

## Reg I protein in healthy and seminoma human testis

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**Summary.** Regenerating gene (Reg), encodes a secretory protein with growth and differentiation stimulating effects mostly in digestive tissues. Overexpression of Reg proteins and specifically of Reg I, one member of the Reg family, is associated with several human diseases and cancers. In the present study we analyzed the expression of Reg I in normal rodent and human testes where germ cells normally proliferate and differentiate into spermatozoa, and in seminoma testis, the most common cancer of young men. Western blot analyses demonstrated the presence of a specific band at 19 kDa in human and rodent testis extracts. Immunofluorescence and deconvolution microscopy demonstrated that Reg I was present within the seminiferous tubules in both Sertoli and germ cells. By using a Sertoli cell line we demonstrated that Reg I was localized at the plasma membrane even in the absence of contact between neighboring cells and appeared before the tight junction associated protein ZO-1 was revealed at this location. Reg I was strongly expressed in human seminoma testis tissue and in a human tumor germ cell line where the immunoreactive signal was mainly detected at the plasma membrane level. These data showing for the first time the weak presence of Reg I in the normal testis and its strong expression in the testis cancer suggest a potential role of Reg I in normal and neoplastic germ cell proliferation.

**Key words:** Reg I, Testis, Seminoma

### Introduction

The Reg family consists of several Reg and Reg-related genes that have been grouped into 4 subclasses: Reg I, Reg II, Reg III and Reg IV (see for reviews Okamoto, 1999; Okamoto and Takasawa, 2002). Reg I

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mRNA and protein are normally expressed in the acinar pancreas (Amouric et al., 1987). We previously reported the presence of Reg I in the healthy human intestinal crypt epithelium (Senegas-Balas et al., 1991) where growth and differentiation take place (Cheng et al., 1974). Reg I was associated with the growth and the regeneration of other tissues, such as endocrine pancreas, brain, stomach or heart (see for reviews Iovanna and Dagorn, 2005; Kiji et al., 2005). It is present or is over expressed in most human digestive cancers or cancer cell lines and its expression appeared correlated with survival prognosis (see for reviews: Zhang et al., 2003a; Iovanna and Dagorn, 2005).

Another member of the Reg family, Reg III, was found to be expressed in human testicular tissue (Nata et al., 2004). Reg I gene disruption results in a decrease in the pancreatic islet cell proliferation, in the gastric mucosa thickness and in the number of proliferating cells in the small intestine mucosa (Unno et al., 2002; Myaoka et al., 2004; Ose et al., 2007). However, in the latter study, no effect on fertility have been reported. Thus, it appeared interesting to analyze the Reg I expression in testis. In the present study, we examined the presence of Reg I in healthy and seminoma human testes and in a human seminoma cell line (JKT-1). We have also analyzed the Reg I expression in the rodent testis and in a rat Sertoli cell line (SerW3).

### Materials and methods

#### Antibodies

Rabbit antiserum against human Reg I protein recognizes Reg I protein but not rodent or rabbit homologues or another member of the Reg family, Reg III (Bernard-Perrone et al., 1999). Rabbit antiserum against mouse Reg I protein recognizes mouse and rat Reg I protein but does not recognize Reg III (Sanchez et

**Abbreviations:** Reg I: Reg I protein; Pephablock: 4-(2-aminoethyl)-benzenesulfonyl fluoride; Chaps: 3((3-cholamido-propyl)-dimethylammonio)-1-propanesulfonate.

al., 2000). The monoclonal mouse anti-human ZO-1 antibody or monoclonal mouse anti-PLAP antibody (clone 8A9) were purchased respectively from Zymed Laboratories (San Francisco, CA) and Dako (Glostrup, Denmark). Monoclonal mouse anti-vimentin was used for human tissues and cell lines (Dako, Clone Vim 3B4). Horseradish peroxidase-labelled F(ab')<sub>2</sub> fragments of goat anti-rabbit IgG antibody and of anti-mouse IgG antibody and Rhodamine Red<sup>TM</sup>-X-labelled F(ab')<sub>2</sub> fragment of goat anti-mouse IgG antibody were purchased from Jackson ImmunoResearch (West Grove, PA), and Alexa Fluor 488 F(ab')<sub>2</sub> fragment of goat anti-rabbit IgG from Molecular Probe (Eugene, OR). Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co (St Louis, MO) and were the highest grade available.

#### Pancreatic juice and tissue procurement

Human pancreatic juices, devoid of free proteolytic activity, were obtained by catheterization of the Wirsung duct of patients without pancreatic disease after informed and written consent, according to Institut National de la Santé et de la Recherche Médicale (INSERM) Ethical Guideline. Patients had no clinical or radiological symptoms of gastrointestinal diseases. Normal human testes were surgically removed from a 19 year old man with irreversible coma after parental agreement and according to France Transplant proceedings. Human adult normal testis and seminoma slides (n=3) were purchased from Biochain Institute (Hayward, CA). Histological analysis confirmed normal seminiferous spermatogenesis. By histological analysis, all tumors have been classified as pure seminoma with placental like alkaline phosphatase (PLAP) positive staining. Rodent pancreas and testes were obtained according to the INSERM recommendations.

#### Cell culture

Rat SerW3 cell line was maintained in Dubelco's Eagle's medium (Gibco BRL, Gercy Pontoise, France) containing 5% fetal calf serum (FSB, Gibco BRL), at 32°C as previously described (Pognan et al., 1997). The JKT-1 cell line, described as a human testicular seminoma cell line (Kinugawa et al., 1999), was routinely cultured in DMEM supplemented with 10% FBS (Gibco BRL, France) as recently reported (Roger et al., 2004).

#### Reg I protein solubilizations

Human, rat and mouse testes were recovered in lysis buffer (0.2 M sodium carbonate/CHAPS) with 1 mM Pephablock (SC plus; Roche, Mannheim, Germany), 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 10 µg/ml STI, 1 µM benzamidin, 100 µM phenanthroline, and 100 µM E64, and Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> and 2 mM Na<sub>3</sub>VO<sub>4</sub> (pH 11.5). SerW3 cells were scraped from the flasks in the

same solution. Samples were further homogenized by 10 strokes in Thomas potter and afterwards in Dounce potter. They were then sonicated and stirred for 60 min. All experiments were performed at 4°C.

#### Western immunoblotting

Electrophoreses (SDS-PAGE) were performed with 15% polyacrylamide gels (Yasuhiro et al., 2001). For human Reg I detection, samples in denaturing solutions (172 mM Tris-HCl, pH 6.8, 10% SDS, and 10% glycerol) were heated for 5 min at 92°C. Denaturing solutions were supplemented with 5% β-mercaptoethanol for western immunoblotting with antibody against mouse Reg I. After transferring the resolved proteins, the PVDF membranes (Millipore, 250 mA, Bredford, MA) were incubated overnight in 5 mM PBS (pH 7.2), containing 5% non-fat milk and 0.1% Tween-20 to reduce non-specific binding. Immobilized proteins were characterized by exposure of some PVDF membranes to antibodies against human or mouse Reg I (1:200) in the same buffer. Non-immune rabbit and mouse whole sera were used as controls. After washing, the blots were exposed to horseradish peroxidase-labelled goat anti-rabbit antibody or goat anti mouse antibody (1:5000). After the blots were stripped, equal loading of proteins was verified by reprobing the same blots with anti-ERK antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed by chemiluminescence (Amersham, Buckinghamshire, UK). Human and rat pancreatic juices, rat and mouse pancreas lysates were used as positive controls.

#### Immunofluorescence analysis

Control and seminoma human testes and rodent testes were embedded in Tissue-Tek OCT Compound (Sakura Finetek USA, Inc, Torrance, CA) and frozen in liquid-nitrogen-cooled isopentane. All immunostaining procedures were performed on 7 µm frozen sections, fixed in methanol (-20°C, 5 min). Cell monolayers at 2, 3, 5 and 7 days of culture were rinsed twice with PBS and fixed in methanol (-20°C) for 5 min. Slides and cells were then incubated with 20% non-immune goat serum to block non-specific binding. We performed individual or double stainings. For individual labelling, testis sections and cell monolayers were incubated with antibodies against human Reg I (1:100) or against mouse Reg I (1:100) and against human ZO-1 (1:100) or against vimentin (1:100) in PBS, 5% goat serum, 0.2% gelatin for 12 h at 4°C. Coverslips were then incubated in an Alexa Fluor 488 conjugated to goat anti-rabbit IgG-F(ab')<sub>2</sub> (1:200) or a Rhodamine Red Tm-X conjugated goat anti-mouse IgG-F(ab')<sub>2</sub> (1:500) for 2 h. For double labelling, testis and cell monolayers were treated by antibodies against human Reg I and against human ZO-1 or against Vimentin (1:100) as described above. Coverslips were then incubated in a mixture of an

Alexa Fluor 488-conjugated to goat anti-rabbit IgG-F(ab')<sub>2</sub> and a Rhodamine Red<sup>Tm</sup>-X-labelled goat anti-mouse IgG-F(ab')<sub>2</sub> for 2 h as described above. To show specificity for each of the antibodies used in the double labelling, several sets of controls were performed. First, the double labelling was compared with the individual labelling for each primary antibody and found to be the same. Second, controls were performed by incubating cells with either a mixture of whole non-immune rabbit serum (1:100) and whole pre-immune mouse serum (1:100) replacing the primary antisera, or PBS replacing conjugated antibodies. Third, immuno-adsorption tests were also conducted by incubating 0.2 mg/ml of human or mouse Reg I (4°C, 24 h) with the 2 primary Reg I antisera respectively. These controls showed that the blocking steps eliminated the binding of antibodies between the first and second antibody incubations, and that the labelling for each primary antibody was specific. Images were captured on the laser microscope (LSM 510, oil immersion lens Zeiss PlanNeofluar, x63, Zeiss, Iena, Germany) with Multy track configuration to avoid bleed-through between channels. Stacks of fluorescence images were collected automatically at 0.38 μm Z-intervals. Images shown were representative of three different experiments.

High resolution deconvolution microscopy analysis was performed using a Nikon TE 2000E microscope, equipped with a piezoelectric translator (PIFOC, PI, Germany) placed at the base of a 100x PlanApo N.A. 1.4 objective, and a 5 MHz Micromax 1300Y interline CCD camera (Roper Instruments, Evry, France) as recently described (Segretain et al., 2003). Stacks of fluorescence images were collected automatically at 0.2 μm Z-intervals using the Metamorph Software (Universal Imaging Corp., Downingtown, PA). For immunofluorescence, exposure times were adjusted to provide circa 3000 grey levels at sites of strong labelling. The deconvolution of each Z-series was automatically computed using a measured Point Spread Function (PSF) and an adapted constrained interactive deconvolution algorithm which yields significantly improved image quality with custom made software package. Images shown were representative of three experiments.

## Results

Western immunoblotting with antibody against a mouse Reg I allowed us to detect a band at approximately 19 kDa in mouse and rat mature testis protein extracts (Fig. 1A). This signal was also present in rat and mouse pancreas, as well as in rat pancreatic juice used as controls (Fig. 1A). The presence of Reg I was confirmed in rat testis by immunofluorescence (Fig. 1B). No specific immunoreactive signal was detected in the interstitial compartment (data not shown). Analysis of the Reg I in rat testis sections demonstrated the presence of an immunoreactive signal, characterized as small dots and lines invaginating the seminiferous

epithelium, and typical of Sertoli cell labelling (Fig. 1B, c). The signal was also detected in specific germ cells near the basal compartment, identified as spermatogonia, and within round spermatids (Fig. 1B, e). No signal was found either in spermatocytes or in myoid cells (Fig. 1B, e). All controls described in "Materials and Methods" were negative and specifically those with replacement of the primary antibody by non-immune rabbit serum (Fig. 1B, b).

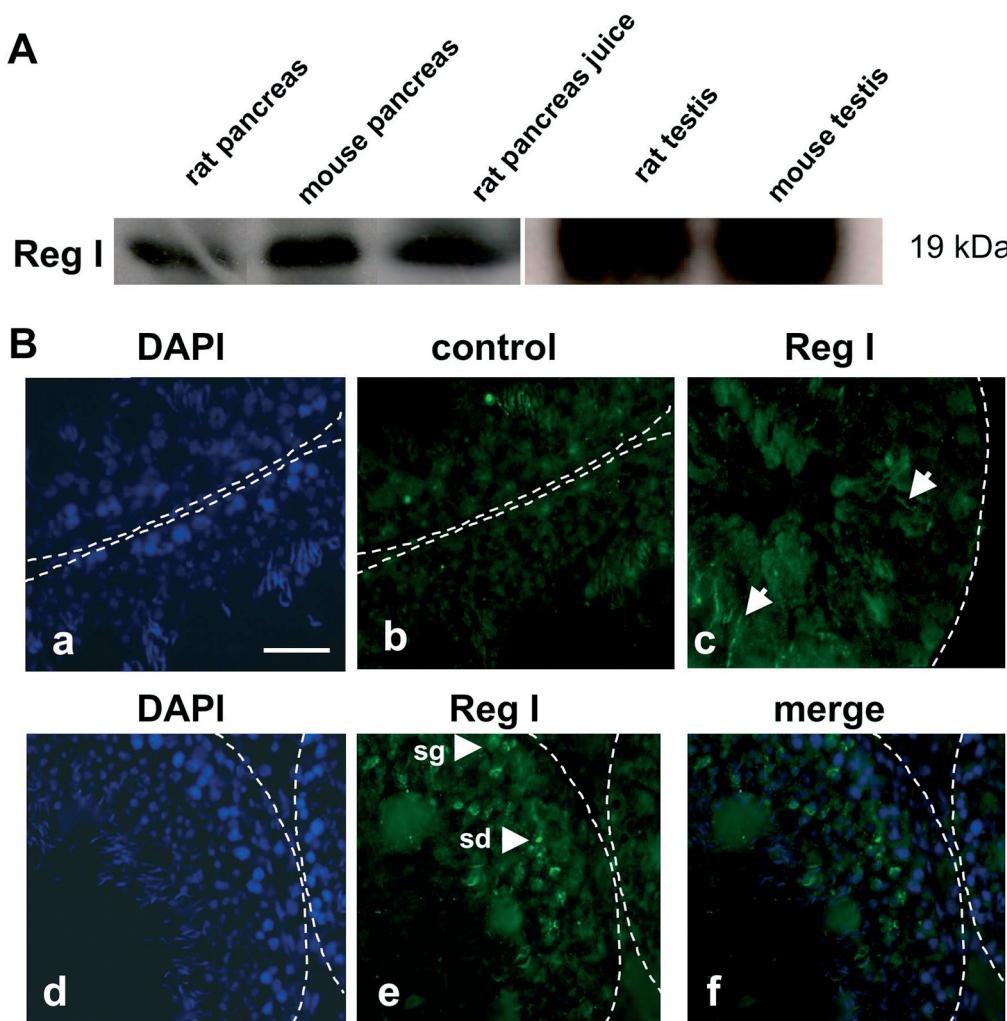
To verify that somatic Sertoli cells express Reg I, the rat SerW3 Sertoli cell line was used. As expected, the presence of Reg I was verified by the observation in SerW3 Sertoli cell extracts of a 19 kDa band that migrated at the same size as in pancreatic juice (Fig. 2A). The intensity of the signal was time-dependent since it increased from day 2 to day 7 of culture whereas the intensity of the signal for ERK remained relatively unchanged whatever the time of culture (Fig. 2A). SerW3 Sertoli cells clearly exhibited a punctuate labelling for Reg I along the plasma membrane (Fig. 2B, a) as we previously reported in Caco-2 cells (Bernard-Perrone et al., 1999). The presence of a weak Reg I staining within the cytoplasm of Sertoli cells, which could be associated with the synthesis, the maturation and/or the degradation of the protein, was also detected. The punctuate labelling localized on the plasma membrane was observed even when cells showed no contact with neighboring cells (Fig. 2B, a, c). In contrast, with ZO-1 antibody, a protein associated with tight junctions, an intense staining was visualized only on the plasma membrane of joining cells (Fig. 2B, b, c). Confocal microscopy revealed the early localization of Reg I to the plasma membrane of SerW3 cells. With the anti-Reg I antibody, a diffuse staining was observed in cytoplasm associated to a punctuate labelling of the plasma membrane of isolated cells, whereas no signal could be detected for ZO-1 (Fig. 2B, d). Later, in cells, which were joined only by a short portion of their plasma membrane, Reg I could already be seen on the entire plasma membrane whereas ZO-1 was only located on the part of the plasma membrane between adjacent cells (Fig. 2B, e, f). Moreover, ZO-1 was localized as small dots still far from the portion of the plasma membrane, which was not associated to those of a neighboring cell (Fig. 2B,e,f). Altogether, these observations revealed that Reg I was expressed in Sertoli cells and appeared to be localized at the plasma membrane before ZO-1.

As for mouse and rat testes, Reg I appeared to be expressed in the human testis. On western blots of human testicular extracts and of control human pancreatic juice, the antibody directed against human Reg I detected a band of approximately 19 kDa (Fig. 3A). Immunofluorescence analysis confirmed the presence of Reg I within the seminiferous epithelium (Fig. 3B). High magnification revealed the presence of immunoreactive dots distributed between cells within the seminiferous tubules (Fig. 3B b, insert). No specific labelling was observed in controls with non-immune

rabbit serum in place of Reg I antibody (Fig. 3B, a). To verify the types of cells that express Reg I, a dual labelling was performed using the antibody specific for human Reg I and an anti-vimentin, which specifically stains the Sertoli cells within the seminiferous tubules (Fig. 3B, c). Application of high-resolution deconvolution microscopy to human testis sections demonstrated that vimentin-positive cells identified as Sertoli cells were also labelled with the Reg I antibody (Fig. 3B, d, e, f). Figure 3B (g, h, i) also revealed that some vimentin-negative cells, identified as germ cells, also appeared to be positive for Reg I. These observations suggest that both cell types, Sertoli and germ cells, express Reg I. Identification of Reg I positive cells and vimentin negative cells according to morphological criteria (size and localization within the seminiferous epithelium) supports a spermatogonia origin for these germ cells (Fig. 3B, c).

We confirmed the diagnosis of testis seminoma for

the slides purchased from Biochaim Institut since tissue sections were positive for PLAP, a classical and specific marker of seminoma cells (Fig. 4a). Tumor cells were also highly labelled with the anti-Reg I (Fig. 4b). The intensity of the Reg I signal observed in three different seminoma appeared to be higher than that observed in normal testis concomitantly stained. Higher magnification demonstrated that the immunostaining was mainly present at the plasma membrane but was also found within the cytoplasm of seminoma cells (Fig. 4b, insert). This latter localization was more obvious when cells were treated with saponin before immunostaining analysis (data not shown). The membrane localization of Reg I was also clearly evidence in JKT1 cells, which formed small clusters (Fig. 4d) but Reg I expression was absent in more confluent cells (Fig. 4f). In the latter condition, the staining was mainly detected in the cytoplasm of these seminoma cells.

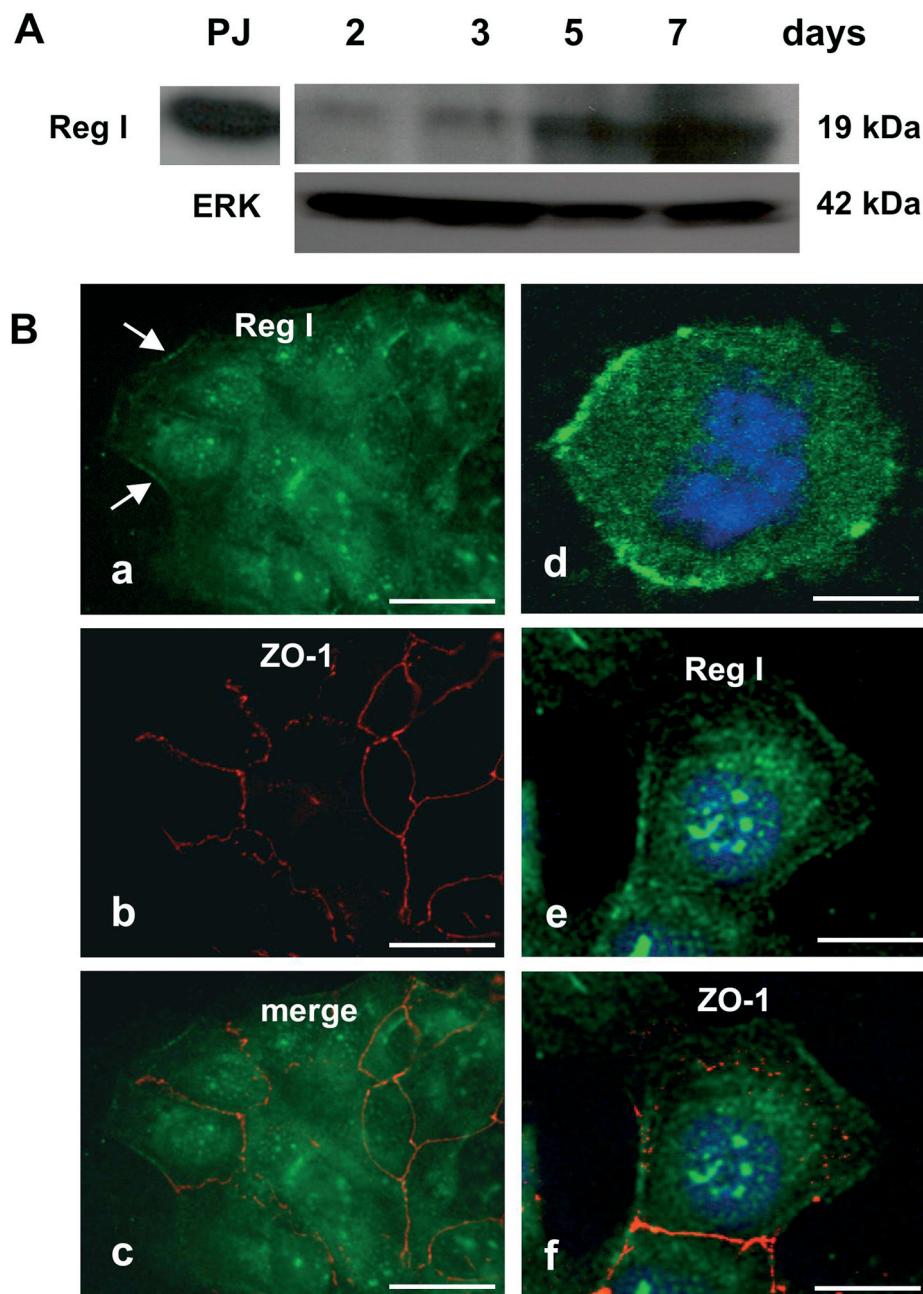


**Fig. 1.** Presence of Reg I in rat and mouse testes. **A.** Western blot analysis detected a band migrating at about 19 kDa in pancreas and testis lysates of mouse and rat and in rat pancreatic juice used as controls. Representative of 3 separate experiments. **B.** Localization of Reg I in seminiferous tubule compartment of mature rats. No specific labelling was observed when the primary antibody was omitted and replaced with pre-immune rabbit serum (**a**, **b**). Specific antibody against Reg I demonstrated the presence of an immunoreactive signal that appeared as small lines within the seminiferous epithelium (**c**, arrows). Identification of nuclei by Dapi staining (**d**) revealed the presence of Reg I in spermatogonia (sg) and in round spermatids (sd) (**d-f**). Bars: a-f, 20  $\mu$ m.

## Discussion

Reg genes are expressed in different tissues, and mRNAs for one of these genes, Reg III, have been recently detected in the human testis (Nata et al., 2004). In the present study, by using polyclonal antibodies specifically raised against mouse and human Reg I, we demonstrated for the first time that this protein is present in the normal testis of human and rodents and is strongly expressed in human seminoma testis. The testicular

expression of Reg I is supported by western blot and immunofluorescence analyses demonstrating the presence of a specific band at the size expected and the detection of the protein in mouse, rat and human testis sections. However, these results are not in agreement with previous observations that Reg I was undetectable in mouse testis by Northern blot analysis (Unno et al., 1993). The reasons for such a discrepancy are presently unknown. Our results also reveal that Reg I was localized in the seminiferous tubule compartment in



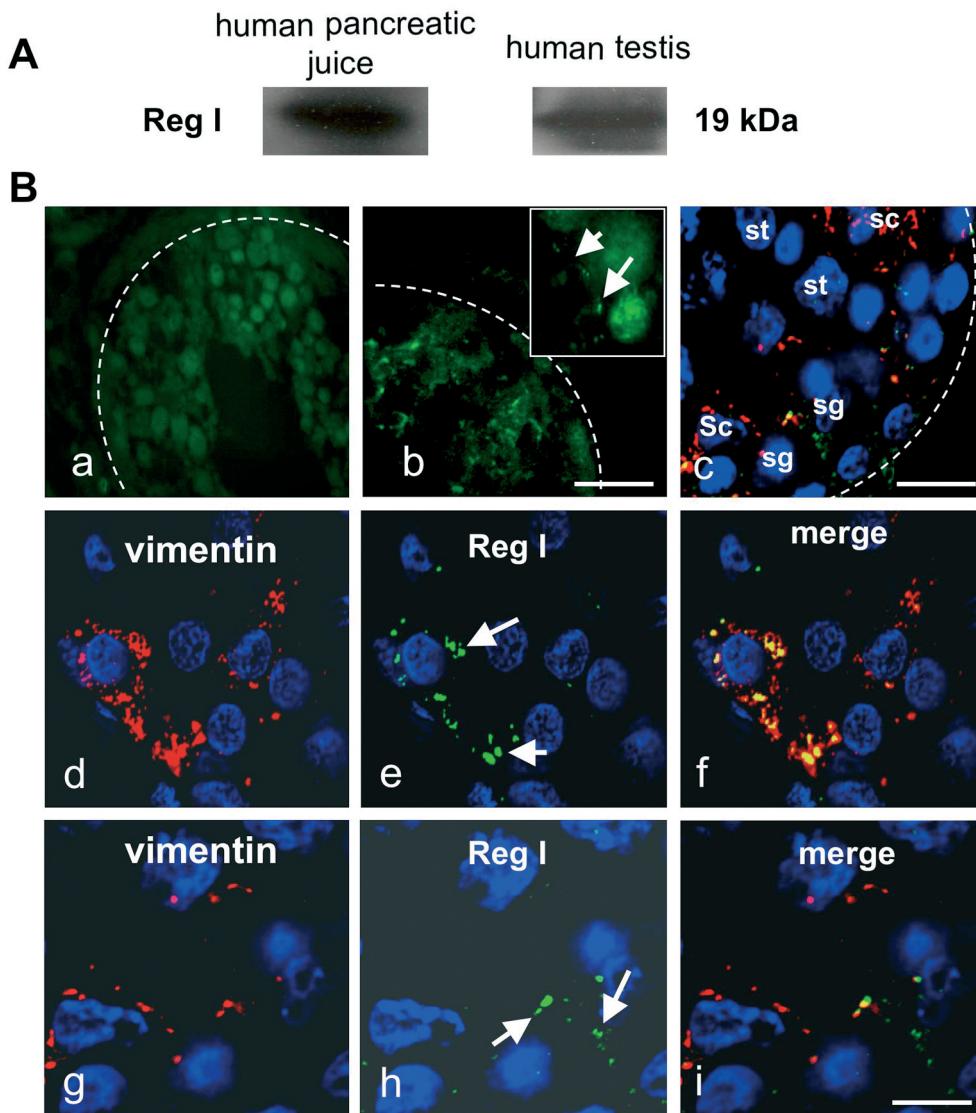
**Fig. 2.** Characterization of Reg I in the SerW3 Sertoli cell line. **A.** Western blot analysis detected a band migrating at about 19 kDa in lysates of Sertoli cells. The intensity of the immunoreactive band increased in a time-dependent manner during cell culture period. Rat pancreatic juice (PJ) served as control. No major modification of the intensity of the bands for ERK used as control was detected. Representative of 3 separate experiments. **B.** Immunolocalization of Reg I and ZO-1 in Sertoli cells. Note that the signal for Reg I protein is present at the plasma membrane even on that without contact with adjacent cells (**a**, arrows). In contrast, the antibody against ZO-1 labelled only the zone of contact between adjacent cells (**b**). Confocal microscopy analysis revealed Reg I labelling in the cytoplasm of isolated cells, and a punctate staining at the membrane plasma level, whereas ZO-1 was not detected (**d**). Later, Reg I could be seen on the entire plasma membrane of adjacent cells (**e**) whereas ZO-1 was only located on the portion of the plasma membrane between adjacent cells (**f**). ZO-1 was localized as small dots still far from the plasma membrane in regions where no contact with adjacent cells occurred (**f**). Bar: a-c, 5 µm; d, 3 µm; e-f, 2 µm.

rodent and human testes. By using high-resolution deconvolution microscopy, we demonstrated that immunoreactive Reg I signal was localized within the human seminiferous epithelium, in both Sertoli cells, the somatic cells that sustained spermatogenesis and in vimentin negative cells, identified as spermatogonia by morphological criteria.

The role of Reg I in the seminiferous epithelium is unknown. Different functions of the Reg proteins have been described, including mitogenic, anti-inflammatory, anti-bacterial effects (see for review Iovanna and Dagorn, 2005). In the testis, within the seminiferous epithelium, germ cells proliferate and differentiate to form spermatozoa. This process is dependent upon the presence of Sertoli cells and is controlled through paracrine factors and direct cell/cell contacts (Pointis

and Segretain, 2005). Thus, it is possible that Reg I, which is localized in plasma cell membranes in Sertoli and germ cells, participates in the local regulation of germ cell proliferation and differentiation. This hypothesis is in agreement with the current data that Reg I was detected in germ cells that proliferate, as spermatogonia, and differentiate as round spermatids.

It should be noted that Reg I was localized in SerW3 cell cytoplasm at the beginning of culture and later on plasma membrane as we previously reported in Caco-2 cells (Bernard-Perrone et al., 1999). Moreover, we demonstrated that this Reg I localization on plasma membrane preceded those of ZO-1. From these observations, we suggest that Reg I may be associated with the growth and the early testis differentiation processes. The present data are in agreement with those

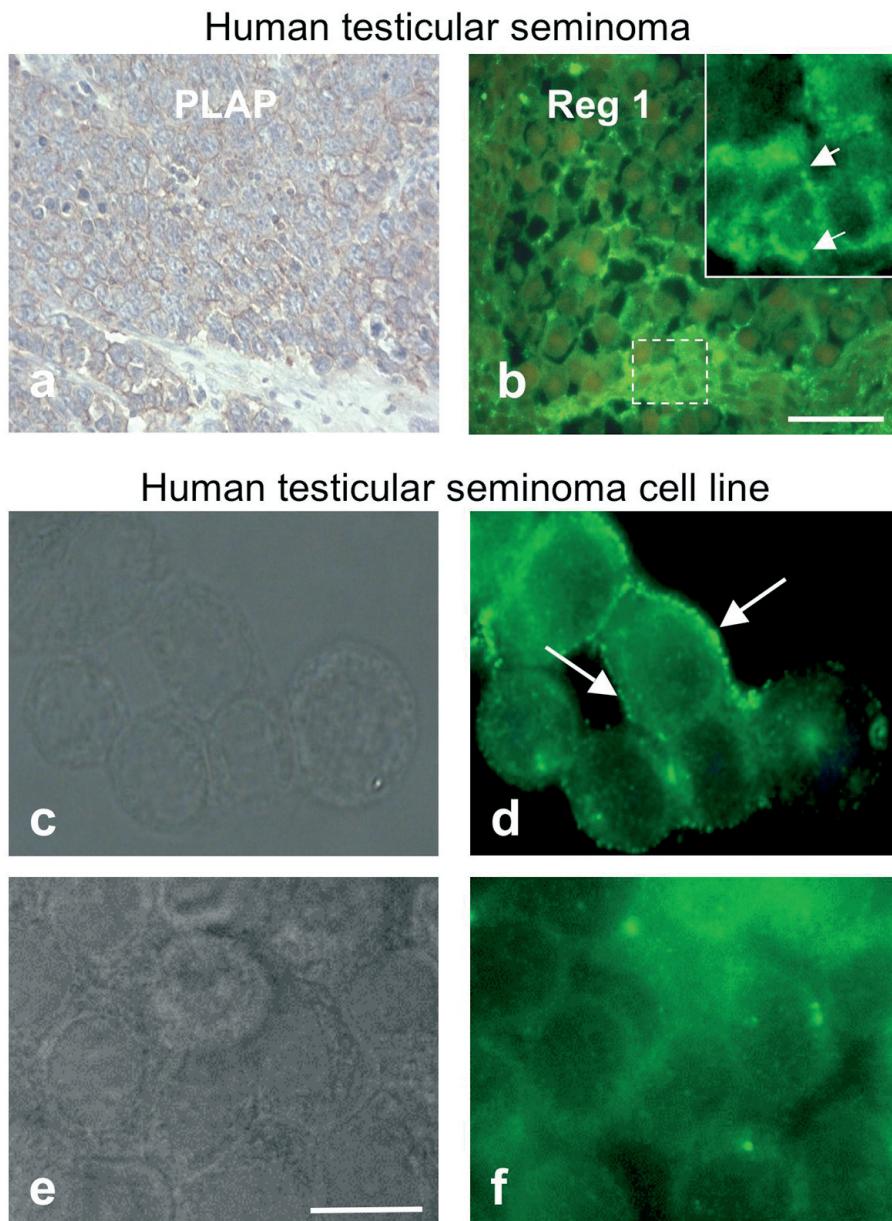


**Fig. 3.** Characterization of Reg I in human normal testis. **A.** Western blot analysis detected a band migrating at about 19 kDa in lysates of human testis. Human pancreatic juice served as control. **B.** Reg I immunolocalization in human testis with histologically normal spermatogenesis. The immunostaining was detected within the seminiferous epithelium (**b**) whereas no labelling was revealed with non-immune serum replacing the primary antibody (**a**). High magnification showed that the signal was present as small dots (insert, arrows). Deconvolution microscopy analysis after double detection for vimentin (red fluorescence) and of Reg I (green fluorescence) showed that within the seminiferous epithelium both vimentin-positive cells and vimentin-negative cells were labelled with the Reg I antibody (**c**). Nuclei of cells were identified by DAPI staining (blue fluorescence). Higher magnifications are presented in **d-i**. Sc: Sertoli cell; sg: spermatogonia; st: spermatocyte. Bars: a, b, 15 µm; c, 3 µm; d-i, 1.5 µm.

reporting Reg I association with early differentiation processes in pancreas, stomach, small intestine or cancer colon cell (Terazono et al., 1988; Sénégas-Balas et al., 1991; Watanabe et al., 1994; Zenilmann et al. 1996; Bernard-Perrone et al., 1999; Miyaoka et al., 2004; Ose et al., 2007).

The biological association of Reg genes with pathological conditions has been widely recognized, particularly for digestive diseases (see for reviews: Zhang et al., 2003a; Iovanna and Dagorn, 2005). Since the expression levels of Reg I, Reg III or Reg IV are consistently found to be much lower in healthy digestive

tissues compared to the corresponding cancer tissues, the usefulness of the Reg family has been recognized for both early diagnosis of digestive cancer and prognostic indicator of tumor survival (Zenilman et al., 1997; Rechreche et al., 1999; Macadam et al., 2000; Hartupee et al., 2001; Harada et al., 2001; Violette et al., 2003; Oue et al., 2005; Motoyama et al., 2006; Nanakin et al., 2007). More recently, the possibility of using Reg IV as a serum biomarker for human pancreatic and gastric cancers has been suggested (Takehara et al., 2006; Mitani et al., 2007). Although few reports are available on the potential association between Reg and tumors



**Fig. 4.** Immunolocalization of Reg I in pure human testicular seminoma biopsies and in the seminoma cell line JKT1. Cells of the testis seminoma sections were PLAP-positive (**a**). The antibody against Reg I revealed that most cells were also Reg I-positive (**b**). High magnification demonstrated that the signal was mainly located at the plasma membrane level (insert, arrows). A positive Reg I signal was also visualized at the edge of the JKT1 seminoma cell line as small spots at the plasma membrane in cells that formed small clusters (**d**, arrows). In contrast, in confluent cells, the immunoreactive signal was only revealed within the cytoplasm (**f**). Phase contrast microscopy of JKT1 cells (**c**, **e**). The scale bars represent 20  $\mu$ m in **a** and **b**, 20  $\mu$ m in **c-f**. Bars: **a, b**, 20  $\mu$ m; **c-f**, 2  $\mu$ m.

outside the digestive system, a potential role of Reg IV in prostate cancer has been suggested (Zhang et al., 2003b). Whether Reg I exhibits similar features in the pathological testis is presently unknown. Indeed, the current results show a weak Reg I presence in the normal testis, whereas most seminoma cells are intensely labelled with the Reg I antibody in the tumor testis.

In conclusion, by using specific antibodies directed against Reg I, the present data demonstrate for the first time that Reg I was present in rodent and human testes within the seminiferous epithelium. Although the potential role of Reg I in the control of spermatogenesis is presently unknown, the elevated expression of this protein in both human testis seminoma and in a human testicular seminoma cell line supports the hypothesis that Reg I could be associated with testis tumor development.

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