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Spatial-temporal protein expression of inhibitor of differentiation-1 (Id1) during fetal embryogenesis and in different mouse and human cancer types

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Summary. Inhibitor of differentiation-1 (Id1) plays a role in cell proliferation, acquisition of epithelial to mesenchymal transition (EMT) features and angiogenesis. Id1 was shown to be expressed in some tumor types, mainly in advanced dedifferentiated stages. However, recent studies using a validated and highly specific monoclonal antibody against Id1 have challenged many of the results obtained by immunohistochemistry. The goal of our work was to perform a thorough analysis of Id1 expression in mouse embryos and adult tissues, as well as healthy and malignant mouse and human samples using this validated antibody (Perk et al., 2006). Our results show that Id1 was highly expressed in the oropharyngeal cavity, lung, cartilage and skin of E14 and E15 mouse embryos, but expression was progressively reduced in more developed embryos. Immunostaining only remained in epithelial cells of the gut and uterus of adult mice. Mammary MMTV-Myc and MMTV-Myc/VEGF transgenic mouse tumors, and squamous cell carcinomas of the lung induced by N-nitroso-tris-chloroethylurea (NTCU) were highly positive for Id1, unlike their respective healthy counterparts. Id1 immunostaining in a human tissue microarray (TMA) revealed strong expression in cancers of the oral cavity, bladder and cervix. Some tumor specimens of esophagus, thyroid and breast were also strongly positive. Our results suggest that Id1 is an oncofetal protein highly expressed in particular tumor types that should be reanalyzed in future studies using large cohorts of patients to reassess its diagnostic/prognostic value. Moreover, MMTV-Myc- and NTCU-induced tumors could serve as appropriate mouse models to study Id1 functions in breast and lung cancer, respectively.

Key words: Inhibitor of differentiation-1 (ID1), Mouse embryonic development, Histocore (H-score), Tissue MicroArray (TMA), Squamous cell carcinomas

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Introduction

The basic helix-loop-helix (bHLH) family of transcription factors is a group of proteins involved in a variety of cell functions. The HLH region of these proteins is responsible for the formation of homo or heterodimers with other transcription factors, whereas the basic region is a key domain for DNA binding and, therefore, for transcriptional regulation of critical genes (Benezra et al., 1990; Ruzinova and Benezra, 2003). The inhibitor of differentiation (Id) proteins, which belong to the HLH family, include four members (Id1 to Id4) that lack the DNA binding domain (Benezra et al., 1990). Id proteins homo or heterodimerize with other HLH transcription factors but, because of the lack of the basic domain, they prevent the binding of the complexes to the DNA. Id proteins have been involved in regulation of cell differentiation, angiogenesis and cancer (Benezra et al., 2001; Ruzinova and Benezra, 2003; Perk et al., 2005).

Id1 to Id4 have been reported to play important roles in embryogenesis, mainly in neural development (Lyden

et al., 1999; Wong et al., 2004). While Id1 and Id3 seem to have overlapping mRNA expression patterns during embryogenesis with high expression in E-12 embryos, Id2 is expressed in a similar spatial pattern but for a longer period of time (Jen et al., 1997). Id4 has a temporal expression pattern similar to that of Id2 but is mainly expressed in the central nervous system (de Candia et al., 2004). Id1 and Id3 double knockout mice are not viable and die during embryogenesis due to important defects in angiogenesis and neural differentiation (Lyden et al., 1999; Perk et al., 2005); however, mice with at least one Id1 or Id3 allele do survive but present critical defects in tumor neovascularization (Lyden et al., 1999).

Id1 is one of the best known members of the Id family, and its expression has been described in several tumor types (Perk et al., 2005; Ling et al., 2006). Diverse studies have proposed different key roles of Id1 in regulation of processes such as hematopoiesis (Cooper et al., 1997), cell cycle control (Zebedee and Hara, 2001), lineage commitment and differentiation (Wong et al., 2004; Jankovic et al., 2007), proliferation and migration (Huang et al., 2010), invasion and tumor progression (Wong et al., 2004), angiogenesis (Benezra et al., 2001) and therapy resistance (Ponz-Sarvise et al., 2011) .

Immunohistochemical studies suggest that Id1 is a useful marker in cancer prognosis and prediction of response to treatment, but its potential clinical value as a therapeutic target is still unclear, due in part to the variable and in some cases contradictory results obtained from these immunohistochemical studies. Recent work (Perk et al., 2006; Lowery et al., 2010) suggests that this discrepancy could be caused by the inespecifity of the polyclonal antibodies used in most of those studies, which utilized an antibody that renders positive bands by western blot in samples from Id1 knockout mice, and unexpected strong cytoplasmatic staining (Perk et al., 2006). A new rabbit monoclonal antibody highly specific for Id1 recently developed (commercially available now from Biocheck, CA, USA) gives a clear nuclear staining and no band in Id1 knockout tissues by western blot (Perk et al., 2006).

Some proteins expressed during embryogenesis lose expression in healthy adult tissues, but are re-expressed during tumorigenesis, particularly in more undifferentiated stages (Ariel et al., 2000; Calvo and Drabkin, 2000; Monk and Holding, 2001). Examples of such oncofetal proteins are the carcinoembryonic antigen (CEA), alpha fetoprotein (AFP) (Wang et al., 1990) or Cripto-1 (Strizzi et al., 2005). This suggests that advanced tumors may acquire embryogenic properties, including mesenchymal features, motility and proliferation. In addition, several studies have proposed some of these oncofetal proteins as tumor biomarkers and prognostic indicators in different tumor types (Bianco et al., 2005; Yang et al., 2005; Xi et al., 2007). Id1 might be included in this group of possible oncofetal tumor biomarkers (de Candia et al., 2004; Kamalian et al., 2008) and possible targets for cancer therapy (Fong et al., 2004). The oncofetal features of Id1 are supported by the fact that its expression is scarce in adult normal tissues but increases in tumors such as oral squamous cell carcinoma (Dong et al., 2010), colorectal cancer (Meteoglu et al., 2008), prostate cancer (Coppe et al., 2004; Forootan et al., 2007), breast cancer (Fong et al., 2003) or non-small cell lung cancer (NSCLC) (Ponz-Sarvise et al., 2011). Moreover, Id1 induces cell proliferation and epithelial to mesenchymal transition (EMT) (Cheung et al., 2011), typical features of embryonic cells.

Given the lack of information of Id1 protein expression in embryos and the conflicting results obtained in tumors, despite its apparent potential as a marker of poor prognosis, the aim of this study was to perform a thorough characterization of Id1 protein expression in mouse embryos and mouse/human adult healthy and cancerous tissues to validate Id1 as an oncofetal protein with a reliable antibody. To this end, we have used the highly specific monoclonal antibody 195-14 (Biocheck, CA, USA).

Materials and methods

Histological samples

Mice fetuses at different stages of development (from 12.5 to 19 days of gestation (E-12.5 to E-19)) and newborn mice (P-1) were obtained from pathogen-free Swiss mice bred at CIFA (Centro de Investigación Farmacológica Aplicada, University of Navarra, Pamplona, Spain) and used to evaluate Id1 expression during mouse development. Samples were fixed in 10% formalin for 24 hours and then embedded in paraffin. Additionally, paraffin sections of mouse prostate tumors from either transgenic mice (TRAMP) (Gingrich et al., 1996) or xenograft models resulting from the injection or Pr14 cells (Calvo et al., 2002) were studied; also, mammary gland tumors from MMTV-Myc and MMTV-Myc VEGF (Calvo et al., 2008) transgenic mice (kindly donated by Michael Johnson, University of Georgetown, USA), N-nitroso-tris-chloroethylurea (NTCU) (Rehm et al., 1991) and urethane-induced lung tumors (Yakovlev and Pavlova, 1997) (generously provided by Marta Larráyoz, CIMA, University of Navarra, Spain) and liver with metastatic nodules from colon carcinomas (Zhang et al., 2010) (kindly donated by Idoia González, CIMA, University of Navarra, Spain) were used as well.

Furthermore, a Tissue MicroArray (TMA) commercially available including normal adult mouse tissues (AMS541; Biomax, Rockville, MD) and a TMA including normal and malignant human tissues (MTU951; Biomax, Rockville, MD) were utilized. Mouse samples from non-commercial origin were obtained under protocols approved at our Institution.

Immunohistochemistry

Paraffin sections 4 μm in thickness were deparaffinized by heating for 20 min at 60°C and

immersion for 30 min in xylene. Endogenous peroxidase was blocked with a 3% hydrogen peroxide solution and sections were then hydrated trough a graded series of ethanol and placed in distilled water. Antigen retrieval was done in a microwave oven with 0.01 M EDTA at pH 8. The rest of the immunohistochemical staining process was different depending on the origin of the samples (mouse or human). Nonspecific binding sites of mouse tissues were blocked with 5% swine normal serum in PBS (phosphate buffered saline) for 30 min and incubated with the primary antibody (rabbit monoclonal anti-mouse/human Id1, clone 195-14; Biocheck, CA, USA) at 1:50 dilution in PBS overnight at 4°C. Slides were then rinsed in PBS and incubated for 30 min with Envision anti-rabbit system (Dako, Denmark). Human samples were incubated with the same primary antibody at 1:100 dilution in Dako Real Antibody Diluent (Dako, Denmark) overnight at 4°C and then rinsed in PBS. Detection of primary antibody was carried out with the Advance™ HRP system (Dako, Denmark), consisting of an initial 30 min incubation with HRP link followed by a second 30 min incubation with HRP enzyme. Peroxidase activity was developed with DAB (3,3'-diamino-benzidine; Dako, Denmark) in all samples, and then slides were counterstained with haematoxylin, dehydrated through graded alcohols and xylene, and cover-slipped with DPX mounting medium (VWR, England).

Immunostaining evaluation

Evaluation of Id1 expression was based on a semiquantification method previously described to score the extension and the intensity of immunostaining (Pajares et al., 2006). The extension was expressed as a percentage of cells which present staining from the total amount of cells in the area of study. The intensity was classified as negative, weak (+), moderate (2+) and strong (3+) by comparing it with a positive control processed in parallel with the experimental samples. A final score was given to each area of study after multiplying the percentage of positive cells by the intensity of the staining.

RNA extraction and real time RT-PCR

Total RNA was extracted from paraffin-embedded wild type E12, E13, E15 and E17 embryos and P1 mice. Id1 KO E12 paraffin-embedded embryos were included as well as controls. 4x20 μm-thick paraffin-embedded unstained sections were cut and placed in 1.5 ml vials. RNA was extracted with the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Applied Biosystems, CA, USA) according to manufacturer's instructions. Briefly, the sections were deparaffinized in 1 mL 100% Xylene and incubated at 50°C for 3 h. Following centrifugation, the pellet was washed twice with 1 mL 100% ethanol and air dried for 10 min. 200 μL of digestion buffer was added to the sample together with 4 μL of protease and incubated for 15 min at 50°C, followed by 15 min at 80°C. For nucleic acid isolation, 240 μL of isolation buffer and 550 μL of 100% ethanol were added, mixed by pipetting and then washed through a filter cartridge. RNA concentration was quantified using a Nanodrop Spectrophotometer (NanoDrop Technologies, Delaware, USA).

cDNA was synthesized with the high-capacity cDNA Archive Kit (Applied Biosystems, CA, USA). Reverse transcriptase reactions were performed with 1 μg of RNA samples using the protocol recommended by the manufacturer. Each cDNA sample was analyzed in triplicate using the Applied Biosystems 7300 Sequence Detection system. Quantification of Id1 mRNA levels was carried out with Power SYBR Green PCT Maxter Mix (Applied Biosystems). The sequence of Id1 primers were as follows: TGGAGCTGAACTCGGAGTCTG (FW); ACGCATGCCGCCTCGGCCGTC (RE). GAPDH was used as an endogenous control and data obtained was represented as mRNA levels ($2^{\Delta Ct}$).

Results

Id1 is highly expressed in E14-15 mouse embryos

We first studied Id1 expression in mouse embryos from day 12.5 of development (day at which the primordial organs can be distinguished) until the end of gestation. Our analysis also included data from P1 (day 1 postnatal). As expected, immunoreactivity was always found in the cell nucleus and, in some cases (always with low intensity) in the cytoplasm. Table 1 shows a summary of the average results obtained after histoscore (H-score) semiquantification for Id1, in at least 3 mice per day of gestation. Immunoreactivity found in embryos reached its highest expression (which includes both intensity and percentage of immunolabeled cells) in E14 and E15 embryos. Strong Id1 expression (H-score >120) was observed for the oropharyngeal cavity and ear regions, cells surrounding the root sheath of the vibrissae (Fig. 1A,B) and hair follicules, basal stratum of the skin (Fig. 1C), and muscle. Other tissues and structures, such as the eye (Table 1, Fig. 1D) and cartilage (Table 1, Fig. 1E,F) also showed strong Id1 labeling. High expression of Id1 mRNA levels in E15 embryos (as compared to other stages of fetal development and P1 mice) was corroborated by real time RT-PCR (Fig. 2A). In addition, both immunohistochemistry (data not shown) and real time RT-PCR (Fig. 2B) in E12 Id1 knock-out mice revealed, as expected, a total absence of Id1 expression, as compared to E12 Wild type embryos.

Immunoreactivity for the mouth area and cells surrounding the root sheath of the vibrissae was maintained throughout the entire gestational period, although was sharply reduced in P1 embryos (Table 1). Id1 expression in the rest of the structures analyzed progressively diminished as development progressed. None of the tissues studied had strong expression (higher than 90 H-score value) in P1 embryos (Table 1).

Expression of Id1 in healthy and malignant mouse adult tissues

We analyzed some cancerous mouse tissues (in at least 3 different specimens per tumor or healthy tissue type) and compared their Id1 staining with their normal counterparts. Results of the semiquantification are summarized in Table 2. Whereas the normal mammary gland was completely devoid of staining (Fig. 3A), mammary tumors from the highly malignant transgenic mouse models MMTV-Myc and MMTV-Myc/VEGF showed an average score of 33.4, and 31, respectively (Table 2). These tumors had extensive areas of intense Id1 staining in the nuclei of both tumor and endothelial cells (Fig. 3B), whereas other areas had no labeling in tumor cells (but were always positive for endothelial cells), thus revealing the heterogeneity of Id1 expression.

Two types of lung cancer models were used in our study: The NTCU-induced model, which gives rise to squamous cell carcinomas and the urethane-induced model, which generates adenocarcinomas. In agreement with what we previously published for human NSCLC (Ponz-Sarvise et al., 2011), squamous cell carcinomas showed much stronger Id1 expression (35.5 H-score) than adenocarcinomas (10.83 H-score) (Table 2, Fig. 3C,D). Nuclear staining of tumor cells was found in some areas of the squamous cell tumors and most malignant endothelial cells within the tumor. Adenocarcinomas showed little immunolabeling in tumor cells but endothelial cells were generally positive. The normal lung displayed occasional weak staining in endothelial cells and, rarely, in neumocytes.

Unlike breast and lung cancer mouse models, both models for prostate cancer and colon cancer metastatic to the liver showed fainter staining (Table 2). The normal prostate was characterized by an occasional weak staining of epithelial cells, whereas the transgenic model TRAMP showed some intense areas of staining in prostate intraepithelial neoplastic (PIN) lesions and tumors (with a focal labeling), whereas other areas were devoid of immunoreactivity. The Pr14 prostate cancer xenograft model showed weak labeling in tumor cells, but both prostate models had positivity for endothelial cells. The MC38 metastatic colon carcinoma model displayed almost no immunoreactivity.

In addition, we used a tissue microarray (TMA) to analyze Id1 protein expression in mouse normal tissues (Table 3). Remarkably, semiquantification of immunostaining revealed high expression in the epithelium of intestine, colon and esophagus (Fig. 3E-G, respectively), despite the lack of staining of these organs in embryos. The epithelial layer of the esophagus, uterus (Fig. 3H), and skin (particularly the basal compartment) was also strongly labeled, whereas staining for glands such as pancreas, salivary glands, prostate, and liver was negative or very weak (in isolated positive cells with a score lower than 30). Brain, cerebellum, testis, lung, muscle (skeletal and cardiac) and lymphoid organs (spleen and thymus), lacked immunostaining as well.

Heterogeneity of Id1 expression in human tissues

Expression of Id1 in healthy and malignant human tissues was studied with a TMA. Based on this TMA, a quite heterogeneous pattern of expression seemed to be a common feature. Id1 expression score in almost all normal tissues was weaker than that found in their

Table 1. Semiquantification data on Id1 protein expression in embryos. In most organs, maximal immunostaining is found in E14-E-15 embryos. ND, Non determined

Organ/Structure	E-12,5	E-13	E-14	E-15	E-16	E-17	E-18	E-19	P-1
Brain	30.83	11.50	60.00	21.79	5.33	4.78	5.33	47.67	3.50
Eye	N.D.	2.50	N.D.	120.00	75.00	60.00	80.83	40.00	N.D.
Ear	N.D.	80.00	75.00	135.21	31.67	20.50	60.00	N.D.	16.67
Mouth (teeth, lips)	146.67	101.88	200.00	88.33	125.00	65.13	N.D.	72.50	62.92
Vibrissae	N.D.	N.D.	130.00	191.25	133.33	82.60	153.33	185.00	55.83
Submaxilary gland	N.D.	70.00	85.00	40.00	42.50	30.30	10.00	1.50	2.33
Heart	0.33	2.00	30.00	4.83	23.33	12.00	N.D.	2.50	10.67
Lung	36.25	40.83	150.00	65.83	60.83	33.20	34.50	1.33	6.25
Liver	1.67	1.00	11.50	10.00	2.83	3.40	0.33	36.67	0.54
Kidney	5.00	21.25	N.D.	30.00	N.D.	N.D.	5.83	31.67	N.D.
Intestine	10.00	7.67	23.33	N.D.	41.67	26.00	N.D.	8.33	39.17
Pancereas	N.D.	8.50	33.75	7.50	33.33	4.67	N.D.	N.D.	1.00
Skin	105.00	0.63	86.67	288.36	98.33	97.50	34.17	8.33	35.90
Cartilage	46.67	50.63	56.67	99.36	3.92	34.50	5.50	Neg.	9.88
Stomach	N.D.	95.00	N.D.	48.33	41.67	30.50	30.50	N.D.	2.33
Nostrils	45.50	38.33	N.D.	183.33	35.33	76.67	0.00	N.D.	49.17
Muscular tissue	15.00	10.88	N.D.	164.64	66.67	81.67	35.00	N.D.	13.67

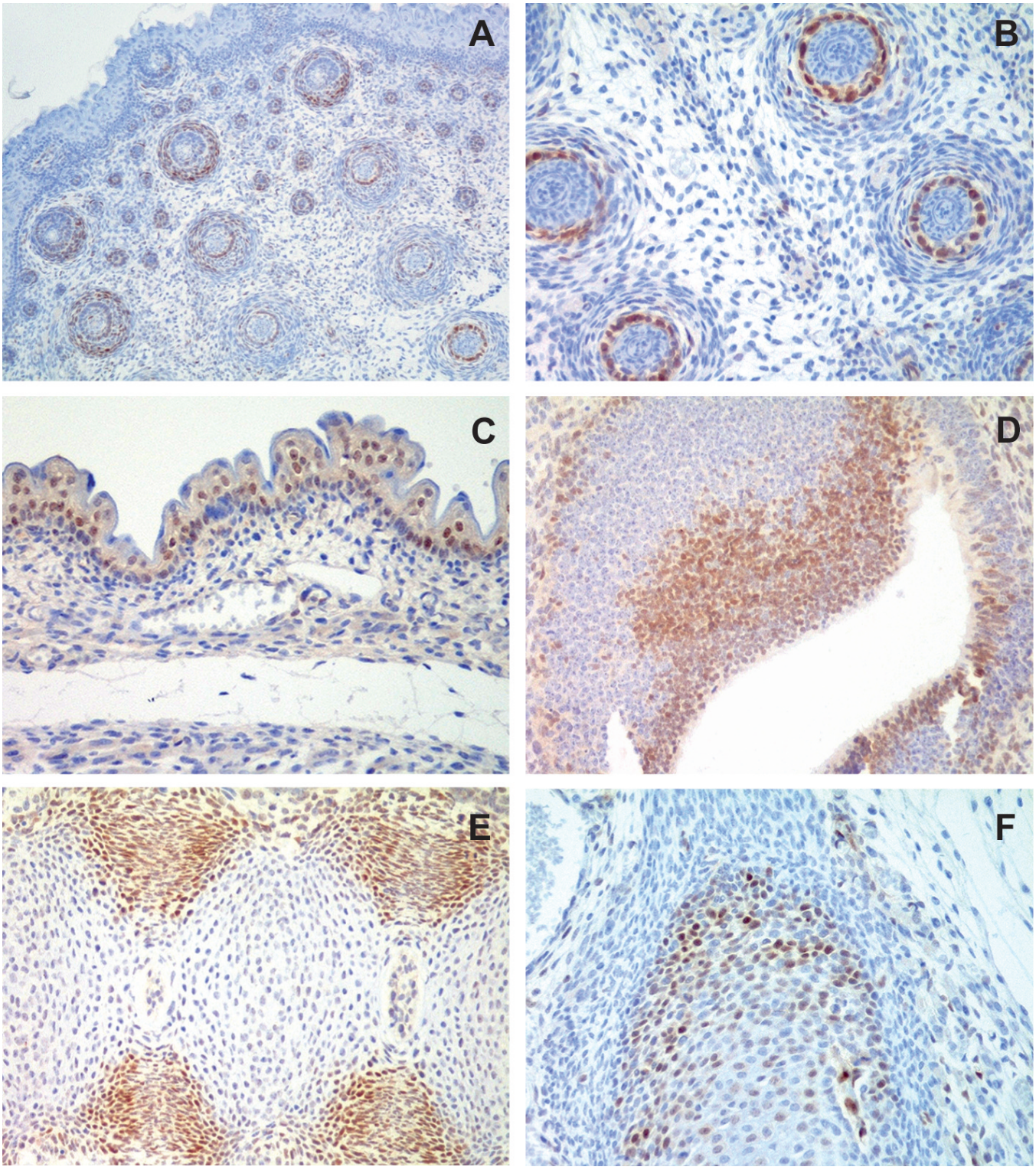

Fig. 1. Immunohistochemical staining for Id1 at different stages of embryo development. **A and B.** Strong immunostaining in vibrissae of an E18 embryo. **C.** Staining in basal stratum of skin of an E17 embryo. **D.** Id1 positivity in developing eye of an E16 embryo. **E.** Id1 expression in tail cartilage of an E16 embryo. **F.** Staining in cartilage of an E18 embryo. A, x 100; B-F, x 200

corresponding tumor counterparts, with the exception of normal stomach, colon and rectum (Fig. 4). In spite of the fact that several normal tissues showed Id1 staining, it was usually restricted to endothelial cells of blood vessels. In cancerous samples, expression was observed, as for mouse tissues, in both tumor and endothelial cells.

Organs with very strong intensity of staining included the epithelia of malignant bladder, cervix and oropharyngeal cavity, all of them lined by squamous or transitional epithelia. The score in tumors was different for each individual case in the vast majority of anatomic sites present in this TMA (Fig. 4), thus reflecting a heterogeneous pattern of expression. For instance, normal cerebellum (Fig. 5A) and cerebellum meningioma (data not shown) showed no staining, while a strong Id1 expression was detected in cerebellum astrocytoma (Fig. 5B). Similarly, nasopharyngeal carcinoma showed a high score, whereas hard palate adenocarcinoma and tongue squamous cell carcinoma displayed very weak Id1 expression (Fig. 4). In non malignant bladder, staining was found to be restricted to the transitional epithelium (Fig. 5C) while one of the two cases of bladder transitional cell carcinoma showed the highest score of Id1 staining (Fig. 5D). One of the two cases of uterine cervical squamous cell carcinoma displayed a strong immunoreactivity (Fig. 5E) whereas normal uterus-cervix showed no significant staining (data not shown).

Consistent with our results found in lung cancer mouse models, Id1 immunostaining was higher in human lung squamous cell carcinoma cores than in lung adenocarcinomas (Fig. 4). The normal lung showed no Id1 immunoreactivity (Fig. 5F) and adenocarcinomas had a very weak staining in some tumor cells and a higher staining in endothelial cells of blood vessels (Fig. 5G), while lung squamous cell carcinomas presented a strong labeling in both cell types (Fig. 5H).

Table 2. Different Id1 expression values in normal and tumor mouse tissues.

Normal tissue	Score (mean ± SEM)	Tumor	Score (mean ± SEM)
Mammary gland	0	MMTV-Myc	33.4±9.96
		MMTV-Myc/VEGF	31±8.46
Prostate	1	TRAMP	13.5±6.5
		Pr14 xenografts	1.5±0.5
Lung	1.25±0.48	NTCU-induced	35.5±6.52
		Urethane-induced	10.83±3.28
Liver	0	Liver metastasis	2.17±0.91

Table 3. Values of Id1 histoscore in normal mouse tissues of the TMA. Neg, Negative (no expression).

Tissue	Score	Tissue	Score
Cerebellum	Neg	Ovary	7
Eye	Neg	Uterus	**37.5**
Esophagus	15	Pancreas	Neg
Stomach	9.3	Prostate	Neg
Small intestine	**33.5**	Salivary gland	Neg
Colon	**57.5**	Skin	20.7
Heart	1	Spleen	4.3
Kidney	2	Striated muscle	Neg
Liver	Neg	Testis	Neg
Lung	Neg	Thymus	Neg

Discussion

The study of oncofetal proteins is providing useful new potential cancer biomarkers, prognostic indicators (Bianco et al., 2005; Yang et al., 2005; Xi et al., 2007) and candidates for novel therapies (de Candia et al., 2004; Fong et al., 2004; Kamalian et al., 2008). Tumors (especially in their advanced stages) and embryonic cells share certain properties, such as a high proliferation rate, cell motility (Ruzinova and Benezra, 2003) and self-renewal capacity (Barrett et al., 2012; Romero-Lanman et al., 2012). We have described in the present study the spatial and temporal expression of Id1 in mouse embryos and in normal and cancerous mouse and human

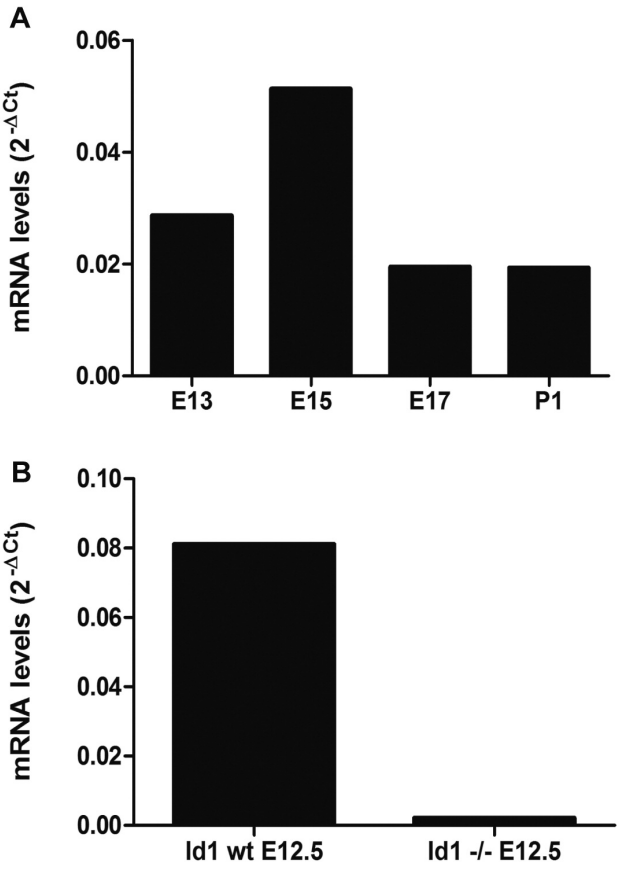

Fig. 2. Expression of Id1 in embryos and P1 mice. **A.** High mRNA Id1 expression was found in E15 embryos. **B.** Real time RT-PCR confirmed the lack of Id1 expression in Id1 KO E12 embryos.

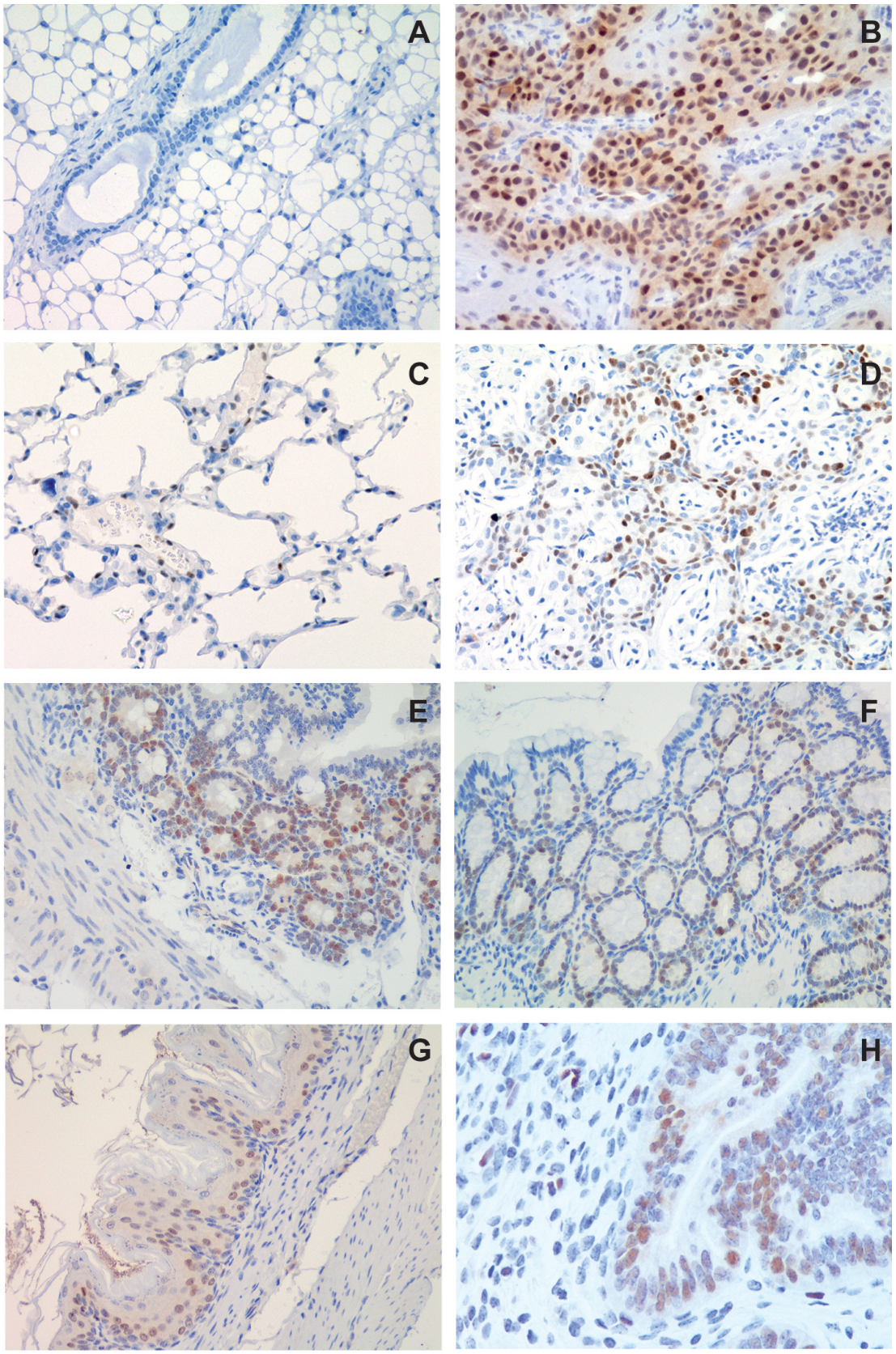

Fig. 3. Immunohistochemical staining for Id1 in different normal and tumor tissues. **A.** Absence of Id1 immunostaining in normal adult mammary gland. **B.** Strong immunoreactivity in MMTV-Myc mammary tumors. Normal lung showed weak staining in some endothelial cells of blood vessels **(C)**, whereas tumor cells of lung squamous cell carcinoma showed a more intense staining **(D)**. Immunostaining for Id1 in basal area of the epithelium of intestinal **(E)**, colon **(F)**, esophagus **(G)** and uterus **(H)** of adult mouse. A-H, x 200

specimens. *In situ* hybridization was previously used to determine the transcriptional patterns of expression of Id proteins in mouse embryos (Wang et al., 1992). We have shown here by immunohistochemistry, in keeping with results reported at mRNA level, a progressive increase in Id1 expression towards day 14-15 of embryogenesis followed by a progressive reduction, with low expression remaining after birth. Most healthy adult mouse and human tissues display absence or low Id1 immunoreactivity, but a variety of malignant tissues reactivate Id1 expression.

Increased Id1 immunostaining in different cancer types compared to their normal counterparts has been previously described (Ruzinova and Benezra, 2003; Perk et al., 2005). However, many of those studies have been carried out with antibodies with limited specificity, as previously demonstrated (Perk et al., 2006). Therefore, a reassessment of the protein expression using more specific antibodies in large cohorts of patients would be necessary to validate the reported results; this is particularly crucial in cases where Id1 has been proposed as a prognostic marker. In our study, using highly specific antibodies (Perk et al., 2006), we found the expected nuclear staining for this transcription factor regulator, in a variety of cancer types. Our study is intended to be, in part, a re-screening process to validate Id1 protein expression in a wide range of human and mouse normal and malignant samples. However, we acknowledge that due to Id1 expression heterogeneity, the use of TMAs in human samples, which include a limited number of cores per tumor type, obviously limits the conclusions about Id1 expression.

In agreement with Id1 being an oncofetal protein, we have found some tissues during embryonic development where strong Id1 expression seems to correlate with high immunoreactivity in their tumor analogs (but not in healthy tissues). For example, a strong Id1 staining in the oropharyngeal cavity remains during practically the whole developmental period, and a high expression was found in oropharyngeal tumors as well. Although our TMA did not include osteosarcoma samples, we found that Id1 was highly expressed in the developing cartilage and Endo-Munoz et al. have described overexpression in osteosarcoma samples (Endo-Munoz et al., 2010). Some healthy adult tissues retain Id1 expression, most likely in cells with self-renewal capacity and responsible for maintaining the tissue proliferative activity, such as the basal layer of skin, and the crypts of the intestine and colon. Indeed, Id1 has been shown to be involved in promoting cell proliferation and inhibiting apoptosis (Nishimine et al., 2003; Hau et al., 2011). Moreover, in some adult tissues, such as the neural stem cells, Id1 confers self-renewal capacity, a characteristic of stem cell identity (Nam and Benezra, 2009). Similar findings have been reported in human prostate samples in which a high proportion of non-tumor glands showed strong Id1 expression in cells located in the basal layer (Perk et al., 2006).

The present study corroborates our previous finding showing high Id1 expression in squamous lung carcinomas (Ponz-Sarvise et al., 2011). In addition, we report here high Id1 immunoreactivity in the NTCU-induced carcinogenic model (with squamous histology) compared to the urethane-induced model (with

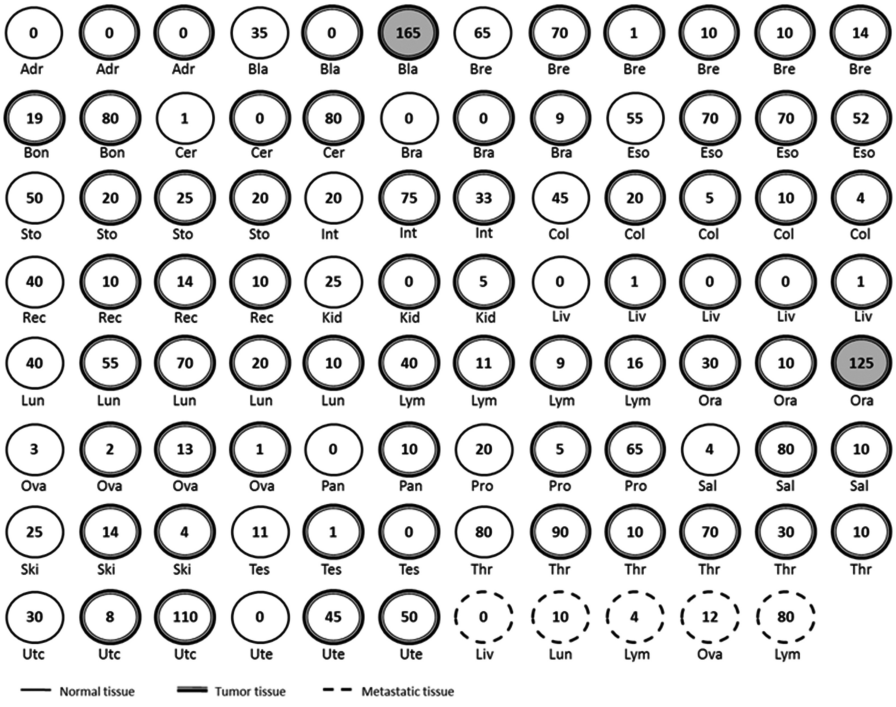

Fig. 4 Semiquantification of Id1 immunostaining in each of the cores of the human TMA (including normal, tumor and metastatic samples). Tumors of the bladder, uterus and oropharyngeal cavity display strong Id1 labeling. Adr- Adrenal gland, Bla- Bladder, Bre- Breast, Bon- Bone, Cer- Cerebellum, Bra- Brain, Eso- Esophagus, Sto- Stomach, Int- Small intestine, Col- Colon, Rec- Rectum, Kid- Kidney, Liv- Liver, Lun- Lung, Lym- Lymph node, Ora- Oronasopharynx, Ova- Ovary, Pan- Pancreas, Pro- Prostate, Sal- Salivary gland, Ski- Skin, Tes- Testis, Thr- Thyroid, Utc- Uterus-cervix, Ute- Uterus-endometrium.

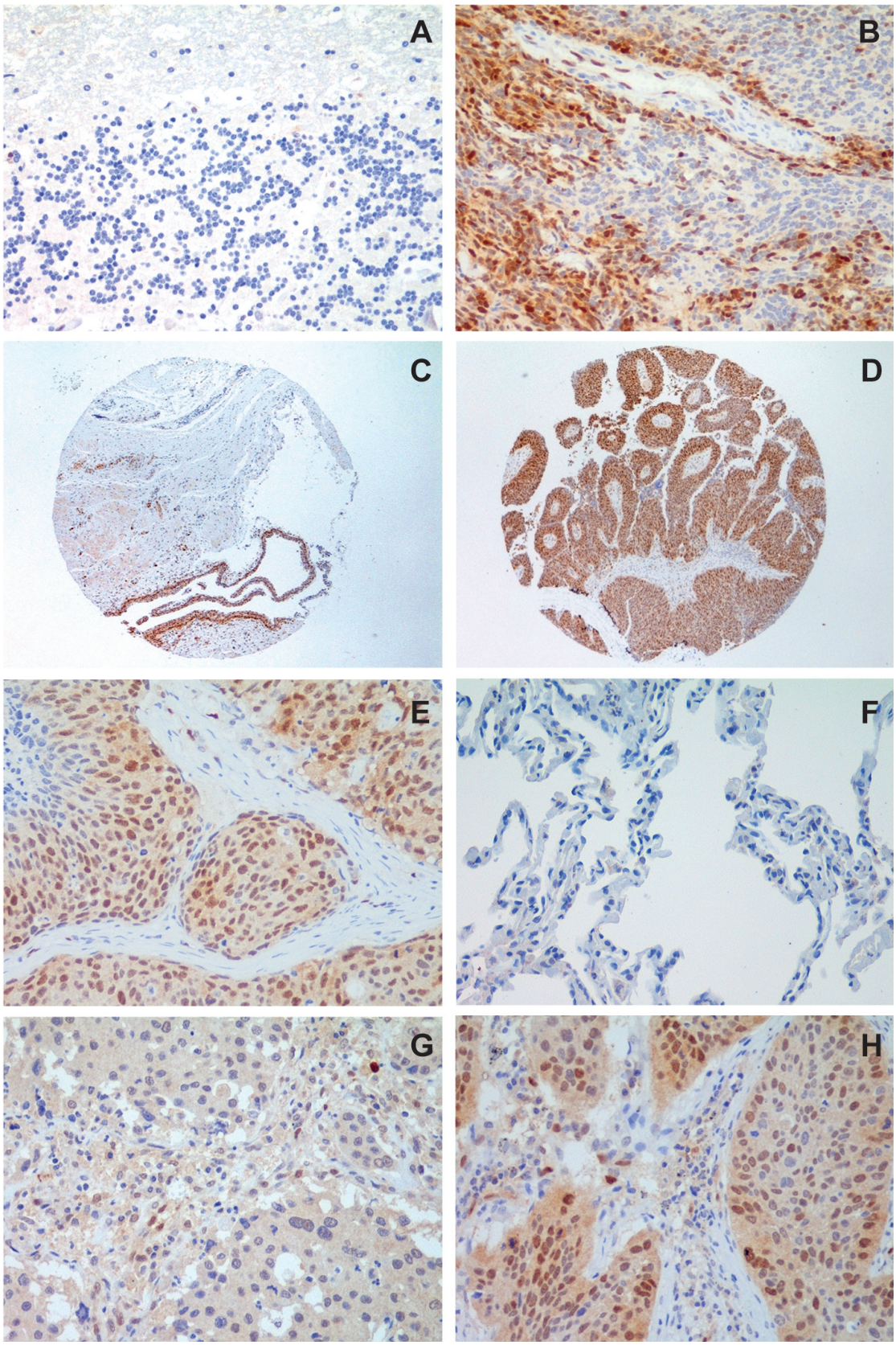

Fig. 5. Immunohistochemical staining for Id1 in different normal and tumor human tissues. **A.** Normal cerebellum lacking Id1 expression. **B.** Cerebellum astrocytoma showing strong Id1 expression in tumor cells and blood vessels. **C.** Expression in non-malignant bladder is restricted to the epithelium. **D.** In bladder transitional cell carcinoma, Id1 expression is clearly increased with respect to the normal bladder. **E.** Cervical squamous cell carcinoma also showed strong Id1 expression. **F.** Normal lung showed no Id1 immunoreactivity. **G.** Adenocarcinomas displayed a weak staining in tumor cells, whereas lung squamous cell carcinomas **(H)** had high Id1 protein levels. A, B, E-H, x 200; C, D, x 40

adenocarcinoma histology). In spite of the lower expression of Id1 in human lung adenocarcinomas with respect to squamous histology, only expression in adenocarcinomas correlates with poor prognosis (Ponz-Sarvise et al., 2011). Pillai et al. have recently demonstrated that cell signaling activated by EGFR and the nicotinic acetylcholine receptor (nAChR) increases Id1 levels, thus facilitating growth and metastasis in NSCLC (Pillai et al., 2011). Id1 levels were found to be elevated in tumors from mice that were exposed to nicotine in this same study. This observation could be a potential explanation for the expression of Id1 protein in non-tumor bladder transitional urothelium in the present study, since the carcinogens of tobacco exposure affect the urinary bladder and, therefore, stimulate Id1 expression long before an apparent tumor lesion is detected. Interestingly enough, other recent pieces of evidence have shown a differential molecular and functional behavior between adenocarcinomas and squamous cell carcinomas of the lung (Hida and Hamada, 2012; Pajares et al., 2012). In fact, the biology of Id1 in lung adenocarcinomas leads to a highly malignant tumor behavior and the molecular mechanisms playing a role in this process are completely unknown. Thus, both NTCU- and urethane-induced mouse models could be useful to address this issue.

Analysis of mammary cancers from both transgenic MMTV-cMyc and MMTV-cMyc/VEGF mouse models in the present work revealed a very strong Id1 immunoreactivity, compared with the normal mammary gland from virgin female mice, which was devoid of expression. Swarbrick et al. (2005) reported that c-Myc regulates Id1 in mammary gland cells. Inhibition of c-Myc with siRNAs results in Id1 downregulation, whereas its ectopic expression triggers a rapid Id1 induction. Interestingly, abrogation of Id1 levels in these cells causes similar effects to those found for c-Myc: decrease in cyclins D1 and E, reduced phosphorylation of pRb and abolition of cyclin-E/Cdk2 activity. It has also been shown (Rothschild et al., 2012) that increased expression of c-Myc represses transcriptionally miR29 (which directly targets Id1) and, as a consequence, causes Id1 upregulation. In lung cancer, reduction of c-Myc levels leads to a parallel decrease in Id1 levels, and both genes are involved in nicotine and EGF-mediated signaling cascades (Pillai et al., 2011). Immunohistochemical studies in breast cancer have shown that both Id1 (Perk et al., 2006; Gupta et al., 2007) and c-Myc (Pietilainen et al., 1995; Xu et al., 2010) are highly expressed in the aggressive triple negative (estrogen-receptor, progesterone-receptor and erbB2/Her-2) tumors. Id1 protein expression has been correlated with poor prognosis in breast cancer (Schoppmann et al., 2003). The relationship between c-Myc protein expression and prognosis produced conflictive results, but Myc gene amplification has been clearly associated with poor outcome (Chen and Olopade, 2008). Therefore, the activity of Id1 and c-Myc proteins seems to be closely related in breast cancer.

In agreement with the key role of Id1 in breast cancer, Massague's group reported that Id1 and Id3 are selective mediators of lung metastatic colonization in triple negative breast tumors (Gupta et al., 2007). Functional studies in mice demonstrated that Id1 and Id3 sustained proliferation during the early stages of metastatic colonization, subsequent to extravasation into the lung parenchyma (Gupta et al., 2007). Other studies have shown that Id1 is involved in epithelial-mesenchymal-transition (EMT) leading to cell migration of breast cancer cells (Tobin et al., 2011). Apart from breast cancer, Id1 has been involved in other aspects of the normal mammary gland physiology, such as normal development and remodeling during pregnancy. Id1 expression was inversely correlated with that of ß-casein, a marker of breast epithelial differentiation (Lin et al., 1999). All these data underscore the importance of Id1 in breast biology.

We have also validated the specific localization of Id1 protein in the nuclei of malignant endothelial cells but not in normal endothelial cells. This pattern was maintained in both human and mouse tumors. Previous work has demonstrated that Id1 plays, together with Id3, a critical function in tumor angiogenesis (Lyden et al., 1999). Indeed, $Id1^{+/-}Id3^{-/-}$ mice challenged with tumors were unable to sustain tumor growth and angiogenesis (Lyden et al., 2001). These properties launched Id1 as a target for therapy. The potential problem for targeting Id1 is its nuclear localization, which makes it difficult for the drugs to access the target. To solve this problem, peptide-conjugated antisense oligonucleotides were designed to target specifically Id1 in endothelial cells (Henke et al., 2008). Efficient delivery, Id1 reduction and antitumor efficacy were obtained by this method in *in vivo* cancer models, with a particularly dramatic antitumor effect when combined with the Hsp90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (Henke et al., 2008). We and others have demonstrated that some chemotherapy and targeted drugs cause a decrease in Id1 levels. Such is the case for 2-methoxyestradiol (Huh et al., 2006), dasatinib and saracatinib (Rothschild et al., 2012), and rapamycin (Jankiewicz et al., 2006). The molecular mechanism involved in Id1 down-regulation is still unknown, but it is likely that an indirect effect through impairment of receptor tyrosine kinases (such as EGFR) (Pillai et al., 2011) and Src (Rothschild et al., 2012) pathways occurs.

In summary, Id1 is widely expressed during embryogenesis and tumorigenesis but not in the majority of healthy tissues. The immunostaining pattern of expression in tumors is heterogeneous, which warrants future studies to determine the clinical implications for this disparity. Id1 is highly expressed in mammary tumors from MMTV-c-Myc and MMTV-c-Myc/VEGF mice, making this mouse model a very convenient tool to analyze the close relationships that exists between c-Myc and Id1 in advanced breast cancer. In addition, both NTCU- and urethane-induced lung cancer mouse models could be useful models to study the role of Id1 in human

lung carcinogenesis.

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