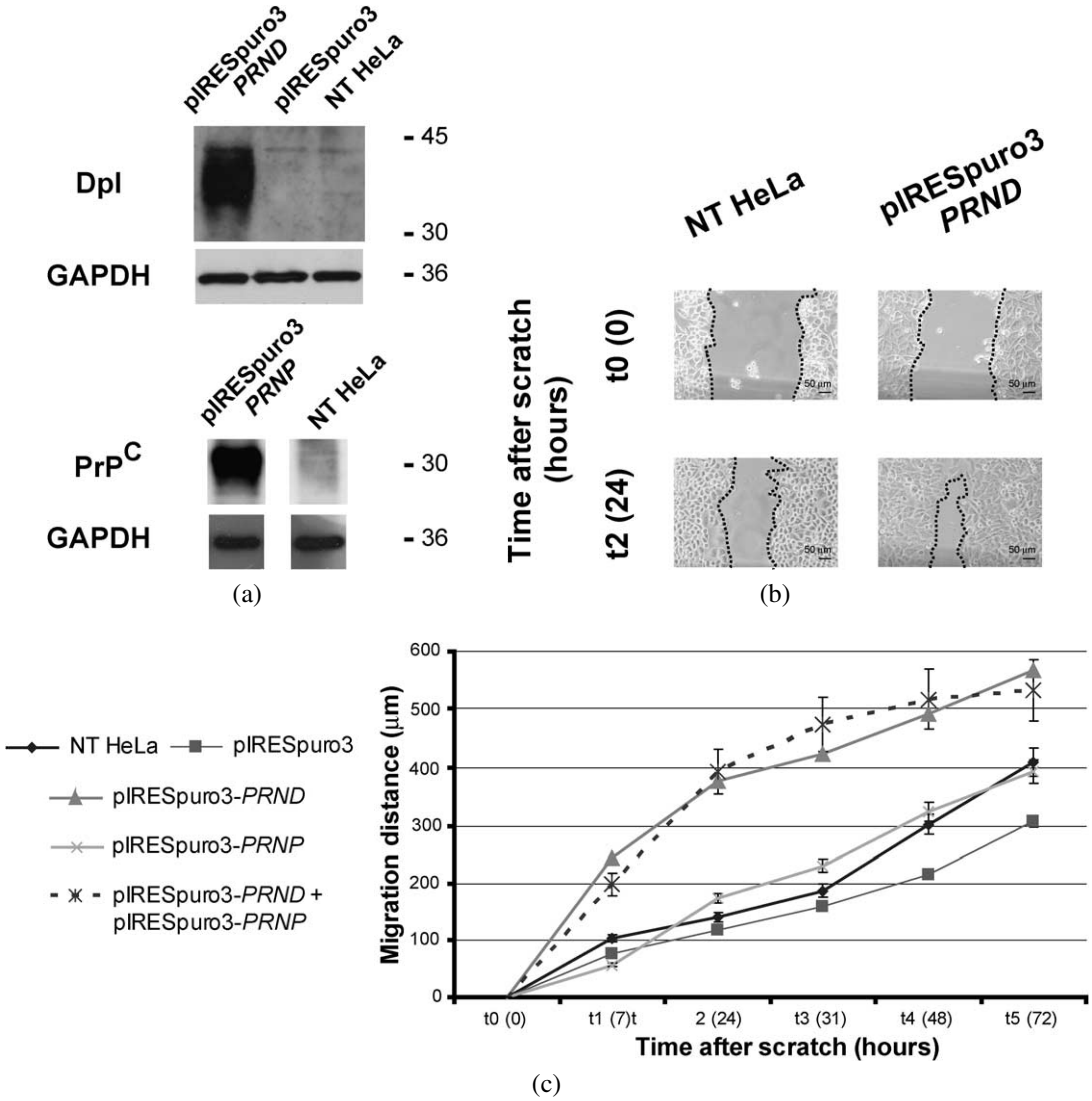

Fig. 2. Stably selected HeLa cells and migratory behaviour analysis. (a) Western blot analysis of HeLa cells overexpressing Dpl (pIRESpuro3-*PRND*) and PrP^C (pIRESpuro3-*PRNP*) respects to the empty-vector transfectants (pIRESpuro3) and the not-transfected (NT HeLa) controls. (b) The snapshots of the cell monolayer scratches indicate the increased migration rate for the overexpressing HeLa (pIRESpuro3-*PRND*) respects to the untreated cells (NT HeLa), observed at 24 h after scratch. Scale bars are reported (50 µm). (c) The migration rate curves of the stably transfected HeLa cells expressing Dpl (pIRESpuro3-*PRND*) or co-expressing both Dpl and PrP^C (pIRESpuro3-*PRND* + pIRESpuro3-*PRNP*) proteins show a higher value respect to, respectively, untreated (NT HeLa), empty vector transfectants (pIRESpuro3) and PrP^C expressing cells (pIRESpuro3-*PRNP*). Statistical analysis was performed by Student's t test ($p < 0.05$).

carried out with a tetrazolium-based method, showed similar curves for all the samples (correlation coefficients average $r = 0.99 \pm 0.012$), within the first 72 h. Figure 4(b) reported HeLa growth curves that were similar for untreated, for empty vector-transfectants and for stably expressing PrP^C cells. On the contrary, Dpl overexpressing cells showed a modified growth curve, particularly at the plateau phase, measurably lower than expected. Cells expressing both Dpl and PrP^C proteins showed a growth curve similar to the control. With regard to morphological aspects reported in Fig. 4(c), Dpl- and Dpl/PrP^C-expressing cells showed relevant alterations in the cellular shape: the typical squared shape of HeLa cells, resulting after empty vector and *PRNP* transfection, changed towards a lengthened and flattened forms in Dpl- and Dpl/PrP^C-expressing cells. In particular, the average length of Dpl-expressing cells varied from 75 to 175 µm com-

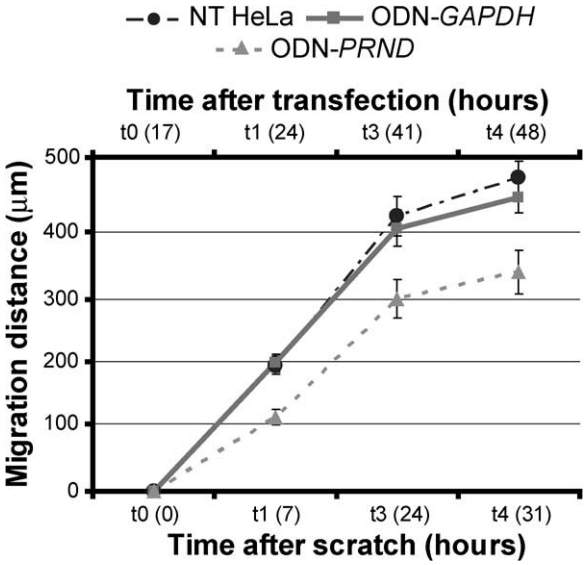

Fig. 3. Migration rate rescue in Dpl-expressing HeLa cells. The *PRND*-specific S-ODN treatment of the stably Dpl-expressing HeLa cells reduced their migration capability respects to untreated (NT HeLa) and to *GAPDH*-specific S-ODN treated cells (ODN-*GAPDH*). Statistical analysis was performed by Student's t test ($p < 0.05$).

pared to the controls (50–75 μm length); moreover, the nuclei underwent a significant dimension increase from about 25–30 μm to 37.5–40 μm in diameter, respectively.

4. Discussion

Astrocytomas are the most common CNS neoplasms; the accepted tumor grading is composed by four classes (WHO I–IV) according to the histological characteristics, but many efforts are performed, at the molecular level, to improve this classification [37]. We have recently proposed a potential novel marker, the doppel gene, *PRND*, the expression of which correlates with the astrocytoma malignancy [10,11]. *PRND* encodes for the Dpl protein, the first discovered paralogue of the cellular prion protein, PrP^C, the agent involved in spongiform encephalopathy diseases [38,43]. Dpl is expressed almost entirely in adult testis where it plays a role in spermatogenesis and in male fertilization but, as we described, it was ectopically and consistently up-regulated in brain tumors [6,9,41]. In this pathological context, some biological variations occurred respects to the physiological conditions: its transcript was retained in the nucleus, underwent an alternative splicing event and might be subjected to post-transcriptional modifications similarly to other genes involved in glioma progression [29]; moreover, the gene product failed to be GPI-anchored to the cytoplasmic membrane and it finally localized in the cytoplasm, undergoing a heavy and unexpected glycosylation [8].

Up to now, our investigations about doppel in the tumor context failed to trace a hypothetical functional role [1,2]. In this work, we analyzed the involvement of Dpl into one of the typical hallmarks of cancer, cell migration. Cells movement is a complex mechanism that is subjected to a rigid control by several genes during various stages of embryonic and adult developmental processes; during carcinogenesis, the motile behaviour reappears without usual cellular controls and this causes tumor cells spreading throughout the healthy tissue [45]; moreover, cancer cells motility plays a primary role in tumor invasion and metastasis formation [57]. In particular, in astrocytomas, infiltration ability of tumor cells constitutes one of the main causes of the unfortunate prognosis for these malignancies and it explains the intensive efforts in aiming the development of novel therapies [27,44]. Several key proteins are described as effectors of migratory ability of astrocytoma cells and several analysis of the transcriptomes are carried out to discovery novel targets [15,16,21,39].

The *in vitro* cellular assay to investigate the role of Dpl in tumor cells migration was performed in an astrocytoma established cell line, i.e. IPDDC-A2, showing detectable levels of Dpl expression, and in HeLa cells, as a Dpl-low expressing cell system. Among several available astrocytoma-derived cell lines (Hu-2 [3], D384-MG [4], U-87 MG and U-373 MG [42]), IPDDC-A2 was employed for the more substantial Dpl expression level and for its highest viability and transfection efficiency of nucleic acids. The migration behaviour was analyzed, respectively, in Dpl-silenced IPDDC-A2 and in stable Dpl-expressing HeLa cells, using an *in vitro* scratch wound healing assay on a cell monolayer, a widely used method to test the migration efficiency of a cell line, as reviewed in [54]. Dpl silencing was performed using two complementary approaches, namely antisense oligonucleotides-based technology and siRNA. These approaches share some similarities in the length molecules and in inducing target degradation after a complementary mRNA hybridization. On the other side, while siRNA adopt a specific cellular machinery (i.e. the RNA induced silencing complex, RISC), antisense technology is mainly based on RNA–DNA duplexes, that obstruct the ribosome processing and induce RNase H-mediated degradation. However, impor-

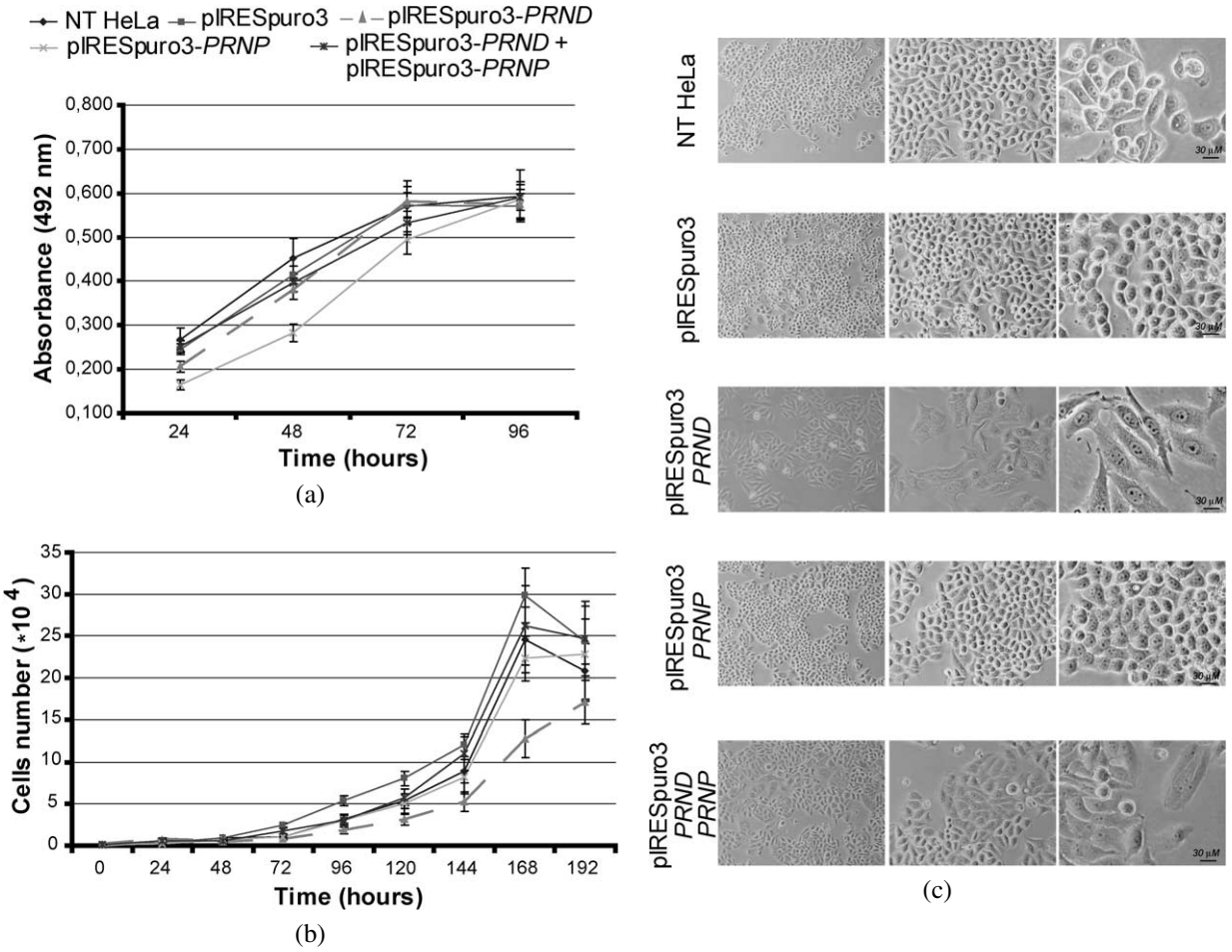

Fig. 4. Growth, viability assays and morphology evaluations. (a) An MTS-based assay was performed to evaluate the cellular viability. Absorbance readouts at 492 nm, evaluated at 24, 48, 72 and 96 h time intervals, revealed a similar behaviour among overexpressing Dpl (pIRESpuro3-*PRND*), PrP^C (pIRESpuro3-*PRNP*), both Dpl and PrP^C (pIRESpuro3-*PRND* + pIRESpuro3-*PRNP*), empty vector transfectants (pIRESpuro3) and untreated cells (NT HeLa). (b) The growth curves of the cells (evaluated for a 192 h time course) were analyzed; Dpl overexpressing cells show a lower plateau threshold compared to other cells. (c) Morphological analysis of HeLa cells, at 10×, 20× and 40× magnifications respectively, show relevant alterations in the cellular shape: the typical squared shape of HeLa cells changes towards a lengthened forms in Dpl- and Dpl/PrP^C-expressing cells. Scale bars are reported (30 µm).

tantly, the *in vitro* or *in vivo* efficacy of antisense *versus* siRNA technology is not theoretically predictable [12]. For the antisense technology, a fully phosphothioate DNA oligonucleotide (S-ODN), widely employed in cancer research [25,35,50], was designed against the first twenty nucleotides of the *PRND* coding sequence. As reported, after transfection, an earlier phenotypic effect on the cell migration after siRNA treatment respect to S-ODNs was detected; however, as immunoblotting revealed, S-ODN molecules showed a more efficient silencing on doppel protein expression than pre-designed siRNA molecules at twenty-four hours after transfection. This phenomenon might be related to an enhanced cellular stability of S-ODN compared to siRNA molecules and, conversely, to an earlier and more transient effect of *PRND*-specific siRNA on cellular migration [49].

To better evaluate the contribution of the doppel gene product in tumor cell migration, stably expressing-Dpl HeLa cells were produced. HeLa cells, that showed a very low endogenous Dpl expression as revealed by real time PCR analysis (data not shown) and western blot analysis (this work), were transfected and stabilized with the eukaryotic expression plasmid pIRESpuro3 containing the *PRND* coding sequence.

Transfected HeLa cells were selected for the stable overexpression of Dpl and the scratch wound healing assay was carried out. Western blot analysis re-

vealed a Dpl diffuse band of about 30–45 kDa, similar to that generally observed in the normal human testis tissue [41]. The migration rate of the Dpl-expressing HeLa cells was sensibly increased, more than two-fold higher, respects to the untreated cells and to the cells stably selected for the empty vector alone, proving that the effect of Dpl on migration capability of the cells was cell-type specific and due to the expression of Dpl. In addition, we treated these transfectants with the *PRND*-decoy ODNs previously tested with IPDDC-A2 cells instead of siRNA whose targets are located outside the *PRND* coding sequence and this make the mRNA transcript derived from the pIRESpuro3-*PRND* construct an "off-target" for our siRNA molecules: the treatment partially restored the migration rate similar to the untreated HeLa, while *GAPDH*-specific S-ODN did not affect this behaviour. Furthermore, we transfected and stably selected HeLa cells with the plasmid containing cellular prion protein coding gene sequence and, as we reported, cells overexpressing PrP^C did not show variations in migratory behaviour neither in morphology. The rationale of using this construct was because PrP^C and Dpl are homologue proteins that display a strong structural similarity [34]. Interestingly, when we examined the HeLa cells stably selected for both the plasmids containing *PRND* and *PRNP* coding sequences, the migration rate became similar to the Dpl-expressing cells. To this regard, our data showed that Dpl and PrP^C might exhibit different functions, following the hypothesis that two paralogue genes, after the duplication from their ancestor, are subjected to mutations that originate two functionally different genes with dissimilar expression patterns [32]. Furthermore, under our experimental conditions that consider cellular migratory behaviour, Dpl and PrP^C did not display any antagonizing role; differently, as previously described, in prion knock-out animal models and cell lines, Dpl neurotoxic effects were counteracted by *PRNP* transgene reintroduction [5,56]. However, in our cellular system, i.e. HeLa cells, the potentially toxic effect determined by the overexpression of doppel was not observed.

To discern the increased migration in Dpl-silenced cells from differences in viability rates, proliferation assays were performed in HeLa cells in a ninety-six growing time interval. Untreated HeLa and Dpl-stable expressing cells had a similar growing behaviour with, however, a significant difference of cell density in Dpl-overexpressing ones: these, differently from HeLa or other tumor cells, failed to reach a complete plate growth density, probably because of an augmented contact inhibition. This phenomenon might be also sustained by the documented differences in cell morphology after Dpl-expression stabilization, possibly explained by the interfering with unknown networks of genes involved in density-dependent cell growth [17, 18,20,22]. An inverted correlation between migration and growth rates, the "go or grow" dichotomy, was already described in tumor cells; however, the molecular and cellular mechanisms involved in these mutually exclusive phenotypes, are up to now not completely elucidated [18,19]. In the case of human astrocytomas, it might be hypothesized that Dpl is expressed mostly in the highly migratory cells of the external edge of the tumor area (work in progress). It is possible to hypothesized that doppel influence cell migration by means of its physiological cellular membrane localization that might be involved in cell-to-cell contacts; however, our knowledge about doppel localization in a tumor scenario could not sustain this speculation since we reported a cytoplasmic ectopic localization of the protein in astrocytoma [8,47]. An additional possibility is that doppel could take part to an up to now unknown signaling network that might influence migration. To this regard, we recently documented RACK1 (Receptor for Activated C-Kinase) as a Dpl interacting protein [2]. RACK1 is an adaptor protein that regulates signaling via Src- and PKC (protein kinase C)-dependent pathways and in particular it has been described to modulate the integrin-mediated cellular adhesion and migration [13,26,48].

Furthermore, as we reported, a forced-Dpl expression induced an irreversible morphological change in HeLa cells. This phenotype was Dpl-specific, since prion and empty expression vector did not induce differences in their cellular shape. Variations in cell motility is often associated with morphological changes and differences in actin expression [55]: to this regard, however, HeLa-treated cells did not exhibit differences in actin expression (data not shown), suggesting that other cyto-architectural proteins might be involved. Finally, neither siRNA nor ODNs treatment of IPDDC-A2 cells showed morphological changes, probably due the transient mechanism of action of these molecules.

In summary, our study highlighted for the first time a functional role of the doppel gene product in inducing differences in astrocytic tumor cell migration and morphology, suggesting a potential antisense therapy against this target protein with the aim of interfering with the described phenotypes.

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