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In vitro effects of oestrogens, antioestrogens and SERMs on pancreatic solid pseudopapillary neoplasm-derived primary cell culture

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Abstract. *Background*: Solid-pseudopapillary neoplasms of the pancreas (SPNs) are uncommon tumours usually frequent in young women. Although the pathogenesis of SPNs is uncertain a potential influence of the sex hormone milieu on the biology of these tumours has been suggested. The controversial expression of oestrogen receptors (ERs) in SPNs, provide a rationale for studying the effects of oestrogenic molecules on SPN development.

Methods: The expression of a large series of hormonal ligands and receptors was evaluated in tissue specimens and in a primary cell culture (SPNC), obtained from a SPN in young female patient. The effects of 17β-oestradiol (17βE$_2$), ICI 182,780 and tamoxifen (Tam) on cell replication and growth were examined.

Results: We have established SPNC primary line. Immunocytochemical analysis was positive for vimentin, cyclin D1 and β-catenin and negative for cytokeratin, CD10 and neuroendocrine markers, in line with the immunostaining features of the tumoral tissue. Expression of ERα, ERβ and progesterone mRNAs was demonstrated in SPNC and tumor tissue. A proliferative and antiproliferative action of 17βE$_2$ and Tam respectively were proved in SPNC.

Conclusion: In conclusion, we provide the first direct evidence that oestrogenic molecules can influence proliferation of SPNC, offering future strategies in the control of this neoplasia via selective ER modulators.

Keywords: Solid pseudopapillary neoplasm of the pancreas, oestrogen receptors, 17β-oestradiol, antioestrogens, SERMs

1. Introduction

Solid-pseudopapillary neoplasms of the pancreas (SPNs) are unusual clinicopathological entities, originally described by Frantz in 1959. From then on, several reports have indicated this neoplasia with various names (Frantz's or Hamoudi tumour, solid and cystic tumour of the pancreas or solid and papillary neoplasm, etc.). Although originally considered a rare tumour, it has being diagnosed with increasing incidence, representing 13% of pancreatic cystic masses and 1–2% of all pancreatic neoplasms [11,12].

The tumour is composed of monomorphous cells making up solid and pseudopapillary structures, and variably expressing epithelial, mesenchymal and endocrine markers. Differential diagnosis includes endocrine tumour, acinar cell carcinoma, pancreatoblastoma, ductal carcinoma and cystic tumours [11,22].

SPNs are reported to be substantially more frequent in young women then in men [15,27,37,38] and some of them have been reported to express oestrogen [2, 14] and progesterone [2,14,34,36] receptors (ER and PR). The gender-related incidence of this neoplasm suggests a potential role of sex hormone in its initiation and progression, although the effects of oestro-

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gen (E) and progesterone (P) on the behaviour of the tumour have never been studied. In fact, retrospective studies failed to evidence significant differences in the clinicopathological features of the lesions in premenopausal and in postmenopausal women [20]. Indeed, confounder factors, such as the use of age alone as a proxy for menopausal status, and the lack of complete information on the use of hormonal replacement therapy or of oral contraceptives, may offer an explanation for these findings. Moreover, the attempt to discriminate SPNs from men and women on the basis of histological and immunohistochemical features of proliferating cells, of sex specific β-catenin gene mutations, or of sex hormones receptors' expression did not evidence any gender difference [30]. In the same study, tumours from men were shown to be prevalently occupied by solid components that lacked prominent pseudopapillary or pseudoglandular degeneration, while those from women showed the typical features of SPNs (i.e., almost complete cystic degeneration, inclusion in thick fibrous capsule and extensive calcification with ossification) [30].

These observations, together with the documented expression of ER and PR in SPNs, provide a rationale for the study of the effects of E and P on this kind of tumour. Because of the rarity of the disease, population-based studies can be difficult to be performed, while *in vitro* cellular and molecular studies could represent a valid approach to demonstrate a direct hormonal influence, which may in turn lead to new strategies for diagnosis, chemoprevention and chemotherapy. This has been made difficult by the fact that often the diagnosis of the tumour is reached after surgery.

We developed primary cell cultures (SPNC) from a pancreatic tumour in a 27-year-old woman, who was later diagnosed by histopathological investigation affected by SPN. This cellular model was used to evaluate the expression of sex hormone receptors and the effects of 17β-oestradiol (17βE$_2$), ICI 182,780 (ICI) and tamoxifen (Tam). The results obtained provided the first evidence that the hormonal milieu can influence cell growth and progression and indicated selective oestrogen receptor modulators (SERMs) as potential chemotherapeutic agents in this pathology.

2. Methods

2.1. Patient's details

Patient is a 27-year-old woman presented with nausea, vomiting and several epigastric pains, but without clinical evidence of any endocrine syndrome. Secretin test was negative with increased basal levels of gastrin and somatostatin. Serum pituitary, parathyroid and gastrointestinal hormones were within the normal range, while serum prolactin levels were elevated (1015–1078 mU/l, normal values 10–650 mU/l). Genetic screening for Multiple Endocrine Neoplasia type 1 was negative. Abdominal magnetic resonance imaging revealed a 22 mm nodular lesion in the junction of body and tail of the pancreas while octreoscan and brain scan excluded, respectively, high-density somatostatin receptor type 2 lesions and pituitary abnormalities. Patient underwent a distal pancreatectomy in 2003; the pancreatic lesion appeared well demarcated and no intra-abdominal metastases were noted. The post-operative course was uneventful, with interleukin-6 (IL-6), dehydroepiandrosterone sulphate, 17βE$_2$, P, sex hormone binding globulin, and testosterone levels in the normal range. Prolactin values were slightly altered (634–699 mU/l), with regular cycles. The patient presented no recurrence three years after surgery.

2.2. Isolation of human primary cell culture

SPNC were isolated from pancreatic tumour tissue obtained after surgical resection. The patient provided informed consent, as dictated by the local Institutional Review Board. Knife biopsy of tumour tissue was immediately placed in sterile McCoy's 5A medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 22 mM HEPES (Sigma Aldrich) and processed in the laboratory within 30 minutes (min) from the excision by mincing into small fragments (0.2–0.5 mm each). The tissue fragments were resuspended in Ham's F12 Coon's modification medium supplemented with 20% foetal bovine serum (FBS, Australian origin, BioWhittaker, Cambrex, Belgium) and 0.3 mg/ml collagenase type I (C-0130, Sigma Aldrich) and digested for 12 hours (h) at 37°C. Then, the fragments were mechanically dispersed and the cells were sedimented by centrifugation at 500g for 5 min. The pellet was resuspended and cultured in Ham's F12 Coon's modification medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin in 100 mm culture plates until confluence. The cell monolayers were detached with trypsin/EDTA solution and plated at the desired density in the appropriate medium, refreshed twice a week. The cells were used for further subculturing or cryopreservation upon reaching 1×10^6 cells/plate. Cells used in the experiments were between the 1st and 5th passage.

2.3. Immunohistochemical and immunocytochemical analysis

The surgical specimen was fixed and processed as described by Galli et al. [5]. The immunohistochemical procedure was performed with a BenchMark® XT autostainer (Ventana, Medical Systems, Tucson, AZ, USA) using the specific antibodies listed in Table 1 and the iVIEW DAB Detection Kit (Ventana) as the revelation system, following the suggested protocol. After the staining run was complete, the tissue sections was counterstained with haematoxylin, dehydrated and mounted with Permount.

Immunohistochemical analysis for ERβ was performed immersing the slides in pre-heated 10 mM/l Citrate Buffer (pH 6.0) for 30 min at 97°C and cooling down at room temperature (RT) for 20 min. Then, the slides were treated with 3% hydrogen peroxide in distilled water for 10 min. After blocking non-specific antigens with normal horse serum (UltraVision, LabVision, Fremont, CA, USA), the sections were incubated at RT for 30 min with mouse monoclonal antibody against the C-terminus epitope of ERβ (Novocastra Laboratories Ltd., Newcastle, UK), diluted 1:50 in antibody diluent (Ventana). Staining was achieved using a biotin-conjugated anti-mouse and anti-rabbit secondary antibody (UltraVision) and streptavidin–peroxidase (UltraVision). The bound antibody was detected using 3,3′-diaminobenzidine (Dako, Glostrup, Denmark) as the chromogen. Nuclei were counterstained with Mayer's haematoxylin.

The analysis of all antibodies was applied to slides with tumour-derived primary cell culture previously fixed in 95% ethanol, using the same protocols above reported.

2.4. Qualitative analysis of gene expression

Total RNA was extracted from SPN tissue, pancreatic normal parenchyma biopsies and 1×10^6 SPNC, using TRIzol reagent (Invitrogen srl, Milano, Italy) according to the manufacturer's instructions. The RNA was purified and retrotrascripted as described elsewhere [28].

Qualitative expression was evaluated both in SPNC and tissues by amplification of cDNA for the following gene transcripts: ERα, ERβ, PR isoform A and B; *aromatase* (*CYP19A1*), *IL-6*, *prolactin receptor* (*PRL-R*), *follicle stimulating hormone receptor* (*FSH-R*), *human chorionic gonadotropin/luteinizing hormone receptors* (*hCG/LH-R*), *human chorionic gonadotropin subunit alpha* (*hCGα*) and *beta* (*hCGβ*) and *β-actin* (housekeeping gene). PCR reactions were performed separately with GoTaqTM DNA Polymerase (Promega, Madison, WI, USA) in a final volume of 25 μl, using a standard thermal profile. The primers' sequences, predicted amplicon sizes and annealing temperatures (T_{ann}) are reported in Table 2A. Identity of each PCR product was confirmed by agarose gel electrophoresis and direct sequencing by using an ABIPrism 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA).

2.5. Quantitative analysis of ERs expression

ERs mRNA and protein expression was evaluated in SPN and normal tissues and in SPNC. β-actin was used as housekeeping gene to normalize expression data.

Table 1
Antibody sources for immunohistochemical and cytochemical tests

Antibodies	Origin	Clone/type
Anti-CD10	Ventana Medical Systems, Tucson, AZ	Clone 270
Anti-CD56	Ventana	Clone 123C3.D59
Anti-β catenin	Ventana	Clone 14
Anti-chromogranin A	Ventana	Clone LK2H10
Anti-cyclin D1	Ventana	Clone DCS6
Anti-cytokeratin	Ventana	Clone AE1/AE3/PCK26
Anti-neuron specific enolase (NSE)	Ventana	Clone E27
Anti-synaptophysin	Ventana	Polyclonal
Anti-vimentin	Ventana	Clone V9
Anti-ERα	Ventana	Clone 6F11
Anti-ERβ	Novocastra Laboratories Ltd., Newcastle, UK	Clone EMR02
Anti-PR	Ventana	Clone 1E2

Table 2A

Sequences and annealing temperatures of primers used for qualitative analysis of gene expression

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product size (bp)	T_{ann} (°C)
ERα	GGCCTTCTTCAAGAGAAGTAT	TCTGGCGCTTGTGTTTCAACATT	194	58
ERβ	CTTACCTGTAAACAGAGAGACAC	TTGCGCCGGTTTTTATCGATTGT	249	58
PR-A/B	AGCCCACAATACAGCTTCGAG	TTTCGACCTCCAAGGACCAT	255	60
PR-B	CCTGAAGTTTCGGCCATACCT	AGCAGTCCGCTGTCCTTTTCT	197	60
CYP19A1	GAATATTGGAAGGATGCACAGACT	GGGTAAAGATCATTTCCAGCATGT	293	58
IL-6	AAGATTCCAAAGATGTAGCCGCCCCACACA	TCTGCCAGTGCCTCTTTGCTGCTTTCACAC	160	60
PRL-R	TGCCTTCTGAATGGACAGTT	TGTACTGCTTGCCAAAGTGG	220	56
FSH-R	AAAAGCTTGTCGCCCTCATG	ACCATATCAGGACTCTGAGG	336	57
hCG/LH-R	GGAGAAGATGCACAATGGAG	CTCTCAGCAAGCATGGAAG	342	55
hCGα	CAGAATGCACGCTACAGGAA	CGTGTGGTTCTCCACTTTGA	218	57
hCGβ	ACCCTGGCTGTGGAGAAGG	TCATCACAGGTCAAGGGGTG	292	57
β-actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG	174	60

Notes: bp – base pairs of amplicon size; T_{ann} – annealing temperature.

Table 2B

Sequences of primers and TaqMan probes used for quantitative analysis of gene expression

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	TaqMan probe (5′–3′)	T_{ann} (°C)
ERα	TGATGAAAGGTGGGATACGA	AGCTCTCATGTCTCCAGCAG	(6-Fam)-AGACCGAAGAGGAGGGAGAATGTTGAA-(BHQ1)	60
ERβ	GTATGCGGAACCTCAAAAGAG	GTTCCCACTAACCTTCCTTTTC	(6-Fam)-CCTGGTGAAGCAAGATCGCTAGAAC-(BHQ1)	60
β-actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG	(6-Fam)-CCGCCGCCCGTCCACACCCG-(BHQ1)	60

Notes: 6-Fam – 6-carboxyfluorescein (reporter fluorochrome); BHQ1 – black quencher (quencher fluorochrome).

2.5.1. ERα and ERβ mRNA expression

Relative abundance of ERα and ERβ mRNAs was analysed by quantitative real-time PCR (QRT-PCR) as described in Tognarini et al. [32]. Exon–exon-spanning forward and reverse primers, labelled internal TaqMan probes, designed by Proligo Primers and Probes (Proligo, Paris, France), and T_{ann} specific for each cDNA, are depicted in Table 2B.

2.5.2. ERα and ERβ protein expression

Cell culture samples were lysed in M-10 PER Mammalian protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL, USA) while tissue samples were homogenised in T-PER Mammalian protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL, USA) with a TissueLyser (Qiagen). About 40 µg of proteins were resolved by SDS-PAGE and electrotransferred onto nitrocellulose membrane. Membranes were blocked in 0.1% Tween-20-ECL Blocking Buffer (GE Healthcare Europe GmbH, Milan, Italy) for 30 min at RT and then incubated for 1 h at RT in a solution of primary antibody (NeoMarkers' mouse monoclonal anti-ERα Ab-10, 2 µg/ml (Lab Vision Corporation, Fremont, CA, USA); Upstate's rabbit polyclonal IgG anti-ERβ, 3 µg/ml (Millipore, Billerica, MA, USA); mouse monoclonal anti-β-actin C-2, 1 µg/ml (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)) in 0.1% Tween-20-ECL Blocking Buffer. Membranes were washed four times for 5 min each at RT in 0.1% Tween-20-PBS and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) (anti-mouse IgG or anti-rabbit IgG (Sigma)) diluted in 0.1% Tween-20-ECL Blocking Buffer for 1 h. Enzyme-linked chemiluminescent detection of protein bands was performed with ECL Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer's instructions. Images were acquired with the ChemiDoc XRS system (Bio-Rad Laboratories, Inc.).

2.6. Analysis of cell growth and viability

E, antioestrogen and SERM molecules effects on cell growth and viability and on DNA synthesis in SPNC were determined by cell counting with a Bürker haemocytometer chamber and by [^{3}H]-thymidine incorporation assay, as described respectively by Tognarini et al. [32] and Rotella et al. [28]. The experiment were performed in appropriate culture medium so composed: Ham's F12 Coon's modification without phenol-red supplemented with 10% charcoal-dextrane stripped (cs) FBS, bFGF 1 ng/ml, 100 IU/ml penicillin and 100 µg/ml streptomycin.

2.7. Statistical analysis

The statistical significance of differences in the cell growth was evaluated by two-tailed Student's t-test in the Excel software (version for PC; Microsoft Corporation, Redmond, WA, USA) on experiments carried out in explicate for [^{3}H]-thymidine incorporation assay and by two-tailed Student's t-test in linear regression on experiments carried out in triplicate for manually cell counting.

3. Results

3.1. Characterization of SPN tissue and primary cell line

Microscopically, the tumour exhibited solid and focally pseudopapillary patterns (Fig. 1A). In both patterns, uniform polygonal cells were arranged around delicate fibro-vascular stalks. The cytoplasm was eosinophilic or vacuolated and the nuclei appeared round to oval with finely dispersed chromatin and inconspicuous nucleoli. The tumour tissue was well demarcated from the adjoining normal pancreas, although a separating layer of connective tissue was missing. Immunohistochemical analysis demonstrated positive reactions for vimentin, cyclin D1 and β-catenin (Fig. 1B), NSE, CD56 and CD10, whereas negative staining for cytokeratins, chromogranin and synaptophysin were obtained (Table 3). The histological and immunohistochemical findings were in keeping with the diagnosis of SPN. The pancreatic resection margin was free of tumour and seven regional lymph nodes were uninvolved.

Cells obtained from the primary tumour were cultured in growth medium and analysed up to the 5th passage for morphological and differentiative properties indicating stable characters of the primary culture. The cells grew as a firmly attached monolayer, showing a homogeneous phenotype with a fibroblast-like appearance. Immunocytochemical analysis was positive for vimentin, cyclin D1 and β-catenin (Fig. 1B) whereas cells were negative for cytokeratin, CD10 and neuroendocrine markers (Table 3), on the whole in line with the immunostaining features of the tumoral tissue. β-catenin immunostaining of cells and tumor tissue yielded characteristic cytoplasmic and nuclear positivity.

The qualitative gene expression was analysed using reverse transcription and PCR revealed the expression of *CYP19A1*, *IL-6*, *PRL-R*, *FSH-R*, *hCG/LH-R*, *hCGα*, *hCGβ* and *β-actin* in both tissue specimens (normal and tumoral) and in SPNC (data not shown).

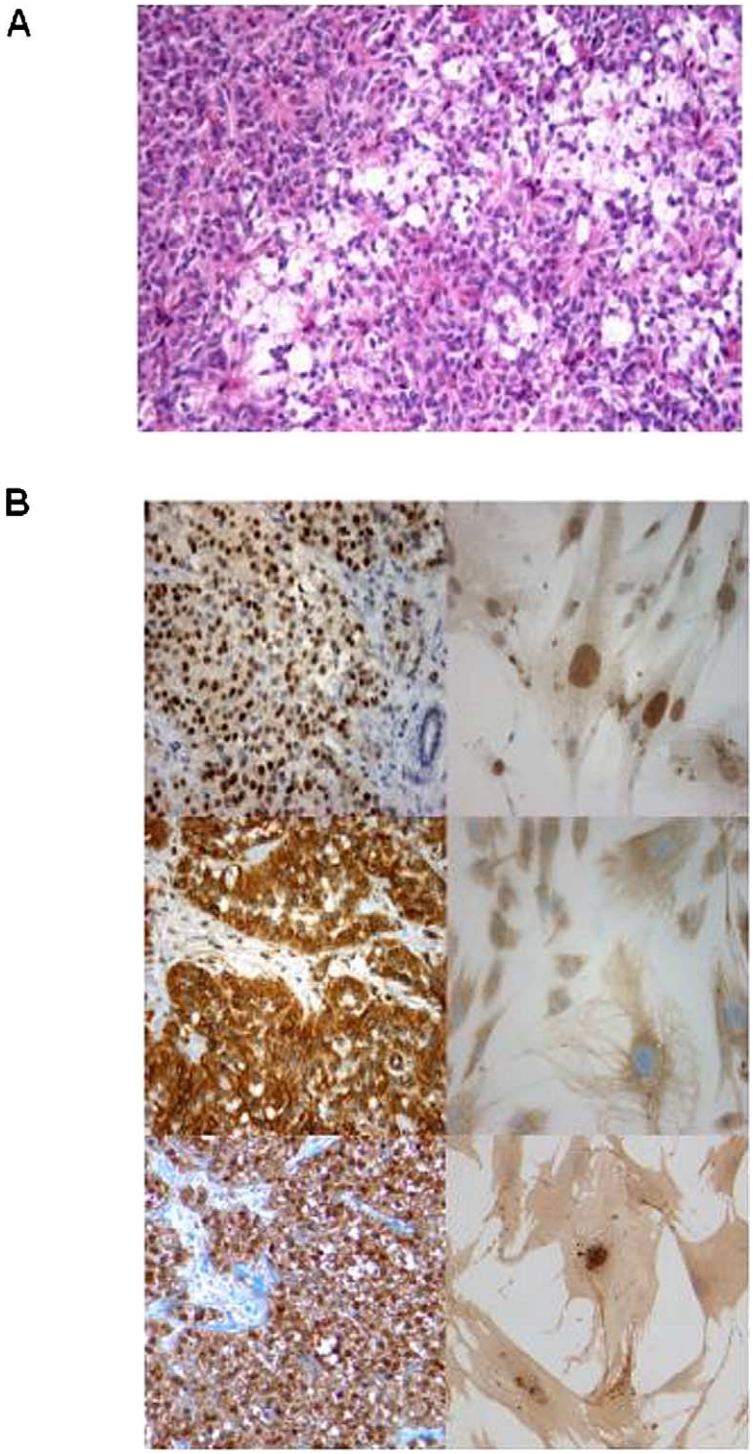

Fig. 1. Characterization of SPN tissue and cells. (A) Hematoxylin–eosin staining of SPN tissue in light microscope. (B) Immunohistochemical (on the left) and immunocytochemical (on the right) analyses of cyclin D1 (upper panels), vimentin (central panels) and β-catenin (lower panels).

Table 3
Immunoprofile of SPNC and tumor tissue

Marker	Tissue	Cells
CD10	+/−	−
CD56	+	−
β-catenin	+	+
Cyclin D1	+	+
Cytokeratin AE1/AE2	−	−
Chromogranin A	−	−
NSE	+	−
Synaptophysin	−	−
Vimentin	+	+
ERα	+	−
ERβ	−/+	−
PR	+	−

Notes: − – no tumour positive cells; −/+ – less than 1/3 positive tumour cells; +/− – 1/3–2/3 positive tumour cells; + – more than 2/3 positive tumour cells.

3.2. ER and PR expression

Immunohistologically, ERα and PR were strongly expressed in the tumour tissue (Fig. 2A and Table 3), while only a few neoplastic cells stained for ERβ (Fig. 2C). ERα, ERβ and PR were tested by immunocytochemistry in SPNC and found negative (Fig. 2B and D), although expression of ERα, ERβ, PR-A and PR-B mRNAs was demonstrated in normal and SPN specimens as well as in SPNC by RT-PCR (data not shown).

ERα and ERβ expression levels were also analysed through quantitative methods in normal and SPN specimens as well as in SPNC at the 1st, 4th and 5th passages in culture (Fig. 3). Results on relative expression, once levels normalized versus *β-actin* expression obtained through QRT-PCR, were consistent with those obtained by quantitative Western blot. ERα was highly expressed in both tissue specimens but its mRNA was three times more abundant in SPN than in the normal parenchyma. These data were also supported at the protein level even if saturation of the image from ERα protein immunoblotting did not allow the relative quantification of the signal. By contrast ERα expression was highly reduced in SPNC at mRNA and protein levels, with a consistent behaviour throughout the culture passages. ERβ mRNA and protein were uniformly expressed at lower levels than ERα. Both QRT-PCR and Western blot analysis revealed apparently higher levels of this receptor in the normal tissue than in the tumour.

3.3. Effects of 17βE$_2$, ICI and Tam on SPTC proliferation

The effect of 10 pM and 1 nM 17βE$_2$ on cell growth was tested during 6 days of culture, and a significant cell proliferation under 1 nM 17βE$_2$ stimulation was observed in SPNC (Fig. 4A). This one was accompanied by a significant ($p < 0.05$) increment in DNA replication after 24 h (Fig. 4B). The pure antioestrogen ICI 182,780 had no effect on cell growth when added to 17βE$_2$ 10 pM (data not shown), while this molecule significantly ($p < 0.05$) reverted the proliferative action of 1 nM 17βE$_2$ when added at 1 μM concentrations (Fig. 4C). Finally, Tam was tested in addition to 1 nM 17βE$_2$ and its effect was clearly antiproliferative at concentrations ranging from 10 nM to 5 μM, with significant growth inhibition after 6 days of culture at 100 nM ($p < 0.05$), 1 μM and 5 μM ($p < 0.001$) concentrations (Fig. 5A). In [^{3}H]-thymidine incorporation experiments, the effect of Tam was significantly evident even after 24 h cell culture, both in the absence ($p < 0.01$) and in the presence ($p < 0.001$) of 17βE$_2$, supporting an inhibitory effect of Tam on DNA replication (Fig. 5B).

4. Discussion

Because of its preferential occurrence in young women within the second and third decade of life, SPN could be considered a hormone-dependent tumour. However, biochemical and immunohistochemical attempts to demonstrate the expression of sex hormone receptors led to discordant results. The expression of PR was demonstrated in the majority of the investigated cases while the analysis of ER expression gave positive results only in half of the studies [2,14, 15,34,35].

In order to sustain the endocrine-dependency of SPN, we evaluated the expression of a large series of hormonal ligands and receptors in this tumoral tissue. Namely, the mRNA expression of bothER isoforms, of both PR isoforms, of *aromatase*, of *PRL receptor*, of both *gonadotropin receptors* and of both *chorionic gonadotropin* subunits was demonstrated in SPN tissue. This characterization unequivocally supports the role of the endocrine milieu on SPN development, offering for the first time an interpretation of the gender differences both in the incidence and in the biology of SPNs [2,14,16,20,21,30,31,36]. In order to uncover the role of sex hormones in SPN we performed

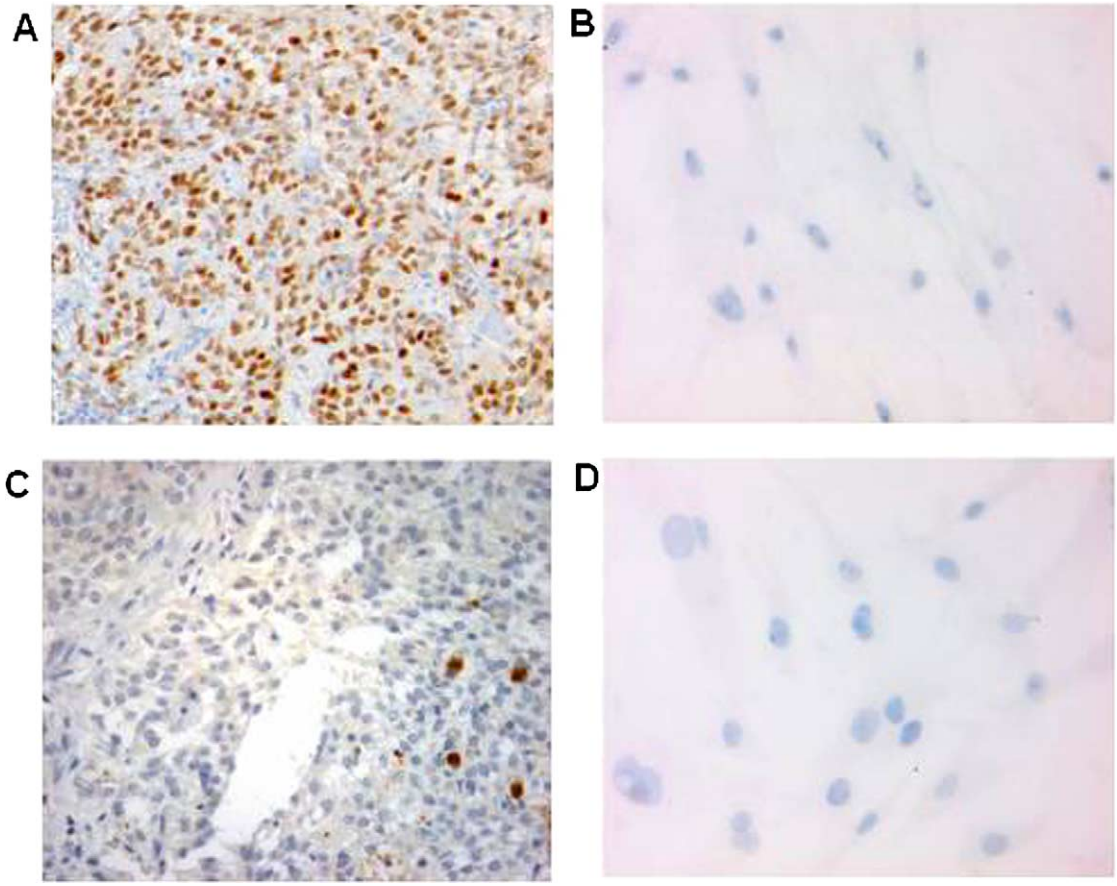

Fig. 2. Immunostaining of ERα (A and B) and ERβ (C and D) in SPN tissue (left panels) and SPNC (right panels).

classic immunohistochemical techniques to detect ER proteins and validated the expression data through of mRNA and protein expression analysis by QRT-PCR and Western blotting. These methods made possible to quantify ER expression and to compare their relative abundance in tissue specimens and in the derived primary cell culture. This analytical approach is necessary in SPN characterization, as observed in other oestrogen-related disorders [3]. The expression of ERα and ERβ was demonstrated in the tumoral tissue with ERα being expressed at very high levels in normal and SPN tissues. ERβ was also clearly present although immunoreactivity was fainter than for ERα. The use of molecular techniques demonstrated that ERβ was expressed in tissue specimens, although less abundant than ERα. The concordance obtained using three different techniques of expression analysis made possible to conclude that ERα is the most abundant ER in this SPN, although ERβ is also expressed. Literature records reported negative immunoreactivity for ER in 93 SPN cases [7,10,13,19,25,26], although radiolabelled oestradiol was shown in the cytosol of SPN cells [2,14]. By immunohistochemistry Geers et al. demonstrated the expression of ERβ and not of ERα in 4 SPNs, partly explaining the presence of radiolabelled oestradiol in SPN cells negative at the immunostaining for ERα [6]. According to the present results we suggest that immunohistochemistry should not be employed as the unique strategy to analyse ER expression and that sensitive methods of investigation should be proposed in the routine examination of these tissues. Interestingly, both protein and mRNA analysis evidenced a reduction of ERβ and an increment of ERα levels in tumour versus healthy tissue, with a consequent higher ERα/ERβ ratio in tumoral with respect to normal tissue. Higher ERα/ERβ ratio in tumours versus their non-tumour counterparts is a common feature of the oestrogen-dependent cancers [3,18], and supports a general mechanism of ERs action, indicating ERα as the mediator of 17βE$_2$ proliferative effect [1] and ERβ as an inhibitor of ERα-mediated proliferative effect [24,29]. Therefore, the selection of

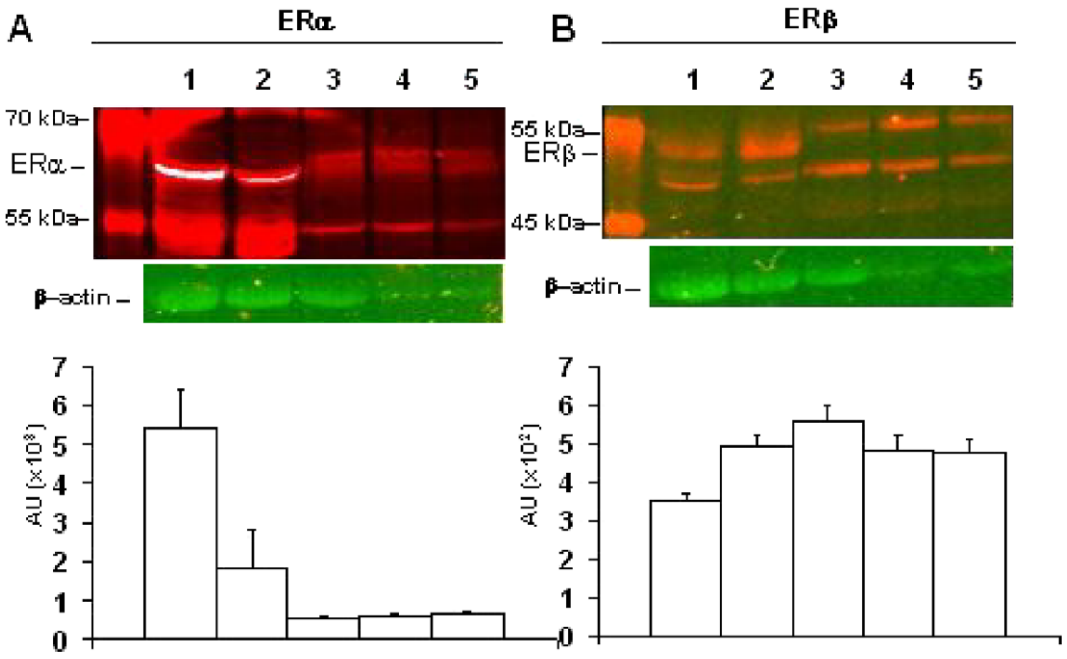

Fig. 3. Western blotting (upper panels) and QRT-PCR (lower panels) of ERα (A) and ERβ (B). *Note*: lane 1 – SPN tissue, lane 2 – pancreatic normal tissue, lanes 3–5 – SPNC at the 1st, 4th and 5th culture passage, respectively. QRT-PCR results are expressed in arbitrary units (AU, mean value $\pm$ SD).

ERα during tumour progression has the obvious consequence of making cancer cells more prone to the proliferative effect of oestrogenic molecules. For the first time we were able to perform a complete immunocytochemical characterization in a primary cell culture system developed from the SPN. Cultured cells were positive to vimentin, cyclin D1 and β-catenin, recognized diagnostic markers for SPN, while they were negative to NSE and CD56, conversely expressed in the tumoral tissue specimens. Lack of staining for cytokeratin AE1/AE3, chromogranin and synaptophysin was concordant in both tissues and cultured cells, while sex hormone receptors' expression pattern was different between tissues and cultured cells. However, using molecular techniques it was possible to demonstrate that cells at early and late culture passages showed a maintained, although reduced, ERα expression while maintaining unaltered ERβ levels, when compared to the results obtained in the tumoral tissue. It is plausible that the tumour mass includes several cellular types, with different phenotypic features and different levels of ERα and ERβ expression. The isolation procedure could contribute to select cells with lower ERα expression but with a high growth potential. This selection would explain both the different expression of ERα and the lack of some of the markers of cell differentiation in SPNC. Therefore, the isolation of cells from SPN tissue led to the enrichment of a culture whose ER expression was maintained, even though phenotypically not fully superimposable to the tumour specimen. On the basis of these findings we embarked in a project of *in vitro* testing of oestrogenic molecules on SPNC proliferation. The reduced expression of ERα in SPNC is the possible explanation for the modest proliferative effect of 17βE$_2$ on these cells. However, the proliferative effect of 17βE$_2$ was specific, because antagonized by the antioestrogen ICI 182,780. Interestingly, the SERM molecule Tam was highly antiproliferative on SPNC showing a dose-dependent inhibition of cell growth with up to 80% inhibition at 5 μM concentration after 6 days of culture with an effect partially due to an early inhibition of DNA replication. The antiproliferative action of Tam was dose-dependent when 17βE$_2$ concentrations were present in the culture medium, but it became dose-independent when 17βE$_2$ was removed. These results are coherent with the well-known complexity of the oestrogen response, especially in a cell model co-expressing both ER isoforms [18]. Indeed, both ERα and ERβ transactivation profiles are similar in response to different ligands when tested at a classical oestrogen responsive element (ERE), but ligand-induced transactivation behaviour is very different at activator protein-1 (AP-1) regulatory sequences, with Tam being a strong activator of AP-1

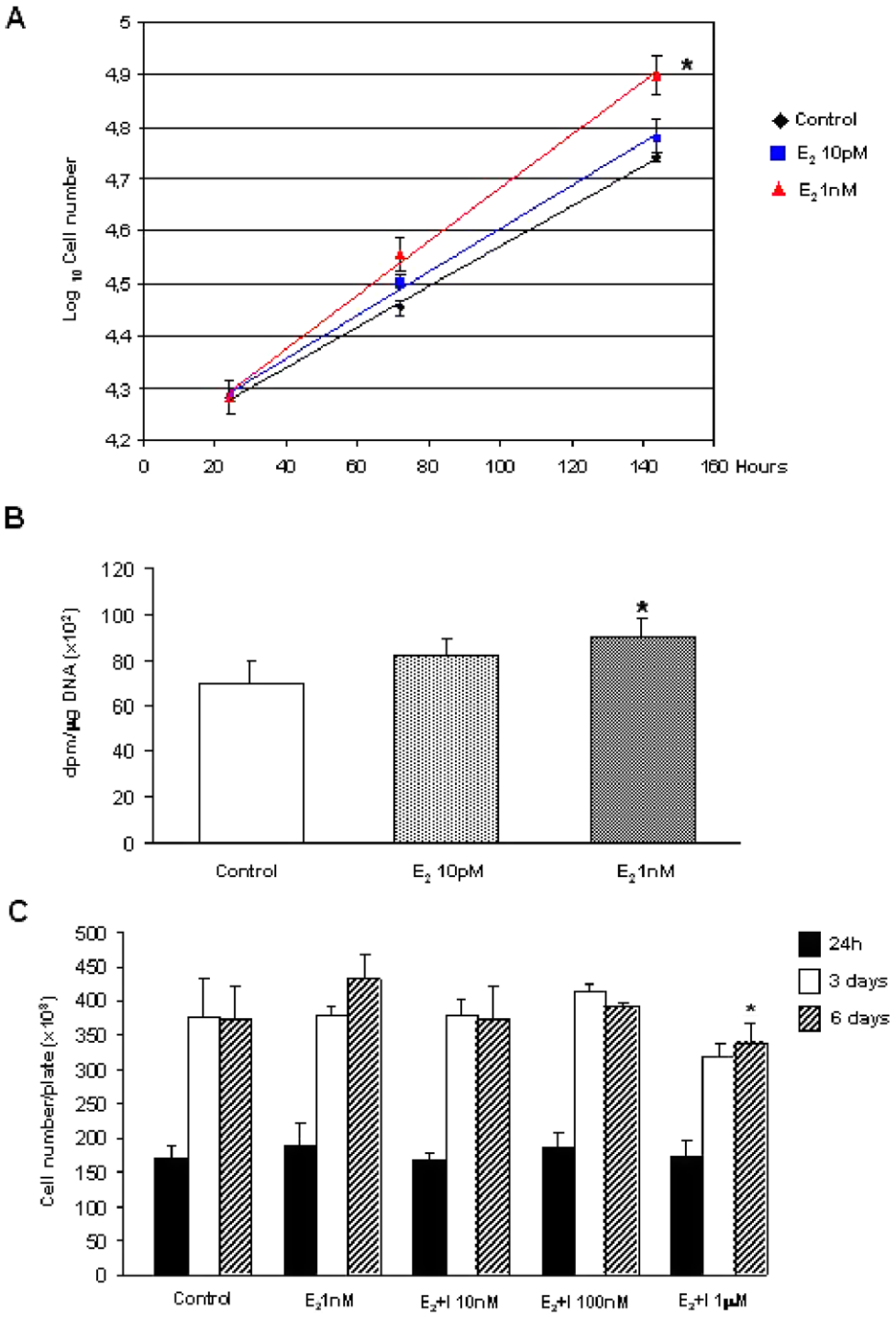

Fig. 4. Analysis of cell growth and viability of SPNC treated with 17βE$_2$ and ICI 182,780. Each bar represents the mean value $\pm$ SD of three experiments. (A and C) Cell number evaluation by a Bürker haemocytometer chamber. *$p < 0.05$ *vs.* 17βE$_2$ 1 nM-treated cells. (B) [^{3}H]-thymidine incorporation assay. *$p < 0.05$ *vs.* untreated cells.

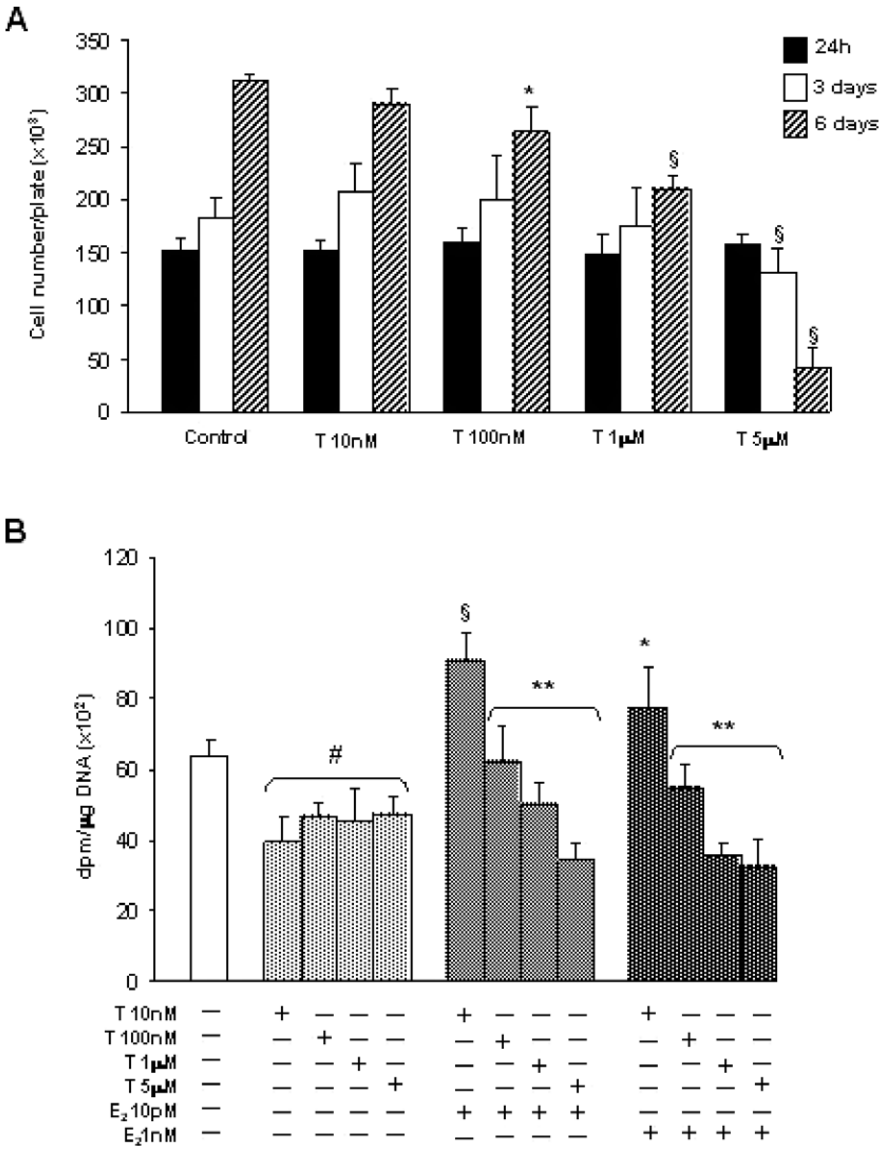

Fig. 5. Effect of $17\beta E_2$ (E_2) and Tam (T) on SPNC. Each bar represents the mean $\pm$ SD of two separate experiments. (A) Cell number evaluation by a Bürker haemocytometer chamber. $^{*}p < 0.05$ *vs.* untreated cells. $^{\S}p < 0.001$ *vs.* 10 nM Tam-treated cells. (B) [^{3}H]-thymidine incorporation assay. $^{*}p < 0.05$ *vs.* untreated cells. $^{\#}p < 0.01$ *vs.* untreated cells. $^{\S}p < 0.001$ *vs.* untreated cells. $^{**}p < 0.001$ *vs.* 10 nM Tam- and 10 pM or 1 nM $17\beta E_2$-treated cells.

through ERβ and a modest one through ERα [23]. The two different biological responses in SPNC, when Tam is added alone or in combination with $17\beta E_2$ could possibly reflect differential responses of ERα and ERβ to $17\beta E_2$ and Tam or to the simultaneous challenge with both stimuli. The biological response to Tam is clearly antiproliferative, whatever acting as the main ligand or acting antagonizing $17\beta E_2$, which is probably the *in vivo* scenario. Accordingly, Tam is employed in pharmacological treatment of tumours in classical E target tissues like mammary cancer [9]. Moreover, indications exist for its use like an antiproliferative agent in non-classical target tumours [8,33,39].

In conclusion, our data supported the antiproliferative effect of Tam on SPNC, and therefore, for a tumour like SPN, in which the benefit of chemotherapy or radiotherapy is uncertain [4,17], these results could offer a new avenue in the treatment of this rare condition with SERM molecules. The possibility to evaluate the potential benefit of Tam, a therapeutical approach

largely used in other conditions, should be tested in the future in patients affected by SPNs.

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