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Inhibitor of DNA binding-1 induces mesenchymal features and promotes invasiveness in thyroid tumour cells

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ABSTRACT

Characterisation of molecular mechanisms that control tumour invasion is a crucial step for the identification of molecular markers to apply in cancer diagnosis and treatment.

In this work, we have investigated the role of Id1 in thyroid tumours.

We demonstrate that Id1 participates to tumour progression by powering the invasion capacity of cancer cells. We prove that the overexpression of Id1 in thyroid tumour cells profoundly alters cell morphology and growth, increasing migration and invasion properties of the cells. Analysis in human thyroid tumours reveals that Id1 is expressed in invading cells and its expression is associated with an increased metastatic potential of non-anaplastic tumours. The gene expression study supports these observations demonstrating that Id1 modulates a number of genes known to control invasion, aggressiveness and pharmacological resistance in different type of human tumours. Finally, we demonstrate that the pro-invasive effect of Id1 is accompanied by the acquisition of mesenchymal features in thyroid tumour cells. This suggests that the trans-differentiation towards a more immature condition is the mechanism through which Id1 promotes thyroid tumour metastatic spreading.

This study identifies Id1 as part of the pro-metastatic programme of thyroid cancer and suggests its possible utilisation as a prognostic marker.

1. Introduction

The inhibitor of DNA binding-1 (Id1) is a transcriptional regulator that belongs to the helix–loop–helix (bHLH) family of transcription factors. Id1 controls the expression of a large number of genes and affects important cellular processes by inhibiting the activity of the bHLH proteins.[1,2] The main role of Id1 is to inhibit cell differentiation.[3] By maintaining the cells under immature condition, Id1 also enhances their proliferation and motility. Loss of differentiation, unrestricted proliferation and increased cell motility are hallmarks of malignancy. Recently, the importance of Id1 in promoting tumour invasion and metastasis has emerged and a role for Id1 as possible molecular marker of tumour aggressiveness has been proposed.[4–6] However, still unclear are the molecular mechanisms through which Id1 exercises its pro-metastatic activity.

Thyroid tumours are the most common malignancies of the endocrine system and their incidence has constantly increased over the past years.[7] Based on histological and

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cytological features, thyrocyte derived tumours are classified into three categories: well-differentiated, poorly differentiated and anaplastic (or undifferentiated). Based on cellular architecture and nuclear features, well-differentiated tumours are sub-divided into papillary (~90% of total) and follicular. Aggressiveness and lethality of tumours decrease with cell differentiation.[8] Nevertheless, the existence of well-differentiated carcinomas that develop diffuse aggressive metastasis is not a rare event and urgent is the need for more accurate diagnostic tools to predict the neoplastic spread of these lesions to distant sites. Indeed diagnosis of thyroid tumours is based exclusively on morphological features, since reliable molecular markers have not been established yet.[9] Thus, the identification of regulatory genes involved in the progression of thyroid tumours towards more aggressive stages, is an obligate step to identify molecular markers thus improving diagnosis and treatment.

We have explored Id1 function in thyroid tumour progression. We show that Id1 increases the invasiveness of thyroid tumour cells by modifying their epithelial phenotype and their interaction with the surrounding microenvironment. We also demonstrate that, *in vivo*, Id1 expression correlates with the aggressiveness and the metastatic potential of non-anaplastic thyroid tumours.

2. Materials and methods

2.1. Cell cultures and Western blot

Sw579 cell line was purchased by ATCC (Manassas, USA). Nthy-ori-3-1 was purchased by ECACC distributed by Sigma–Aldrich (St. Louis, MO, USA), B-CPAP, TPC1 and WRO cell line were kindly provided by the laboratory of Dr. Santoro M. University Federico II of Naples (Italy). All cell lines were grown at 37 °C, 5% of CO_2 in DMEM supplemented with 10% FBS (Gibco-Invitrogen, Paisley, Scotland, UK).

For Western blot, cells were lysed on 1× SDS sample buffer. Protein extracts were analysed in SDS–PAGE. The gel running and transfer were carried out using the Bio-Rad apparatus according to manufacturer instructions. The following antibodies were used for Western blot: mouse Anti-Actin (Sigma–Aldrich, St. Louis, MO, USA); Rabbit Anti-Id1 clone 195-14 (Biocheck, Foster City, CA USA); Mouse Anti-Vimentin (Dako, Denmark). Anti-Mouse and Anti-Rabbit secondary antibody HPRT conjugated were purchased by GE-healthcare (Uppsala, Sweden). All the primary antibodies were used at a dilution 1:1000 except anti-Id1 that was used 1:500. Secondary antibodies were used 1:5000. The ECL Western blot Detection Reagent (GE-healthcare; Uppsala, Sweden) was used to develop the HPRT signal, according to manufacturer instructions.

2.2. Clones isolation

For stable clone derivation, B-CPAP cells were transfected with Id1-pCDNA 3.1 or with pCDNA 3.1 plasmids using Lipofectamine 2000 (Invitrogen Paisley, Scotland, UK) according to manufacturing instruction. Twenty-four hours after transfection cells were seeded at very low density and G418 (Geneticin) (Invitrogen Paisley, Scotland, UK) was added to the regular growth medium at a concentration of 800 μg/ml. Two weeks after selection, single clones were picked and expanded. Thirty-two Id1 overexpressing clones and seven control clones were obtained and characterised for Id1 overexpression by using Western blot and qRT-PCR. *In vitro* assays are described in the Supplementary information.

3.2.1. Immunohistochemistry

All samples were retrieved from the archive of the Pathology Unit of Arcispedale S. Maria Nuova. Tissue specimens were fixed in 10% formalin, paraffin embedded and processed for light microscopy. For immunohistochemistry 5 μm sections were cut and stained with a rabbit monoclonal anti-Id1 antibody clone 195-14 (Biocheck, Foster City, CA USA) according to previously published protocol[2] then counterstained with haematoxylin. Images were captured using a Nikon Eclipse E80 microscope. This study was approved by the Arcispedale Santa Maria Nuova ethical committee.

2.3. Microarray analysis

The microarray analysis was performed by the Microarray Unit of Cogentech (Milano). A full description of microarray procedure is provided in the Supplementary information. We have deposited the raw data at GEO/ArrayExpress under accession number E-MEXP-2832, we can confirm that all details are MIAME compliant.

2.4. Quantitative real time PCR

One microgram of total mRNA was retrostrancribed with SuperScript VILO cDNA kit (Invitrogen; Paisley, Scotland, UK). qRT-PCR was conducted using the Light Cycler DNA SYBR Master Mix and a Light Cycler thermocycler (Roche Diagnostics, Mannheim, Germany). Sequence of primers used will be provided upon request. Relative expression of target genes was calculated using ΔΔCT method and normalised to GAPDH mRNA content.

2.5. Immunofluorescence

Cells were seeded in 4 well Lab-Tek Chamber slide (Nunc; Roskilde, Denmark), in regular growth medium. Twenty-four hours after seeding, cells were fixed in 4% PFA in PBS1× for 15 min at room temperature, permeabilised with 0.1% Triton, in PBS1× for 2–5 minutes, blocked with 20% FBS and 2% BSA in PBS1× for 1 h, incubated with a Mouse anti-β-Catenin antibody (BD Bioscence) or with a Rabbit anti-Id1 clone 195-14 (Biocheck, Foster City, CA USA) in a humidify chamber. Antibody binding was revealed with a secondary anti-mouse FITC conjugated antibody (Sigma–Aldrich, St. Louis, MO, USA) or anti-Rabbit Alexa568 conjugated antibody (Invitrogen Paisley, Scotland, UK). Actin filaments were stained with an Alexa 488 conjugated Phalloidin (Invitrogen, Paisley, Scotland, UK), according to the manufacturer instructions. Cell nuclei were stained with DAPI (Invitrogen, Paisley, Scotland, UK). Slides were mounted using the SlowFade mounting medium

(Invitrogen, Paisley, Scotland, UK) and observed using an Axiophot fluorescent microscope (Zeiss, Germany).

2.6. TGFβ time course

TGFβ was purchased from Peprotech (Rocky Hill, NJ, USA). Nthy-ori-3-1 cells were seeded in a 12 well plate in regular growth medium. After adhesion, cells were serum starved overnight and maintained in a medium containing DMEM + 2% of BSA (Sigma–Aldrich, St. Louis, MO, USA). Cells were stimulated with 5 ng/ml of TGFβ for different time (30, 90 min, 3, 6, 24 h). Protein extracts were collected at the indicated time point lysing the cells in SDS-sample buffer. Id1 protein levels were analysed by SDS–PAGE and Western blot as described above.

3. Results

In order to investigate Id1 function in thyroid tumours we have overexpressed Id1 in a thyroid tumour cell line. The B-CPAP cells, derived from a well-differentiated papillary thyroid carcinoma[10] were chosen as a cellular model. B-CPAP cells had the lowest Id1 expression (Fig. 1A) and are less compromised in terms of mutations and functional characteristics than other thyroid tumour derived lines.[11] The B-CPAP cells were transfected with an Id1-expressing vector or with an empty vector and single clones were generated by antibiotic selection. Two clones, expressing different levels of Id1, were chosen: Id1A (high Id1 expression) and Id1B (medium Id1 expression) (Fig. 1B and C). We noticed that the overexpression of Id1 drastically changes the morphology of the

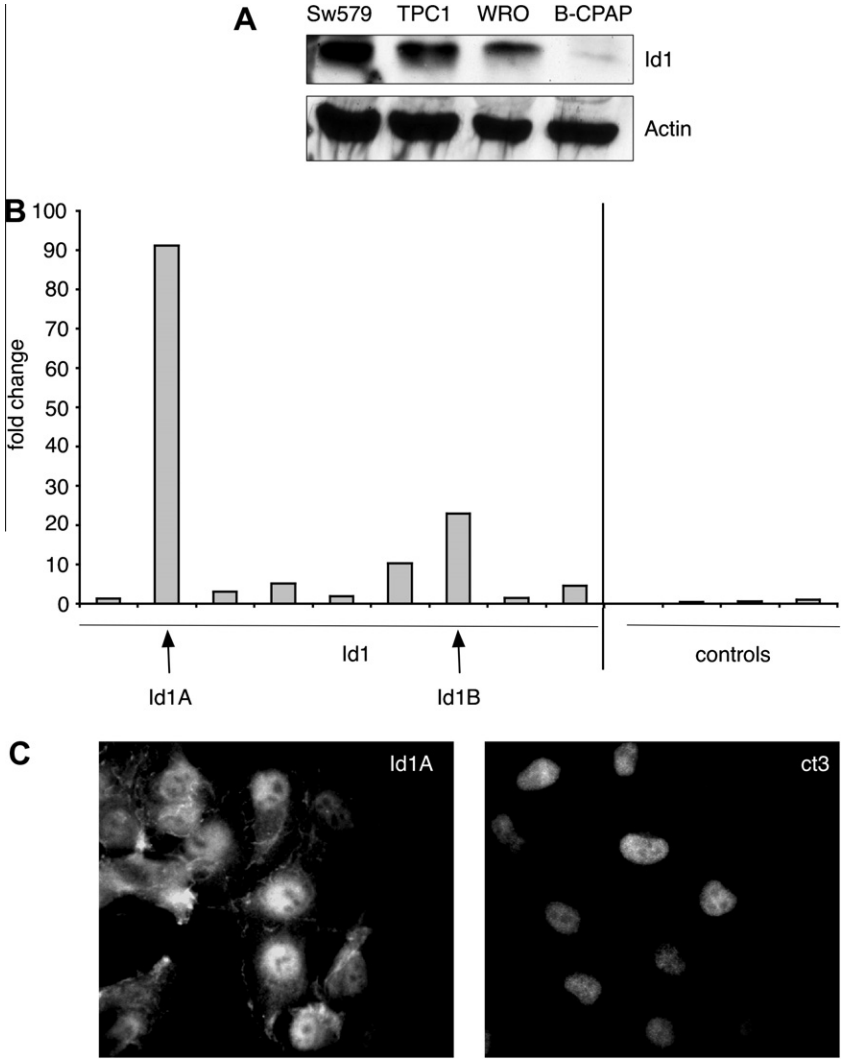

Fig. 1 – Establishment of Id1 overexpressing thyroid tumour cells. (A) Western blot analysis of Id1 (upper panel) and actin (lower panel), on protein extract obtained from the indicated cell lines. Sw579 are undifferentiated thyroid tumour cells; TPC-1 and B-CPAP are well-differentiated papillary tumour cells; WRO are well-differentiated follicular tumour cells. (B) qRT-PCR analysis of Id1 mRNA levels in a representative set of B-CPAP derived clones. The histograms represent the fold induction, of Id1 levels, in nine clones transfected with Id1 (left part) and four controls clones (right part) relative to the Id1 levels on the last control clone represented in the graph. Arrows indicate the levels of Id1 expression in Id1A and Id1B cells that were used in the following experiments. (C) Immunofluorescent staining of Id1 in Id1A (left panel) and ct3 (right panel) cells. Magnification 400×.

cells (Fig. 2A). Control cells are large, polygonal, with abundant cytoplasm growing in clusters, with the epithelial characteristics of the parental B-CPAP cell line[10] (ct2-4). In contrast, the Id1 overexpressing cells showed a fibroblast-like elongated structure, did not grow in clusters but distributed through out the plate (Id1A-B). A cell growth assay showed that proliferation does not correlate with Id1 expression levels since Id1B cells proliferate faster than Id1A and controls (Fig. 2B). Afterwards, a single cell suspension of Id1A and ct3 cells was seeded in a six well plate. Two weeks after, the colonies were analysed. No significant difference in the number of formed colonies was found. However, a striking difference in the morphology of the colonies could be observed (Fig. 2C). In the control plate large isolated colonies were visible and no migrating cells could be detected. In contrast, in the Id1-overexpressing plate numerous cells were spreading from their colony of origin and moving through out the plate. Therefore, we carried on a scratch wound healing assay (Fig. 3A). Following adhesion, part of the cells were scrubbed leaving on the plates scratches of comparable dimensions.

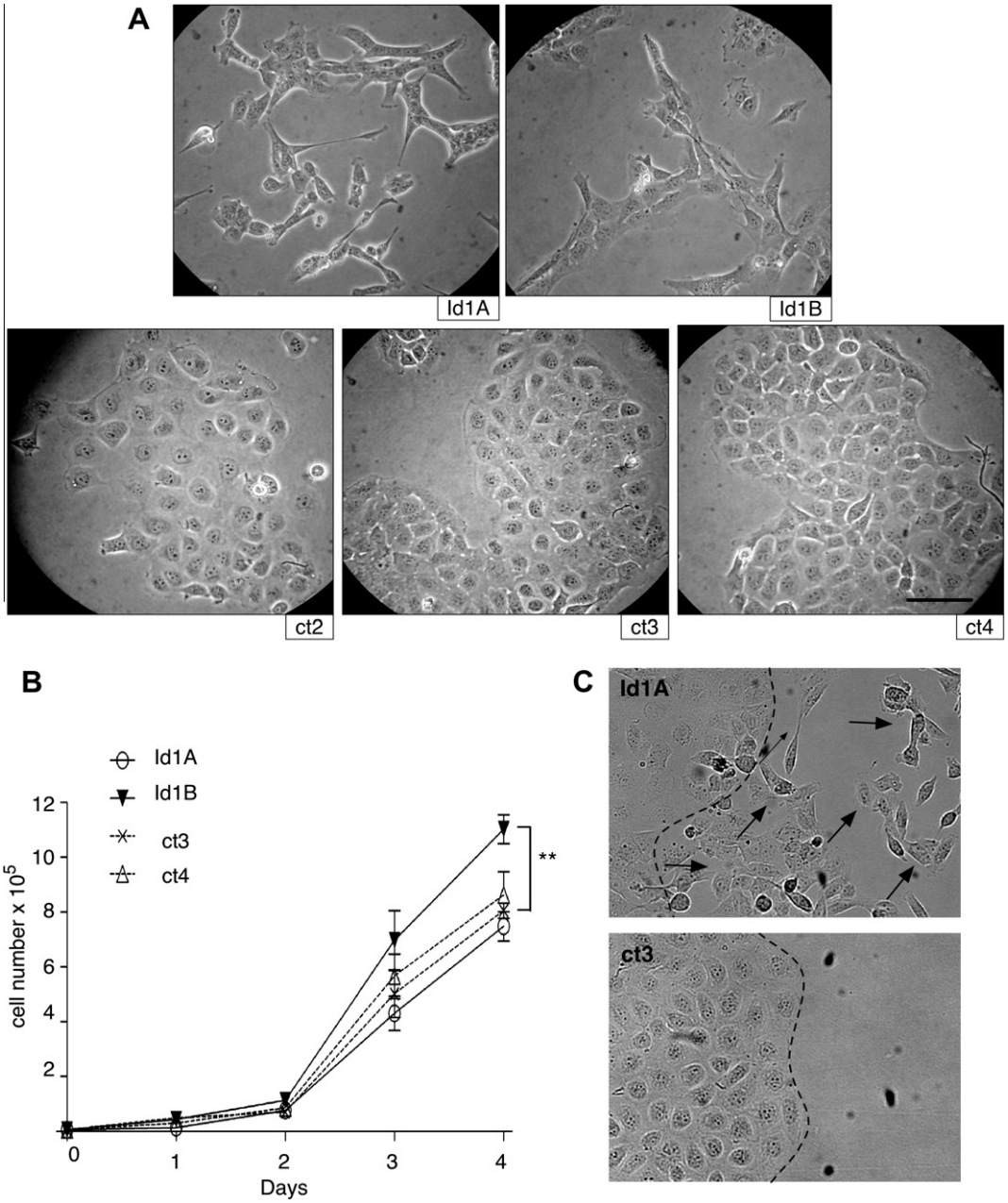

Fig. 2 – Effects of Id1 overexpression on thyroid cancer cell morphology, proliferation and growth. (A) Light microscopy images of the Id1A and Id1B clones (upper panels) and of the controls clones ct2, 3 and 4 (lower panels). Magnification 400×. Scale bar 50 μm. (B) Proliferation curves of Id1A, Id1B, ct3 and ct4 clones. Curves represent the averaged number of cells per well for each clone at each time point ± SEM. $p = 0.0036$. (C) Light microscopy images of the Id1A (upper panel) and ct3 (lower panel) cells. Broken line underlines the borders of the colonies. Arrows indicate migrating cells that are moving from their colony of origin in the Id1A cell plate. Magnification 400×. Scale bar 50 μm.

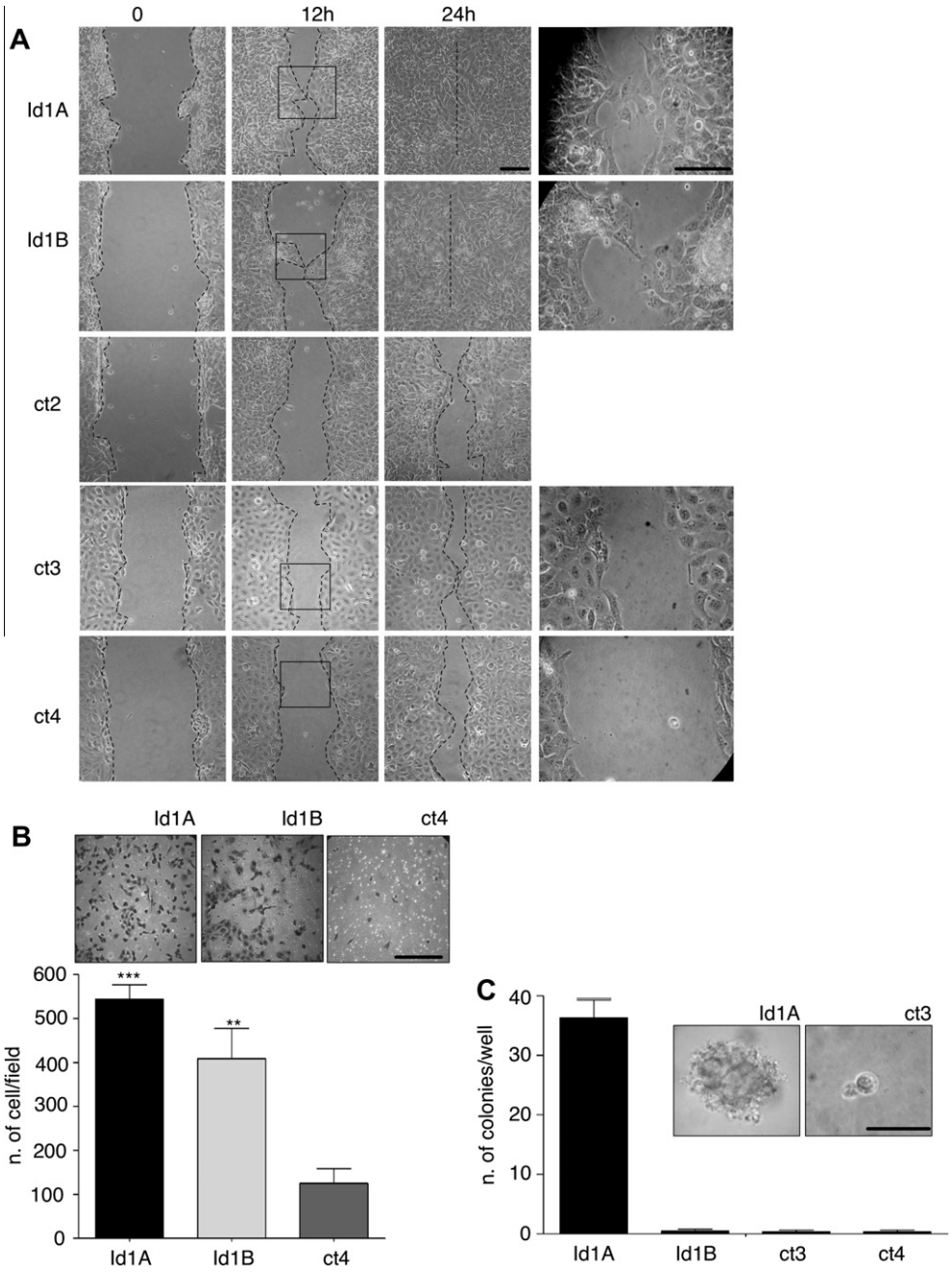

Fig. 3 – *In vitro* analysis of Id1 overexpression effects on B-CPAP invasion and anchorage independent growth property. (A) Light microscopy images of Id1A, Id1B, ct2, 3, 4 clones during a representative scratch wound healing assay at time 0, 12 and 24 h after scratch application. Magnification 200×. Inserts show magnification of scratch central regions in Id1A, Id1B, ct3 and ct4 clones at 12 hours. Magnification 400×. In the inserts invading cells are visible in the healing area for the Id1A and Id1B clones but not for the controls. Four scratches for each clone were analysed in this experiment obtaining the same results. The experiment has been replicated twice obtaining comparable results. (B) Results of the invasion chamber assay. Each clone was tested in triplicate. After migration invading cells were stained and pictured with a light microscope at a magnification 100×. Three independent fields for each replicate were counted. The histograms represent the averaged number of invading cell/ field obtained in two separated experiments. For each clones, the mean values ± SEM are indicated. ***$p < 0.001$; **$p = 0.0042$ (unpaired *t* test; GraphPad Prism). Inserts show pictures of representative fields of migrating cells (arrows) for each of the indicated clones. The pictures were captured at a magnification of 200×. (C) Results of the anchorage independent growth assay. Each clone was tested in triplicate. Histograms represent the averaged number of colonies/ well ± SEM. Insert show pictures of a representative colony formed by Id1A clone (left insert) and single cells of ct3 clone pictured four week after seeding (right insert). Magnification 400×. Scale bar 50 μm.

The scratches were pictured after 12 and 24 h to check the healing process. After 12 h the scratches were reduced in all the clones. The healing was more pronounced in the Id1A and Id1B as compared to the controls. Higher magnification showed that Id1A and Id1B cells had invaded the scratch and some parts of it were already healed. By contrast, the margins of the scratches in control clones were still neat without sign of invading cells (right column). After 24 h, the scratches were completely healed in the Id1A and Id1B plates and still visible in all controls. To confirm this observation, Id1 overexpressing and control cells were tested in an invasion chamber assay. Each clone was seeded in triplicate and after 22 h the invading cells were counted. As shown in Fig. 3B, the number of invading cells was higher for the Id1 overexpressing cells as compared to the controls. These experiments demonstrate that, *in vitro*, high levels of Id1 increase the invasiveness of thyroid cancer cells.

In order to colonise a new site, metastatic cells must acquire the capacity to grow in the absence of physical anchorage. We seeded a single cell suspension of each clone in a semisolid soft agar medium. Under these conditions, cells are isolated and cannot adhere to the plate. Four weeks after plating, the colonies were counted. Only the Id1A line was able to form colonies while no colonies were detected in the plates of the other lines (Fig. 3C). We concluded that high Id1 expression is sufficient to confer anchorage independent growth properties to thyroid cancer cells. This observation, together with the increased invasiveness of Id1 overexpressing cells, shows that Id1 may mediate thyroid cancer metastatic spreading.

Next, we have investigated Id1 expression in human thyroid cancers, to verify *in vivo* the pro-invasive function of Id1. Fifty-one human thyroid cancers were stained by immunohistochemistry with a specific anti-Id1 antibody[2] (Table 1). Twenty-nine out of the 51 samples showed Id1 nuclear staining in tumour cells. Despite the anti-differentiation function of Id1, no major correlation with the differentiation stage of the tumours emerged. 62.5% of the well differentiated and 55.5% of the poorly differentiated tumours showed Id1 positive cells, while only 40% of the anaplastic tumours were positive to Id1. Fig. 4A shows Id1 staining in a representative subset of the analysed samples. In most cases Id1 was detected in sporadic cells scattered in the tissue (less than 2%) (a, d, e). One-third of the positive samples showed a higher frequency of positive cells (10–30%) (b, c, f). We also analysed benign lesions and normal thyroids as control but none of these samples displayed detectable levels of Id1 ($n = 10$).

It is known that non-anaplastic thyroid tumours are usually indolent lesions, with a low incidence of recurrence and low metastatic potential. The set of tissues analysed included 8 non-anaplastic highly aggressive tumours, which developed diffuse metastasis within 3 years after the primary lesions. Noticeably, seven of these tumours were positive for Id1. A high frequency of Id1 positive cells was observed at the edge of invading regions (Fig. 4B). Panel g shows a representative staining of the tumour boundary where Id1 positive cells are visible at the interface between tumours and non-neoplastic tissue (i) or at invading nests within the surrounding capsule (h). This observation indicates that Id1 is expressed in invading tumour cells and it is in accordance with a pro-invasive function of Id1 in thyroid tumours.

Since Id1 controls biological processes by regulating gene expression, we used the microarray technique to identify its target genes in thyroid cancer cells.

The gene expression profile of the Id1A cells was compared to the profiles of the control lines 2, 3 and 4. The expression of 444 genes was modified, more than 2-fold, by Id1: 314 were upregulated and 130 were repressed (Supplementary Table 1). We grouped the genes in five functional categories (Table 2). Noticeably, genes that mediate cell migration and genes coding for ECM components were significantly higher in the Id1A cells as compared to the controls. In contrast, genes coding for proteins involved in adherent junction or tight junction regulation were down regulated following Id1 overexpression. This observation suggests that Id1 modifies the interaction of cells with the microenvironment favouring interaction with the ECM and cell motility versus cell–cell interaction. We confirmed the microarray analysis by qRT-PCR on selected genes. Fig. 5 shows the expression level of the indicated genes in the Id1A and Id1B cells compared with the control lines 2, 3 and 4. The modifications observed in the microarray analysis were confirmed both in Id1A and Id1B cells suggesting that these genes are Id1 targets and that their de-regulation is not due to clone selection. In conclusion, the microarray analysis shows that Id1 controls the expression of proteins that are involved in invasion, aggressiveness and pharmacological resistance in thyroid cancer cells.

Table 1 – Tabulated results of Id1 immunohistochemistry in thyroid cancers.

Tumours	Number of samples (total = 61)	Number of Id1 + samples (total = 29)	Percentage (%)
Well differentiated	32	20	62.5
Poorly differentiated	9	5	55.5
Anaplastic	10	4	40
Non-malignant[a]	10	0	
Non-anaplastic with aggressive metastasis[b]	8	7	87.5

[a] Non-malignant tissues analysed comprised: 1 hyperplasia, 4 follicular adenomas, 4 normal thyroid from contro-lateral lobe of patients with carcinoma (including 1 medullary and 3 papillary carcinomas) and 1 normal thyroid from healthy patient.

[b] Non-anaplastic tumour with aggressive metastasis analysed comprised: 4 well-differentiated tumours (histotype: 3 papillary, 1 follicular) and 4 poorly-differentiated tumours (histotype: 3 papillary, 1 follicular). Eight out of 8 patients developed diffuse metastasis within 3 years after surgical removal of primary lesions. Seven out of 8 patients died because of thyroid tumour dissemination.

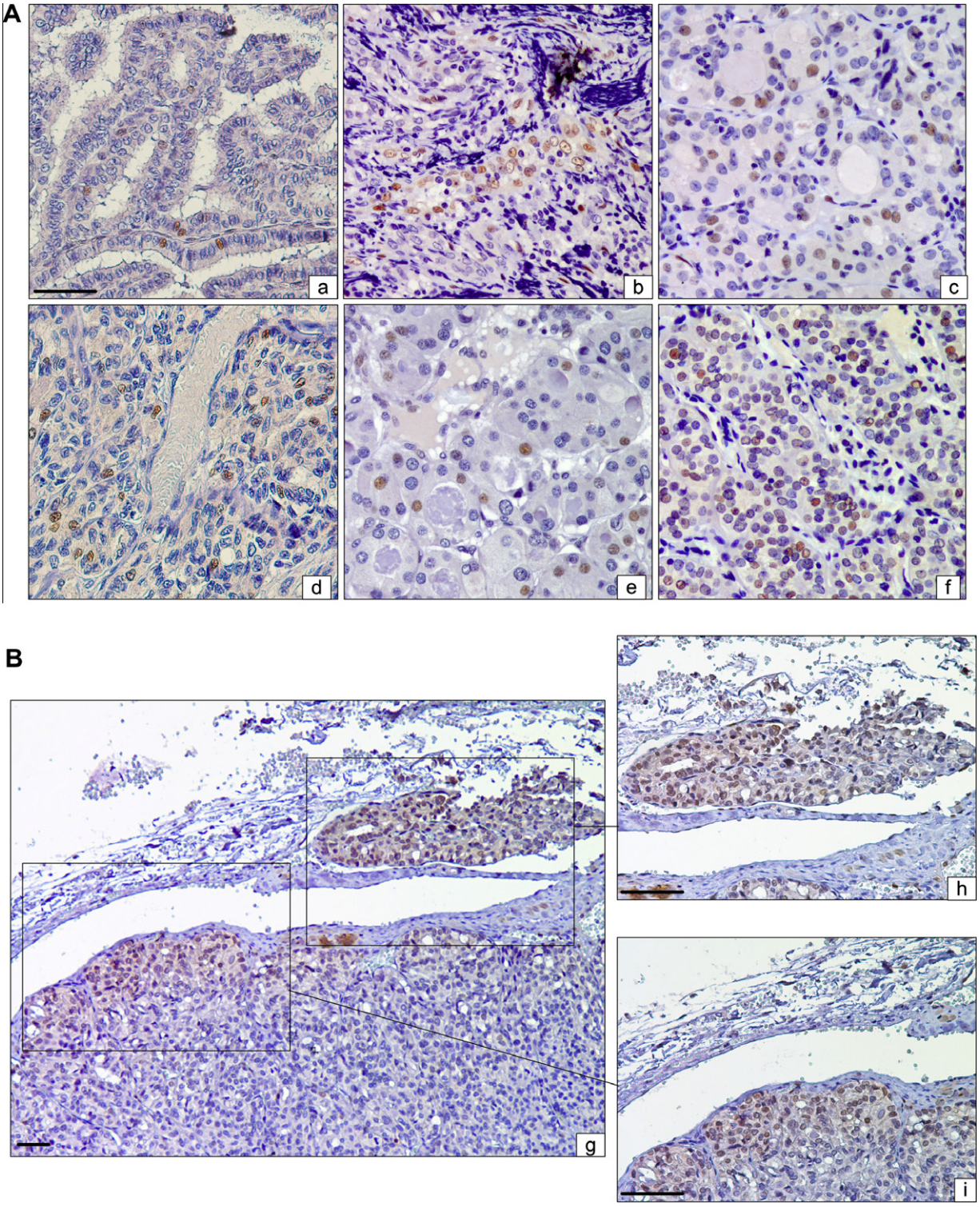

Fig. 4 – Immunohistochemical analysis of Id1 expression in human thyroid tumours. (A) Id1 staining (brown) in different types of thyroid carcinomas. Haematoxylin (blue). (a) Papillary carcinoma, tall cell variant; (b) papillary carcinoma, sclerosing diffuse variant; (c) follicular carcinoma, Hürtle cell variant; (d) poorly differentiated insular carcinoma; (e) classic follicular carcinoma; and (f) papillary carcinoma solid variant. Magnification 200×. (B) Id1 staining at the edge of invasive region in a case of poorly-differentiated insular papillary carcinoma (g). Magnification 100×. The inserts show higher magnification of tumour cells pushing on the neoplastic capsule (i) and of an invading nest within the capsule (h). Magnification 200×. Scale bar 100 μm.

Table 2 – Functional categories of Id1 target genes. The numbers indicate the gene expression fold change in the Id1A clone compared to ct2, ct3 and ct4 averaged value. Highlighted in bold genes that have been described to partake or to be de-regulated in EMT.

Cell–cell/cell–ECM interacting protein		Cytoskeleton organisation		Transcription factor		Cell signalling	
Gene name	Fold change	Gene name	Fold change	Gene name	Fold change	Gene name	Fold change
TIMP 3	13.1	SCIN	9.3	**RUNX2**	8.7	**SFRP1**	7.7
K-CDH	6.6	SNTG1	6.3	EYA4	5.3	**LPHN2**	7.4
P-CDH10	4.9	MTMR8	3.7	MEIS2	4.6	IL1 α	5.4
TNC	4.6	AFAP1L2	3.4	MEST	3.7	c-KIT	4.9
ITGβ8	4.3	MAP2	2.6	MYB	2.8	TNIK	4.8
KRT19	4.3	MYLK	2.5	CREB3 L1	2.8	EREG	4.5
NCAM 2	4.1	SNTB 1	2.2	**LEF1**	2.6	SHC4	4.3
AJAP1	4.0	MFAP 5	2.0	PBX3	2.4	BLK	4.2
KRT13	3.9	DTNA	2.0	RARG	2.4	PTGFR	4.1
LAMC2	3.7	CALD1	–2.0	BHLHB3	2.3	TNFRSF9	3.9
ITGα4	3.7	EML1	–2.3	TBX3	2.5	ELTD1	3.5
NLGN1	3.6	CTTNBP2	–2.3	EYA1	2.3	EPHA5	3.3
SPOCK3	3.3	AFAP1L1	–2.6	BHLHB2	2.1	LIF	3.3
DSC2	3.2			BHLHB9	2,0	PIK3R3	3.1
CLDN4	3.1			ETV1	2.0	HTR 7	3.1
SPINT2	3.1			GATA6	2.0	LTBP3	2.9
ADAMTS3	2.9			**NKX2-1**	–2.2	DUSP4	2.7
NRP1	2.8			MYC	–2.5	**TGFβ1**	2.7
LAMA3	2.7			LMO7	–3.1	PTHLH	2.6
MMP 14	2.6			LMO3	–3.2	PTGER2	2.5
M-CAM	2.5					**FGF2**	2.5
CLDND1	2.5	**ABC transporter**				PTPN6	2.5
JAM3	2.4					MAPK13	2.4
SEMA3C	2.4	ABCA1	3.1			**BMP2**	2.4
MMP13	2.4	ABCB1	2.7			CSF1	2.4
SPP1	2.3	ABCG2	2.4			IL17RD	2.4
MMP 2	2.3					EGF	2.3
ADAM 12	2.3					IL1β	2.3
SEMA7A	2.3					LIFRα	2.2
Serpin E 1	2.2					EPHRB1	2.1
VCAN	2.2					EFNB2	2.1
CTHRC1	2.1					NOS	2.0
F11R	2.1					IGFBP6	2.0
SEMA3B	2.1					**TGFBR3**	–2.0
ColV α1	2.0					PRKCη	–2.3
KRT4	2.0					PEL1	–2.3
GJA1	–2.2					TLR 4	–3.4
CAV1	–2.2					FGF 1	–3.4
SEMA6D	–2.4					**NOG**	–3.5
MMP3	–2.6					IGFBP5	–4.3
EPB41L4A	–3.1					**HHIP**	–5.2
GPC 5	–3.6						
EPB41L3	–4.7						

We noticed that the morphological changes (e.g. fibroblast-like morphology) and the increased invasiveness, induced by Id1 are characteristic features of the epithelial–mesenchymal transition (EMT). The EMT is a complex process by which epithelial cells lose their characteristics to acquire mesenchymal properties. The acquisition of mesenchymal features is considered one of the leading mechanisms mediating invasion and metastatic spreading of epithelial tumours.[12] To test whether Id1 overexpression induces a mesenchymal phenotype in thyroid tumour cells we analysed several mesenchymal markers[13] (Fig. 6). First we showed that Id1 induced a profound rearrangement of the cytoskeleton. The mesenchymal intermediate filament Vimentin was more abundant in the Id1A and Id1B cells as compared to control cells (Fig. 6A and B). In addition the organisation of the actin filaments was completely different in Id1A cells as compared to ct3 cells (Fig. 6C). In control cells a cortical organisation of the actin filaments, characteristic of the epithelial cells, was visible (red arrowhead). In contrast, in Id1A cells the actin filaments had lost their polarity (yellow arrowheads) and formed large aggregates in the cytoplasm, named stress fibres (white arrowheads). In epithelial cells β-catenin is associated to the membrane and is released in the cytoplasm following transition towards a mesenchymal condition. Fig. 6D shows the immunofluorescent staining of β-catenin in Id1 overexpressing and control cells. In ct3 cells β-catenin is detected

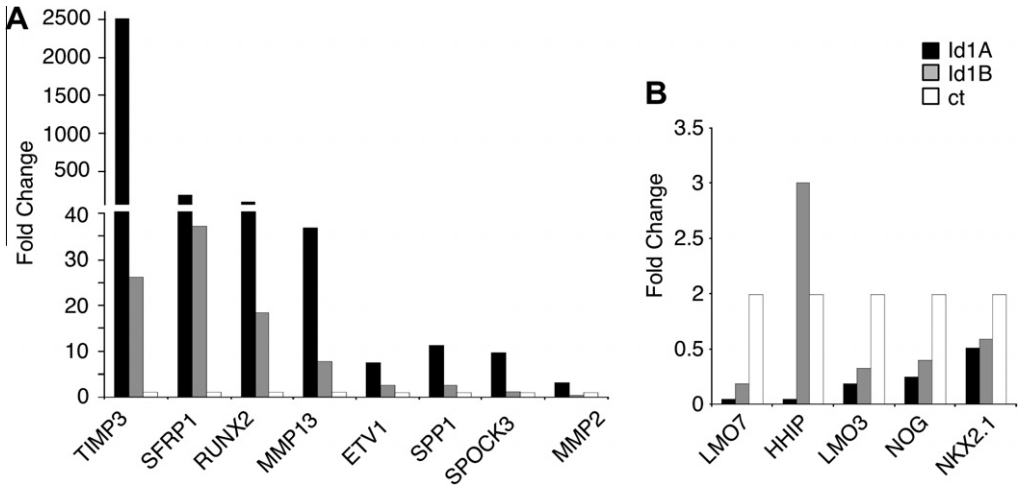

Fig. 5 – Validation of Id1 target genes identified in the microarray analysis. (A) Quantitative RT-PCR analysis of a representative set of genes that were upregulated upon Id1 overexpression. (B) Quantitative RT-PCR analysis of a representative set of genes that were downregulated upon Id1 overexpression. For each of the indicated genes the results were normalised to the GAPDH levels. The bars represent the averaged fold change of the indicated gene relatively to the value of the gene in the control. The control represents the average value of the gene in ct2, ct3 and ct4 clones.

predominantly at the cell membrane, concentrated at the cellular junctions. In contrast, a diffuse cytoplasmic β-catenin staining is visible, in the Id1A cells. Finally, we noticed that several of the Id1 target genes identified by the microarray analysis in thyroid tumour cells are de-regulated or partake to the EMT process (highlighted in bold in Table 2).[12–15] Both Id1A and Id1B cells show a significant upregulation of the mesenchymal markers K-CDH and TNC as compared to control cells (Fig. 6E). These results, together with the increased invasiveness and the fibroblast like morphology of the cells, indicate that Id1 induces the acquisition of mesenchymal features in thyroid tumour cells. Since TGFβ is the major regulator of EMT[16] and since Id1 has been shown to be a TGFβ target in other cell types, we have investigated whether TGFβ controls Id1 expression in thyroid cells. Fig. 6F shows that TGFβ caused an early and strong induction of Id1 expression in normal thyroid cells, suggesting that the upregulation of Id1 could be a crucial event in the TGFβ-induced mesenchymal transformation in the thyroid. Overall, these experiments demonstrate that Id1 is a mediator of thyroid cancer invasion and malignancy and implicate that the pro-invasive function of Id1 is mediated by the trans-differentiation of thyroid tumour cells towards a mesenchymal status.

4. Discussion

The characterisation of molecular mechanisms that control the invasiveness and the metastatic potential of a tumour is a major effort of molecular oncology. In this work we propose Id1 as a new factor controlling the invasive characteristics of thyroid tumours. By combining *in vitro* and *in vivo* approaches we demonstrate that Id1 promotes thyroid tumour cell invasion and that its expression correlates with the development of aggressive metastasis in non-anaplastic thyroid cancer. Id1 overexpressing cells show higher motility and invasive capacity as compared to control cells (Figs. 2 and 3), in different *in vitro* assays. Moreover, the overexpression of Id1 profoundly alters the ability of the cells to interact with the surrounding microenvironment, reducing the cell–cell interactions (Fig. 2A and C) and conferring to the cells the ability to grow in the absence of physical anchorage (Fig. 3C). The analysis of Id1 expression *in vivo* strongly confirms the cytochemical experiments (Fig. 4). It is interesting to notice that, so far, the use of non-specific antibodies against Id1 has been misleading in the investigation of Id1 expression *in vivo*. In 2004, Kebebew and colleagues have shown that Id1 is expressed in all kinds of thyroid conditions (from normal to very aggressive tumours), with a higher intensity and frequency, in anaplastic cancer.[17] In contrast, our analysis was carried out using a highly specific monoclonal antibody[5], shows that Id1 is expressed only in malignant thyroid tumours and does not correlate with tumour differentiation (Fig. 4). However, we have found a high frequency of Id1 positive tumours in a group of non-anaplastic tumours, which develop aggressive metastasis. Moreover, we have shown that Id1 positive thyrocytes are localised mainly at the invading regions of the tumour and that Id1 is highly expressed in thyroid tumour cells that have invaded the adjacent non-neoplatic tissues (Fig. 4B). This observation is in agreement with Id1 promoting invasiveness and metastatic dissemination in thyroid cancer. The microarray data strongly support this hypothesis, showing that, in thyroid tumour cells, Id1 controls the expression of a number of genes known to confer aggressiveness and metastatic potential in other tumour types (Table 2). Several of the identified Id1 target genes code for proteins that promote cell migration and infiltration.[18–21] In particular, metalloproteinases (MMP2, MMP13 and MMP14), ADAMs, semaphorins (SEMA3C, SEMA7A and SEMA3B), NRP1, TNC, ITGβ8 and ITGα4 were significantly higher in Id1 overexpressing cells as compared to the control cells. During migration and invasion, tumour cells undergo profound morphological changes that have to be sustained by cytoskeleton remodelling. Accordingly several genes involved in the cytoskeleton organisation are de-regulated in the Id1 overexpressing cells.

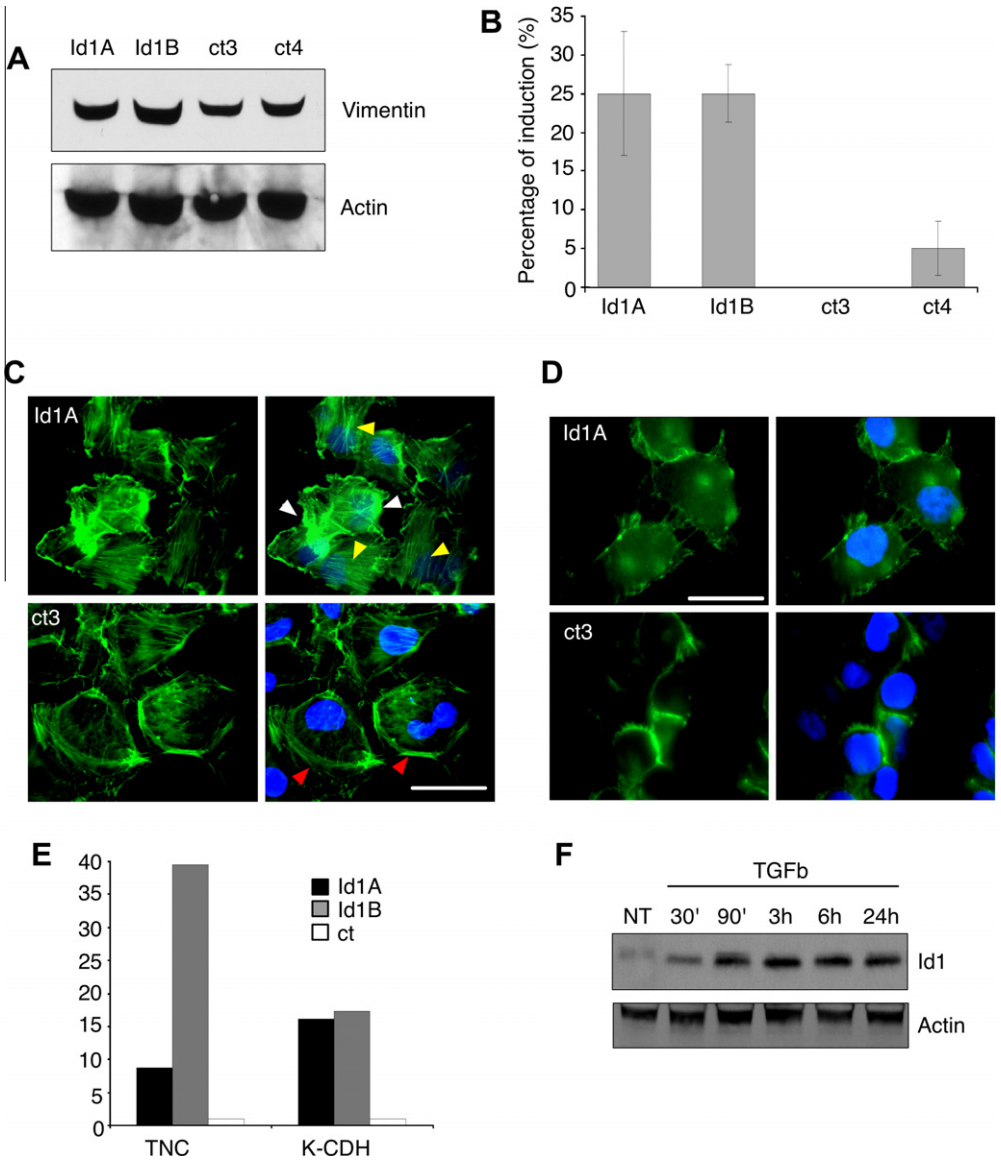

Fig. 6 – Analysis of mesenchymal markers in thyroid tumour cells upon Id1 overexpression. (A) Western blot analysis of Vimentin (upper panel) and Actin (lower panel) proteins in the indicated clones. (B) Quantification of the Western blot signals. The histograms represent the averaged percentage of Vimentin upregulation ± SD relative to ct3 clone ($n = 2$). (C) Immunofluorescence staining of the actin filaments in Id1A (upper panels) and ct3 (lower panels) cells, with Alexa 488-conjugated phalloidin (green). Red arrowheads indicate the cortical localisation of the actin filaments, below the cell membrane, in the ct3 cells. Yellow arrowheads indicate disorganised actin filaments that have lost their cortical localisation in Id1A cells. White arrowheads indicate large stress fibres formed by the actin filaments in Id1A cells. On the left panels for both Id1A and ct3 cells the nuclei position is shown (blue). Scale bar 50 μm. Magnification 1000×. (D) Immunofluorescence analysis of β-Catenin localisation in Id1A and ct3 clones. Green β-Catenin, blue DAPI. Scale bar 50 μm. Magnification 1000×. (E) Quantitative RT-PCR analysis of TNC and K-CDH in Id1A, Id1B and control clones. The results were normalised to the GAPDH levels. The bars represent the averaged fold induction of the indicated gene relatively to the value of the gene in the control. The control represents the average value of the gene in ct2, ct3 and ct4 clones. (F) Western blot analysis of Id1 expression in NthyOri 3.1 cells at different time points after TGFβ (5 ng/ml) exposure. The amount of loaded proteins is shown by the Western blot with anti-Actin antibody. We also analysed the E-cadherin levels but we were not able to detect any E-cadherin expression in either Id1 overexpressing or control clones.

In accordance with these microarray data we have shown a profound modification in the organisation of the actin filaments and in the expression of the intermediate filaments in Id1A cells as compared with control cells (Fig. 6A–C). We think that the altered interaction with the microenvironment and the rearrangement of the cytoskeleton mediates the morphological and growth modifications found in Id1 overexpressing cells. The gene expression analysis indicates that

Id1 upregulates three different ABC transporters (ABCA1, ABCB1 and ABCG2) described as responsible of the multidrug resistance, causing relapse of tumour after chemotherapy.[22,23] Further studies are required to define the molecular mechanisms underlying Id1 control of this large set of genes. However, several transcription factors were found in the microarray analysis as Id1 targets (RUNX2, MYB, LEF1, RARG, ETV-1, GATA6, MYC, etc.). RUNX2 is a member of the runt-related family of transcription factors whose expression is regulated by a number signalling pathways.[24–26] Several RUNX2 target genes are modified in the microarray list: MMP13, SPOCK3, SPP1, VCAN and GPC5[27–30] (Fig. 5). Vasko and colleagues, in an attempt to define mechanisms that control invasion of papillary thyroid carcinomas, have identified RUNX2 and its target SPP1/osteopontin as genes that are upregulated at the invading front of the tumours.[15] This observation is in agreement with Id1 controlling Runx2 expression, since we have observed that Id1 expression is enhanced at the invasive front of tumours.

We have also shown that upon Id1 overexpression thyroid tumour cells acquire a mesenchymal phenotype. The elongated-fibroblast morphology, the loss of cell–cell interaction and the increased motility observed in Id1 overexpressing cells, as compared to control cells, are all distinctive features of mesenchymal cells. Moreover, we have demonstrated that, five mesenchymal molecular markers were induced by Id1 in thyroid tumour cells (Fig. 6) and that Id1 is an early target gene of TGFβ, the most powerful inducer of EMT, in thyroid cells.

The major function of Id1 (which also means Inhibitor of Differentiation) is to inhibit cell differentiation. This does not seem the case in thyroid carcinomas. The overexpression of Id1 in thyroid tumour cells does not make the cells less mature but induces a trans-differentiation toward a mesenchymal phenotype. *In vivo*, we found that the undifferentiated (anaplastic) lesions have the lowest levels of Id1. However, Id1 expression is a common feature of all the non-anaplastic highly aggressive tumours that we have analysed. In these tumours Id1 positive cells tend to be localised predominantly at the edge of invasive regions. In agreement with our observations and in a similar localisation, evidence for epithelial–mesenchymal transition in thyroid tumours has been reported.[15,31] On the other hand, anaplastic tumours are characterised by the presence of very immature cells that have lost the epithelial characteristics, and it seems likely that this type of cell does not require mesenchymal features and, thus, Id1 function, to invade and metastasise other tissues.

Overall these experiments indicate that Id1 powers invasiveness of tumour cells by inducing their trans-differentiation toward a mesenchymal condition and identifies Id1 as a crucial mediator of thyroid tumour invasion and metastatic dissemination.

Authors' contribution

A.C. designed and performed the experiments, interpreted the results and wrote the manuscript. S.P. analysed the immunohistochemistry results and wrote the manuscript. G.G. and R.V. performed correlation of the *in vivo* analysis with the clinical information, and B.C. interpreted the *in vitro* results and supported technically the work.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.11.009.

REFERENCES

1. Ruzinova MB, Benezra R. Id proteins in development, cell cycle and cancer. *Trends Cell Biol* 2003;**13**(8):410–8.
2. Perk J, Gil-Bazo I, Chin Y, et al. Reassessment of id1 protein expression in human mammary, prostate, and bladder cancers using a monospecific rabbit monoclonal anti-id1 antibody. *Cancer Res* 2006;**66**(22):10870–7.
3. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. *Cell* 1990;**61**(1):49–59.
4. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature* 2005;**436**(7050): 518–24.
5. Perk J, Iavarone A, Benezra R. Id family of helix–loop–helix proteins in cancer. *Nat Rev Cancer* 2005;**5**(8):603–14.
6. Gupta GP, Perk J, Acharyya S, et al. ID genes mediate tumour reinitiation during breast cancer lung metastasis. *Proc Natl Acad Sci USA* 2007;**104**(49):19506–11.
7. Davies L, Welch HG. Increasing incidence of thyroid cancer in the United States, 1973–2002. *JAMA* 2006;**295**:2164–7.
8. Kondo T, Ezzat S, Asa SL. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. *Nat Rev Cancer* 2006;**6**(4):292–306.
9. Volante M, Collini P, Nikiforov YE, et al. Poorly differentiated thyroid carcinoma: the Turin proposal for the use of uniform diagnostic criteria and an algorithmic diagnostic approach. *Am J Surg Pathol* 2007;**31**(8):1256–64.
10. Fabien N, Fusco A, Santoro M, et al. Description of a human papillary thyroid carcinoma cell line. Morphologic study and expression of tumoral markers. *Cancer* 1994;**73**(8):2206–12.
11. Meireles AM, Preto A, Rocha AS, et al. Molecular and genotypic characterization of human thyroid follicular cell carcinoma-derived cell lines. *Thyroid* 2007;**17**(8):707–15.
12. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009;**9**(4):265–73.
13. Zeisberg M, Neilson EG. Biomarkers for epithelial–mesenchymal transitions. *J Clin Invest* 2009;**119**(6):1429–37.

14. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer* 2004;**4**(2):118–32.
15. Vasko V, Espinosa AV, Scouten W, et al. Gene expression and functional evidence of epithelial-to-mesenchymal transition in papillary thyroid carcinoma invasion. *Proc Natl Acad Sci USA* 2007;**104**(8):2803–8.
16. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell Res* 2009;**19**(2):156–72.
17. Kebebew E, Peng M, Treseler PA, et al. Id1 gene expression is up-regulated in hyperplastic and neoplastic thyroid tissue and regulates growth and differentiation in thyroid cancer cells. *J Clin Endocrinol Metab* 2004;**89**(12):6105–11.
18. Moschos SJ, Drogowski LM, Reppert SL, Kirkwood JM. Integrins and cancer. *Oncology* 2007:2113–20.
19. Murphy G. The ADAMs: signalling scissors in the tumours microenvironment. *Nat Rev Cancer* 2008;**8**:929–41.
20. Capparuccia L, Tamagnone L. Semaphorin signaling in cancer cells and in cells of the tumour microenvironment – two sides of a coin. *J Cell Sci* 2009;**122**:1723–36.
21. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumour microenvironement. *Cell* 2010;**141**:52–67.
22. Kimura Y, Morita SY, Matsuo M, Ueda K. Mechanism of multidrug recognition by MDR1/ABCB1. *Cancer Sci* 2007;**98**:1303–10.
23. Bertolini G, Roz L, Perego P, et al. Highly tumourigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci USA* 2009;**106**:16281–6.
24. Tou L, Quibria N, Alexander JM. Transcriptional regulation of the human Runx2/Cbfa1 gene promoter by bone morphogenetic protein-7. *Mol Cell Endocrinol* 2003;**205**: 121–9.
25. Gaur T, Lengner CJ, Hovhannisyan H, et al. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* 2005;**280**: 33132–40.
26. Li YL, Xiao ZS. Advances in Runx2 regulation and its isoforms. *Med Hypotheses* 2007;**68**:169–75.
27. Sato M, Morii E, Komori T, et al. Transcriptional regulation of osteopontin gene in vivo by PEBP2alphaA/CBFA1 and ETS1 in the skeletal tissues. *Oncogene* 1998;**17**:1517–25.
28. Wang X, Manner PA, Horner A, et al. Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. *Osteoarthritis Cartilage* 2004;**12**:963–73.
29. Gordeladze JO, Noël D, Bony C, et al. Transient down-regulation of cbfa1/Runx2 by RNA interference in murine C3H10T1/2 mesenchymal stromal cells delays in vitro and in vivo osteogenesis, but does not overtly affect chondrogenesis. *Exp Cell Res* 2008;**314**:1495–506.
30. Teplyuk NM, Haupt LM, Ling L, et al. The osteogenic transcription factor Runx2 regulates components of the fibroblast growth factor/proteoglycan signaling axis in osteoblasts. *J Cell Biochem* 2009;**107**:144–54.
31. Bai Y, Kakudo K, Nakamura M, et al. Loss of cellular polarity/cohesiveness in the invasive front of papillary thyroid carcinoma and periostin expression. *Cancer Lett* 2009;**281**:188–95.