Transcription Factor AP-2γ Is a Developmentally Regulated Marker of Testicular Carcinoma *In situ* and Germ Cell Tumors

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ABSTRACT

Purpose: **Transcription factor activator protein-2γ (*TFAP2C,* AP-2γ) was reported previously in extraembryonic ectoderm and breast carcinomas but not in the testis. In our recent gene expression study we detected AP-2γ in carcinoma *in situ* testis (CIS, or intratubular germ cell neoplasia), precursor of testicular germ cell tumors. In this study we aimed to investigate the expression pattern of AP-2γ and to shed light on this factor in germ cell differentiation and the pathogenesis of germ cell neoplasia.**

Experimental Design: **We analyzed expression pattern of AP-2γ at the RNA and protein level in normal human tissues and a panel of tumors and tumor-derived cell lines. In the gonads, we established the ontogeny of expression of AP-2γ in normal and dysgenetic samples. We also investigated the regulation of AP-2γ by steroids and retinoic acid.**

Results: **We detected abundant AP-2γ in testicular CIS and in testicular germ cell tumors of young adults and confirmed differential expression of AP-2γ in somatic tumors. We found that AP-2γ expression was regulated by retinoic acid in an embryonal carcinoma cell line (NT2). The investigation of ontogeny of AP-2γ protein expression in fetal gonads revealed that it was confined to oogonia/gonocytes and was down-regulated with germ cell differentiation. In some prepubertal intersex cases, AP-2γ was detected outside of the normal window of expression, probably marking neoplastic transformation of germ cells.**

Conclusions: **AP-2γ is developmentally regulated and associated with the undifferentiated phenotype in germ cells. This transcription factor may be involved in self-renewal and survival of immature germ cells and tissue-specific stem cells. AP-2γ is a novel marker of testicular CIS and CIS-derived tumors.**

INTRODUCTION

Carcinoma *in situ* testis (CIS, also known as intratubular germ cells neoplasia; ref. 1) is the preinvasive stage of testicular germ cell tumors of adolescents and young adults. There is growing evidence that testicular CIS may be initiated *in utero* by an arrest in differentiation of early germ cells (primordial germ cells or gonocytes), which later undergo malignant transformation (2). The etiology of neoplastic transformation of germ cells is unknown, but according to our hypothesis this may be due to the action of adverse exogenous/environmental influences acting on a genetically susceptible fetus (3). The fetal origin hypothesis was initially based on a morphologic similarity between the CIS cells and gonocytes and later supported by a similarity in the protein expression pattern (2, 4). In a recent large-scale microarray analysis of gene expression in testicular tissues containing CIS compared with normal testis tissue (5), we identified a high abundance of genes linked to the embryonic stem cell phenotype. Among others, we found a high expression of the transcription factor activator protein-2 (AP-2γ, 50 kDa, also known as estrogen receptor factor 1, ERF-1, and AP2.2) and established that the transcript localized to CIS cells. This caught our attention, because the expression of AP-2γ was not described previously in the testis. By contrast, a large number of studies explored this factor in other tissues, especially in mammary gland and in breast cancer.

AP-2γ is encoded by the *TFAP2C* gene mapped on chromosome 20q13.2 (6) and is a member of a family of DNA-binding transcription factors, which includes AP-2α, AP-2β, AP-2γ, TFAP-2δ, and TFAP-2ε (7–12; reviewed in ref. 13). During vertebrate embryogenesis, these proteins play important roles in the development and differentiation of the neural tube, neural crest derivatives, skin, heart, and urogenital tissues and show overlapping, but distinct, patterns of expression within these tissues (10, 14, 15). AP-2γ is required within the extraembryonic lineages for early postimplantation development (16), but apparently does not play an autonomous role within the embryo proper. Mice lacking AP-2γ die at day 7.5 to 8.5 after coitum due to malformation of extraembryonic tissues (17). In humans, expression of AP-2γ has been associated with neoplasia, particularly in breast cancer. AP-2γ protein (and AP-2α) was significantly overexpressed in breast carcinomas compared with normal mammary tissue (18) and in breast tumor-derived cell lines that expressed proto-oncogene *ERBB2* (19). AP-2 proteins have been reported to interact with regulatory elements associated with the estrogen receptor gene, and AP-2γ expression was reported to be induced by estrogens (20). Both the promoter and an intronic enhancer within the *ERBB2* gene contain binding sites for AP-2 proteins, and AP-2γ is involved

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in the estrogenic regulation of *ERBB2* gene expression (21–23). In addition, the list of genes proposed to be regulated by AP-2 proteins is composed of the universal cell-cycle inhibitor *CDKN1A*, a tyrosine kinase receptor for the stem cell factor *KIT*, insulin-like growth factor I (*IGF1*), retinoblastoma protein (*RB1*), c-myc (*MYC*), and E-cadherin (*CDH1;* refs. 24–27). AP-2γ has been recognized as a retinoic acid (RA)-inducible gene in a screen of murine embryonal carcinoma cells (9).

The aim of this study was to explore the possible role of AP-2γ in the pathogenesis of reproductive tumors, especially in the testis, and to additionally investigate the timing of neoplastic transformation of germ cells. To that end, we determined a comprehensive expression profile of AP-2γ in a large panel of normal tissues, tumors, and tumor-derived cell lines; established the ontogeny of AP-2γ in normal and dysgenetic human gonads; and studied regulation of the AP-2γ expression in selected cell lines.

MATERIALS AND METHODS

Tissue Samples. The Regional Committee for Medical Research Ethics in Denmark approved the use of human tissue samples for the studies of novel genes expressed in germ cell cancer. The tissue samples from adults with testicular neoplasms were obtained directly after orchidectomy and macroscopic pathological evaluation. Each testicular sample was divided into several tissue fragments, which were either snap-frozen at −80°C for nucleic acid extraction or fixed overnight at 4°C in Stieve's fluid, buffered formalin, or paraformaldehyde, and subsequently embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or with antibodies against placental alkaline phosphatase for histologic evaluation (28, 29). A series of 31 overt testicular tumors was analyzed by immunohistochemistry, including classical seminomas, various nonseminomatous tumor components, spermatocytic seminomas, Leydig cell tumors, and a testicular B-cell lymphoma (listed in Table 1). Moreover, 14 samples of testicular CIS were analyzed: 12 that were from tissue adjacent to a testicular germ cell tumor, and 2 that had not progressed to an overt tumor but only had signs of microinvasion. Furthermore, we included 8 samples containing normal testicular tissue from either patients with prostate cancer or from tissue surrounding a GCT. A representative panel of tissues adjacent to the microscopically examined samples (seminomas, nonseminomatous tumors, and specimens with CIS) was analyzed by reverse transcription-PCR (RT-PCR) and *in situ* hybridization (ISH). RNAs for RT-PCR were isolated from homogeneous tumors and from specimens containing the largest possible number of tubules with CIS, as judged by microscopic examination of adjacent tissue fragments.

The series for immunohistochemical analysis of the ontogeny of expression of AP-2γ in normal and dysgenetic gonads included paraffin-embedded specimens from tissue archives of the Rigshospitalet, Copenhagen University Hospital. The 40 normal fetal tissue samples (19 testicular and 21 ovarian specimens) were obtained after induced or spontaneous abortions and stillbirths, mainly due to placental or maternal problems. The developmental age was calculated from the date of the last menstrual bleeding, supported by the foot size of the fetus. Normal postnatal testicular samples ($n = 16$) were obtained either from autopsies of infants who died suddenly of causes unrelated to the reproductive system or as testicular biopsies performed in boys with acute leukemia for monitoring the spread of disease. Pathology specimens were composed of a series of 20 overtly dysgenetic gonads from individuals with intersex disorders or other components of the testicular dysgenesis syndrome (30) and 12 testicular biopsies with mild dysgenetic features (*e.g.,* undifferentiated tubules and/or microliths) with or without CIS, performed for diagnostic reasons in young adult men with subfertility or with contralateral germ cell tumors. Some of the normal/dysgenetic fetal and prepubertal tissues were used in our previous studies (31–34).

In addition 130 samples from extragonadal tissues were examined. Panels of normal tissues and mammary gland tissues with or without neoplastic lesions were investigated using commercial tissue arrays (MaxArray Human Breast Carcinoma and Human Normal Tissue Microarray slides, Zymed, South San Francisco, CA). All of the specimens are listed in Table 1.

Studies in Cell Lines. The following established cancer cell lines were grown in standard conditions (5% CO_2, 37°C): embryonal carcinoma NT2 and 2102EP lines and a mammary tumor-derived MCF7 line. Total RNA was isolated for RT-PCR as described below, whereas for the immunohistochemical staining of the protein, the cells were spun in a cytocentrifuge or directly grown on microscopic slides. For analysis of the effect of retinoic acid on the AP-2γ expression (35), NT2 cells were grown in DMEM (10% fetal bovine serum, 2 mmol/L L-glutamine, 25 IU/mL penicillin, and 25 μg/mL streptomycin) and stimulated with 10 μmol/L RA (Sigma-Aldrich, St. Louis, MO) for 0 to 15 days to induce differentiation; 2102Ep cells were grown in DMEM with added 100 μmol/L β-mercaptoethanol and stimulated with 10 μmol/L RA for 0 to 10 days. A possible estrogen regulation of AP-2γ was analyzed in MCF7 cells, which were grown in steroid-depleted DMEM (5% dextran-charcoal stripped fetal bovine serum, 1 × nonessential amino acids, 1 nmol/L insulin, 2 mmol/L L-glutamine, 25 IU/mL penicillin, and 25 μg/mL streptomycin) for 6 days and then exposed to 1 nmol/L estradiol, ICI-182,780 (antiestrogen), or EtOH (vehicle) for 24 or 48 hours.

RT-PCR. Total RNA was purified using the NucleoSpin RNAII kit as described by the manufacturer (Macherey-Nagel, Düren, Germany). RNA samples were DNase digested, and cDNA was synthesized using a dT_{20} primer. Specific primers were designed for *TFAP2C* (TFAP2C-ex6: ATCTTGGAGGACGAAATGAGAT, TFAP2C-ex7: CAGATGGCCTGGCTGCCAA), spanning intron-exon boundaries. RT-PCR was performed in duplex in 30 μL of (final concentrations): 12 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.9 mmol/L $MgCl_2$, 0.1% Triton X-100, 0.005% gelatin, 250 μmol/L deoxynucleotide triphosphate, and 30 pmol of each primer. For control of PCR load and cDNA synthesis, the expression of the marker gene *ACTB* was analyzed with the following primers (ACTB-ex4: ACCCACACTGTGCCCATCTA, ACTB-ex6: ATCAAAGTCCTCGGCCACATT). Cycle conditions for all of the PCR reactions were as follows: 1 cycle of 2 minutes at 95°C; 40 cycles of 30 seconds at 95°C, 1 minute at 62°C, and 1 minute at 72°C; and finally 1 cycle of 5 minutes at 72°C. PCR products were run on 1.5% agarose gels and visualized by ethidium bromide staining. Fragment size was 205 bp for *TFAP2C* and 815 for *ACTB*.

Table 1 Summary of the immunohistochemical staining for AP-2γ

Age	*n*	Histology/diagnosis	AP-2γ *	Remarks
Normal testes				
10–22 wg	11	Normal fetal testes	− to ++ (75–100%)	3 samples negative
23–26 wg	4	Normal fetal testes	+/− to + (5–50%)	
27–42 wg	2	Normal fetal testes	+/− (<10%)	
0–2.5 months	2	Normal infantile testes	− to + (25%)	
3–4 months	5	Normal infantile testes	+/− (single cells or <10%)	
0.5–2.5 years	3	Normal infantile testes	−	
3–11 years	7	Normal prepubertal testes	−	
16 years	1	Normal testis with complete spermatogenesis	−	
Normal ovaries				
9 wg	1	Normal fetal ovary	+ (single cells)	Oogonia+
12–16 wg	5	Normal fetal ovaries	+/− to +	Oogonia+, oocytes−
17–26 wg	7	Normal fetal ovaries	+/− to ++	Oogonia+, oocytes−
27–41 wg	9	Normal fetal ovaries	+ to ++	Oogonia+, oocytes−
Adult	1	Normal adult ovary	−	
Dysgenetic and intersex gonads				
15 wg	1	Fetal testis (mosaic isochromosomeY)	−	AMH+, OCT3/4-
20 wg	2	Fetal testes (AIS, 46,XY females)	+ (10%)	
4 months	1	Infantile testis (AIS, 46,XY females)	+ (5%)	
14 months	1	Ovotestis (46 XX hermaphrodite)	+/−	Gonocytes +, oocytes−
9 months–6 years	3	Prepubertal testes (AIS, 46 XY females)	+ to +++ (2–10%)	Gonocytes with CIS characteristics, PLAP+ or −
2–17	6	Prepubertal testes (AIS, 46 XY females)	−	
2 years	1	Infantile testis with decreased number of germ cell (idiopathic genital ambiguity)	−	
9 years	1	Pre-pubertal testis (Prader-Willy's syndrome)	−	
15 years	1	Leydig-cell hyperplasia (adrenogenital syndrome)	−	
15 years	1	46,XY female, 17βHD mutation	−	
18 years	1	Dysgenetic testis with CIS and gonadoblastoma (45,X/46XY male)	++ (90–100%)	
25 years	1	Klinefelter, bilateral testicular cryptorchidism	−	
20–39 years	9	Adult testes with TDS (undifferentiated tubules, microliths) but without CIS or TGCT	−	
30–48 years	3	Adult testes with TDS (undifferentiated tubules, microliths) and contralateral TGCT	−	
Testicular CIS and tumor samples				
Adult	5	CIS adjacent to seminomas	+ to +++ (90–100%)	
Adult	5	CIS adjacent to nonseminomas	+ to +++ (90–100%)	
Adult	2	CIS adjacent to mixed TGCT	++ (90–100%)	
Adult	2	CIS without overt TGCT	+++ (90–100%)	
Adult	12	Classical, homogenous seminomas	+ to+++ (90–100%)	
Adult	12	Nonseminomas	+/− to ++	Various epithelial and stromal elements positive
Adult	2	Spermatocytic seminomas	−	
Adult/6 years	3	Leydig cell tumors	− to +	Cytoplasmic, background?
14 years	1	Gonadoblastoma	+++	
Adult	1	Testicular B-cell lymphoma	−	
Other tissue samples				
Fetal 9–20 wg	1 of each	Liver, heart, thymus, thyroid gland, kidney, and adrenal gland	−	
Adult	11	Normal breast tissues	+ to ++ (90–100%)	Subpopulation of basally situated duct cells
Adult	47	Infiltrating ductal breast adenocarcinomas	7 samples +/− (<10%) 7 samples + (50%) 33 samples −	
Adult	1	Breast mucinous carcinoma	−	
Adult	4	Breast medullary carcinomas	2 samples +/− (90–100%)	
Adult	3	Ductal breast carcinoma *in situ*	+/−	Single basal cells
Adult	2	Tubular breast cancers	−	
Adult	1	Infiltrating nodular breast adenocarcinoma	−	

Table 1 Continued

Age	*n*	Histology/diagnosis	AP-2γ *	Remarks
Adult	4	Lung	+	Epithelial pneumocytes +
Adult	7	Skin	+/− (25%)	Basal cells
Adult	1	Kidney	+/− (50%)	Single cells in canaliculi, glomeruli−
Adult	1–3 of each	Skeletal muscle, heart muscle, stomach, esophagus, small intestine, colon, liver, spleen, pancreas, salivary gland, pituitary gland, adrenal gland, thyroid gland, parathyroid gland, thymus gland, tonsil, epididymis, uterus, cervix, ovarian serous adenocarcinoma, prostate gland, prostatic cancer, omentum, peripheral nerve, cerebral cortex, cerebellum	−	
Cell lines				
NT2 cells	Day 0,3,15 of retinoic acid treatment		− to +	In all samples a subpopulation of cells were stained
2102Ep	Day 0,1,3,7,10 of retinoic acid treatment		++	In all samples most cells were stained
MCF7	Exposed to ICI-182,780, ethanol or 1 nM estradiol for 24 or 48 hours		− to +	In all samples a subpopulation of cells were stained

Abbreviations: N, number; wg, weeks of gestation; AIS, androgen insensitivity syndrome; AMH, anti-Müllerian hormone; TDS, testicular dysgenesis syndrome; TGCT, testicular germ cell tumor; CIS, carcinoma *in situ*. Staining intensity: +++, strong staining; ++, moderate staining; +, weak staining; +/−, very weak staining; −, no positive cells detected.

* Staining intensity and approximate % of positive cells in a section.

Representative bands were excised, cloned (with TOPO Cloning Invitrogen, Carlsbad, CA), and sequenced for verification.

***In situ* Hybridization.** Probes for ISH were prepared by use of specific primers (first primer combination: AAGAGTTTGTTACCTACCTTACT and CATCAATTTGACATTTCAATGGC, second primer combination: *AATTAACCCTCACTAAAGGG*TTAAAGAGCCTTCACT and *TAATACGACTCACTATAG*GGCTAAGTGTGTGG) containing an added T3- or T7-promoter sequence, respectively (promoter sequences italicized). PCR conditions included the following: 5 minutes at 95°C; 5 cycles of 30 seconds at 95°C, 1 minute at 45°C, 1 minute at 72°C; and 20 cycles of 30 seconds at 95°C, 1 minute at 65°C, 1 minute at 72°C, and finally 5 minutes at 72°C. The resulting PCR product was purified on a 2% low melting point agarose gel and sequenced from both ends, using Cy5-labeled primers complementary to the added T3 and T7 tags. Aliquots of ~200 ng were used for *in vitro* transcription labeling, using the MEGAscript-T3 (sense) or MEGAscript-T7 (antisense) kits, as described by the manufacturer (Ambion, Houston, TX). The composition of the 10×-nucleotide mix included the following: 7.5 mmol/L ATP, GTP, and CTP; 3.75 mmol/L UTP; and 1.5 mmol/L biotin-labeled UTP. To estimate quantity and labeling efficiencies, aliquots of the labeled RNA product were analyzed by agarose gel electrophoresis, dotted onto nitrocellulose filters, and developed. ISH was performed on three to six different samples as described previously (36, 37).

Immunohistochemistry. A commercially available monoclonal anti-AP-2γ antibody (6E4/4:sc-12762, kindly provided for testing by Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used. The antibody was validated by Western blots by the manufacturer. In addition, several other antibodies were used as controls, in particular to detect CIS cells (antiplacental alkaline phosphatase, a monoclonal antibody from BioGenex, San Ramon, CA; anti-OCT3/4, C-20, and sc 8629, Santa Cruz Biotechnology) and to control for integrity of fetal and infantile testis samples obtained at autopsies (anti-AMH, a monoclonal antibody kindly provided by R. Cate, Biogen, Cambridge, MA, US). The immunohistochemical staining was performed using a standard indirect peroxidase method, as described previously for other antibodies (28, 32–34). Briefly, the dewaxed and rehydrated sections were heated in a microwave oven to unmask the antigen. Sections fixed in formalin or paraformaldehyde were heated in a 5% urea (pH 8.5), whereas sections fixed in Stieve's fluid were heated in TEG buffer (pH 9.0; Tris 6.06 g/5L and EGTA). Subsequently, the sections were incubated with 0.5% H_2O_2 to inhibit the endogenous peroxidase, followed by diluted nonimmune goat serum (Zymed) to block unspecific binding sites. The incubation with the primary anti-AP-2γ antibody diluted 1:25 to 1:100 was carried out overnight at 4°C. For tissues fixed in Stieve's fluid the primary antibody was diluted in Background Reducing antibody diluent (DakoCytomation, Glostrup, Denmark). For negative control, a serial section from each block was incubated with a dilution buffer. Subsequently, a secondary biotinylated goat-antimouse link antibody was applied (Zymed), followed by the horseradish peroxidase-streptavidin complex (Zymed). Between all of the steps the sections were thoroughly washed. The bound antibody was visualized using aminoethyl carbazole substrate (Zymed). Most sections were lightly counterstained with Mayer's hematoxylin to mark unstained nuclei.

The sections were examined under a light microscope (Zeiss) and scored systematically by two investigators (C. E. H-

H. and E. R-D. M.). The staining was assessed using an arbitrary semiquantitative score of the percentage of cells stained in a section and their staining intensity: +++, strong staining; ++, moderate staining; +, weak staining; +/−, very weak staining; −, no positive cells detected.

RESULTS

AP-2γ Is a New Marker for Testicular Carcinoma *In situ* and Derived Germ Cell Tumors. After the discovery of the high expression of AP-2γ in testicular tissues harboring CIS cells (5), we extensively mapped the expression at the mRNA level by RT-PCR in a panel of testicular neoplasms and tumor-derived cell lines. Subsequently, we determined cellular localization of AP-2γ transcripts by ISH and at the protein level by immunohistochemistry in a comprehensive panel of normal and neoplastic tissues.

RT-PCR showed a high expression of AP-2γ in samples of seminoma, teratoma, embryonal carcinoma, and CIS as compared with normal testicular tissue, which showed only a trace of reaction in 1 biopsy from a patient with an adjacent testicular germ cell tumor and could encompass a few CIS or microinvasive tumor cells (Fig. 1). Expression in CIS cells was approximately equivalent in tissue containing CIS adjacent to overt tumors and in CIS at the microinvasive stage. For a few CIS and tumor samples the expression was confirmed in the database of our earlier study by differential display PCR (36; results not shown). ISH revealed cytoplasmic staining in CIS cells, seminoma, embryonal carcinoma, and to lesser extent in some components of teratomas (Fig. 2). ISH in normal testicular tissue was not completely conclusive; in contrast to IHC, which did not show any detectable protein in normal germ cells, a reaction was observed in some spermatocytes and spermatids (results not shown). The presence of *TFAP2C* transcripts in the normal germ cells with a simultaneous lack of the protein product cannot, therefore, be excluded. This could be due to translational regulation of AP-2γ in the testis. A similar discrepancy between expression at the mRNA and the protein level was described previously for AP-2α in colon cancer (38). Immunohistochemistry demonstrated a high abundance of AP-2γ protein in CIS. CIS cells adjacent to testicular germ cell tumors were uniformly strongly positive for AP-2γ regardless of the tumor type and equally strong in CIS present in the testis and as an isolated premalignant lesion. Gonadoblastoma, a CIS-like premalignant lesion of severely dysgenetic gonads, was also strongly positive (Fig. 3). Differential expression was, however, observed in the overt testicular germ cell tumors: seminomas were strongly positive, whereas a heterogeneous pattern was detected in the nonseminomas (Fig. 2). Embryonal carcinoma demonstrated strongest staining, teratomas displayed staining in some epithelial and stromal elements, but many highly differentiated components were negative. In all of the testicular germ cell tumor specimens staining was present exclusively in the nucleus. No expression was detected in spermatocytic seminoma. In non-germ-cell–derived testicular tumors no expression was detected in a B-cell lymphoma and two Leydig cell tumors (1 testicular and 1 adrenal). One Leydig-Sertoli cell tumor was unexpectedly positive; however, as the staining was confined to the cytoplasm, this may be an unspecific reaction, which is quite frequently observed for various antibodies in the protein and glycoprotein-filled Leydig cells. A similar but much weaker unspecific staining in Leydig cell cytoplasm was also observed in a few specimens of testicular parenchyma. In general, the intensity of staining varied somewhat depending on the fixative used, with stronger staining in tissues fixed with paraformalde-

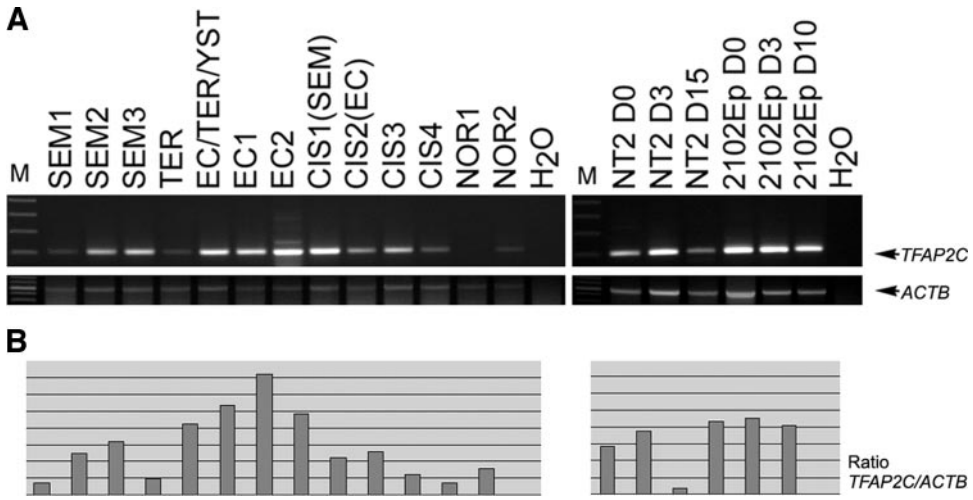

Fig. 1 Expression of AP-2γ at the RNA level in testicular tissue and cell lines by RT-PCR. *A,* agarose gel displaying RT-PCR analyses of *TFAP2C* (205 bp) and the control gene *ACTB* (815 bp; both gene products marked with *arrows*) in various tissues (*left*) and cell lines (*right*). *TFAP2C* mRNA is expressed in all of the analyzed neoplastic tissues at variable levels and to some degree in the tissue samples containing normal testicular tissue. A down-regulation is seen in the analyzed NT2 cells induced to differentiate with RA (at day15), whereas no change occurred in the nullipotent 2102Ep cells. *B,* the semiquantitative ratio of *TFAP2C:ACTB* for each sample shown in *A* determined by agarose gel scanning. *SEM,* seminoma; *TER,* teratoma; *EC,* embryonal carcinoma; *YST,* yolk sac tumor; *CIS (SEM/EC),* carcinoma *in situ* in the vicinity of seminoma/embryonal carcinoma; *CIS,* carcinoma *in situ*, which has not proceeded to an overt tumor; *NOR,* normal testicular tissue; *NT2,* NT2 cells at various days of RA treatment; *2102Ep,* 2102Ep cells at various days of RA treatment; H_2O, water control; *M,* 100-bp marker.

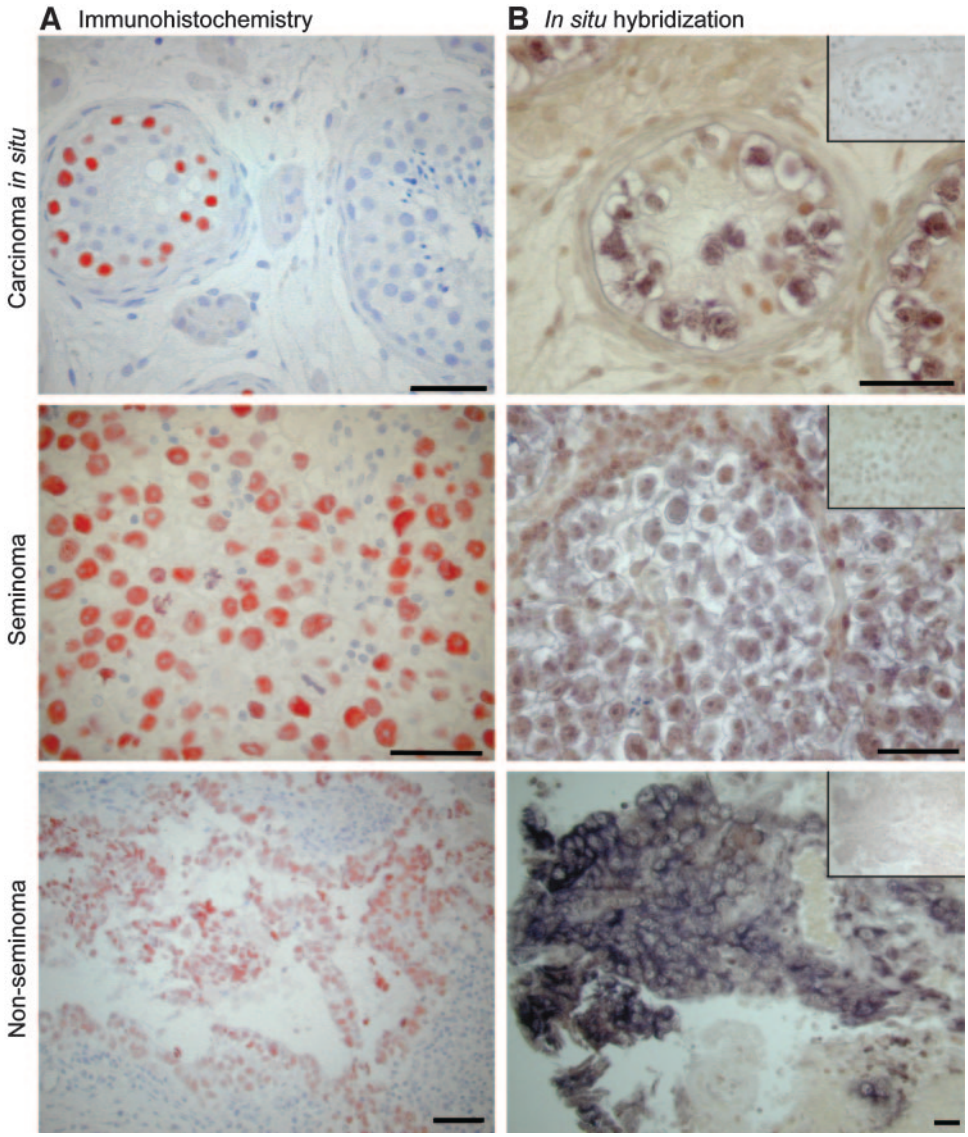

Fig. 2 Analysis of AP-2γ at the protein level by immunohistochemistry (*A*) and at the RNA level by ISH (*B*) in testicular neoplasms. ISH was performed with antisense and control sense (*insets*) RNA probes on serial histologic sections of the same tissue sample. CIS shows nuclear immunohistochemical staining. An adjacent tubule with normal spermatogenesis is visible with no staining. By ISH, CIS cells show a stained cytoplasmic ring with a stained nucleus in the center, whereas the rest appears empty because of large amounts of glycogen washed out during the tissue processing. Seminoma (*middle*) stained similarly to the CIS cells. Specimens of nonseminoma (*bottom*) show the presence of AP-2γ only in undifferentiated embryonal carcinoma cells, whereas some stromal or differentiated elements of the tumor are not stained. *Scale bar,* 50 μm.

hyde and formalin compared with those fixed in Stieve's and Bouin. In addition, in samples fixed in Stieve's fluid or in high-percentage formalin, there was a tendency for an unspecific staining of erythrocytes. The complete results of the immunohistochemical staining are listed in Table 1, and representative examples are shown in Figs. 2, 3, and 4.

The Expression of AP-2γ Is Developmentally Regulated in Human Gonads. To investigate whether the high expression of AP-2γ in CIS cells is linked to their gonocyte-like phenotype, as is the case for a number of other markers for CIS cells (4), we established the ontogeny of expression of AP-2γ by immunohistochemistry in a panel of specimens of normal human fetal testis and ovaries during development.

The AP-2γ protein was detected in almost all of the gonocytes in testes from 10 weeks of gestation (earlier samples were not available) until ~22 weeks of gestation (Fig. 3), although a few of our analyzed tissue samples failed to show expression. Expression was gradually reduced after week 22 but persisted in a few cells until ~4 months of postnatal age. Afterward, AP-2γ protein was not detected in spermatogonia in normal prepubertal, postpubertal, or adult testes.

In fetal ovaries AP-2γ expression was seen in oogonia in high amounts in early gestational ages and to a lesser extent in the third trimester. The number of positive oogonia was decreasing with the increasing number of oocytes that entered the meiotic prophase, which were AP-2γ negative. No expression was seen in adult ovary, but only a few samples were analyzed.

Changes of the Expression Pattern of AP-2γ in Intersex and Dysgenetic Gonads. Subsequently, we asked the question of whether or not the normal developmental pattern of the expression of AP-2γ is changed in testicular dysgenesis and disorders of sex differentiation, conditions with a markedly increased risk of germ cell neoplasia. Expression of AP-2γ in dysgenetic gonads was analyzed only by immunohistochemistry, because the speci-

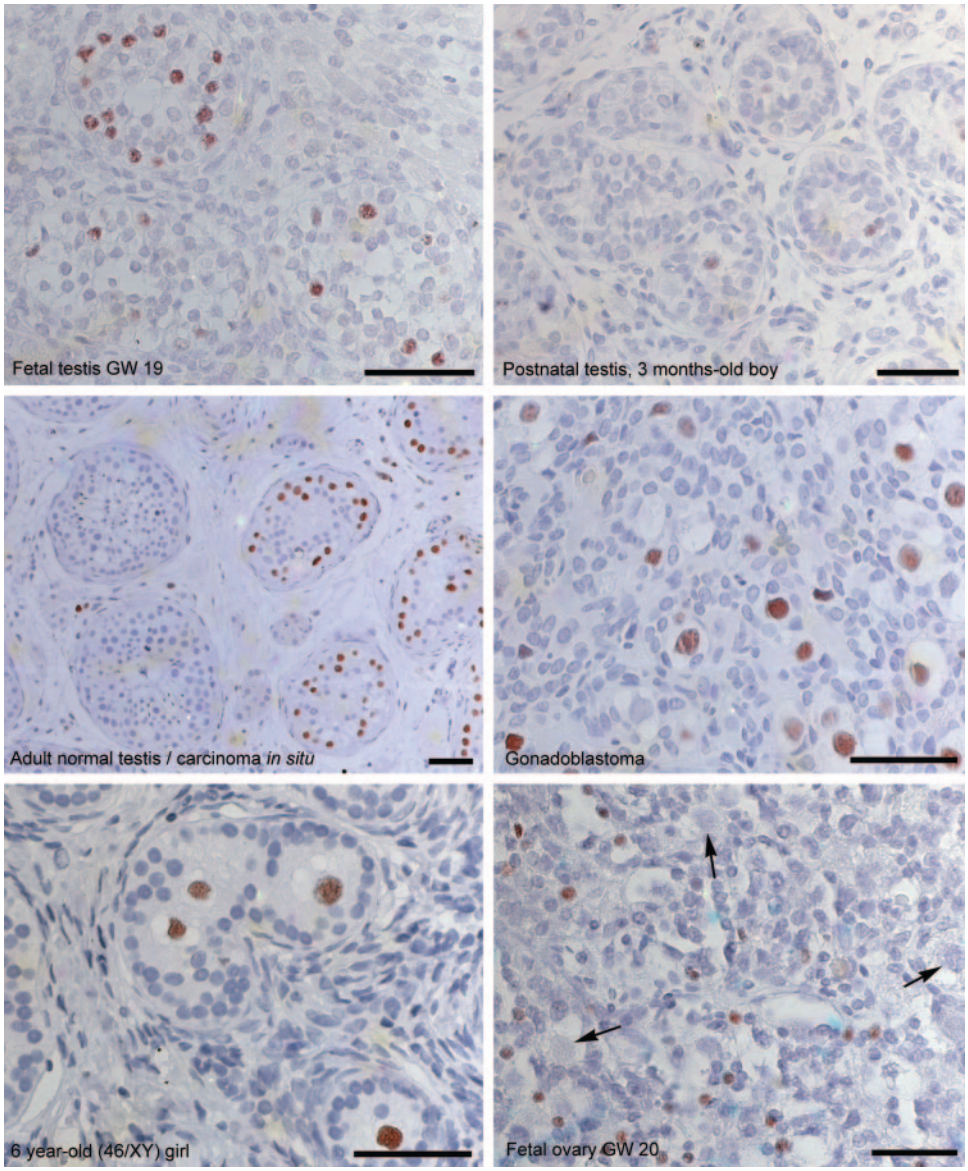

Fig. 3 Examples of the immunohistochemical expression of AP-2γ protein in testicular tissues and ovary. Fetal testicular tissue of gestational week 19 showing expression in almost all of the gonocytes. Normal postnatal testicular tissue from a 3-month-old boy showing expression in only a subset of gonocytes. In the image with adult testicular tissue there are tubules containing CIS with abundant nuclear staining at the *right* and at the *left* tubules with normal spermatogenesis. Gonadoblastoma with staining in the CIS-like cells. Dysgenetic testicular tissue from a 6-year-old girl (*46, XY*) with the androgen insensitivity syndrome (*AIS*) showing expression in atypical spermatogonia partially resembling CIS cells. Fetal ovarian tissue of gestational week 20 with expression in oogonia, and no expression in dictyate oocytes (*arrows*). *Scale bar,* 50 μm.

mens were available only as paraffin-embedded blocks from tissue archives. The pattern of expression in these specimens was in general similar to that in the normal testes, but in some samples expression deviated from the observed pattern. Expression of AP-2γ was not observed in a sample from a male fetus at gestational age 15 weeks with mosaic isochromosome Y, similar to a previously observed lack of OCT3/4 (34). Staining for AMH was positive, suggesting that the integrity of proteins should be preserved in the sample. Prolonged expression of AP-2γ was seen in cells resembling gonocytes in testicular cords in an ovotestis from a 14-month-old XX hermaphrodite but not in the oocytes present in the ovarian part. In a few of the dysgenetic samples neoplastic, preinvasive CIS, or gonadoblastoma was present, and these showed a high expression of AP-2γ. In 3 cases staining was observed in foci of germ cells that morphologically were classified as somewhere in between gonocytes and CIS (see Fig. 3). In 2 of the cases (girls with androgen insensitivity syndrome of age 9 months and 6 years, respectively) placental alkaline phosphatase was also positive. The third was from a testis removed from a 2-year-old girl with androgen insensitivity syndrome, where a very small focus of AP-2γ–positive cells was detected. We were not able to locate this focus morphologically in adjacent sections cut before and after, and stainings with placental alkaline phosphatase and OCT3/4 were accordingly negative.

Expression of AP-2γ Is Associated with Cell Differentiation or Proliferation and Is Cell-Type Dependent. To define the extent of expression and a possible link to hormonal stimulation, expression of AP-2γ was analyzed in some tissues that are endocrinologically activated and in a panel of normal somatic tissues. In normal female mammary tissue expression was seen in a subpopulation of basally situated epithelial duct cells, and expression was also detected in some, but not all, of

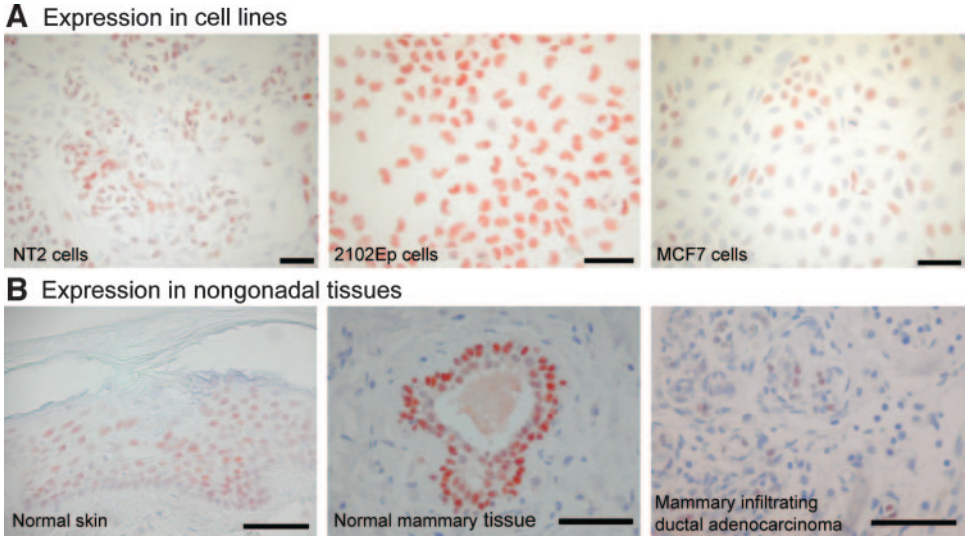

Fig. 4 Examples of the immunohistochemical expression of AP-2γ in (*A*) cell lines and in (*B*) various nongonadal tissues. The cell lines show staining in a subset of NT2 cells (Day 0), strong staining in all 2102Ep cells (Day 0), and staining in a subset of MCF7 cells (estrogen-treated 24 hours). The nongonadal tissues shown are normal epidermis and dermis, normal mammary tissue, and mammary infiltrating ductal adenocarcinoma. *Scale bar,* 50 μm.

the premalignant or manifest malignant changes in the female breast (Fig. 4 and Table 1). In samples from skin, the basal layer of keratinocytes was positive, and weaker staining could be seen in the adjacent layers of epidermis (Fig. 4), which is in concert with the pattern reported previously (39). In normal adult kidney AP-2γ expression was seen in a subset of cells in proximal tubules. Finally, AP-2γ expression was present in lung tissue, in pneumocytes lining alveoles. All of the other analyzed tissue samples were negative (Table 1). We cannot exclude that the AP-2γ expression may be developmentally regulated in some tissues, in analogy to the testis. We could not gain access to a full spectrum of human fetal tissues at different developmental stages, but we have examined and found no AP-2γ expression in the following fetal tissues from the first or second trimester of gestation: epididymis, liver, heart, thymus, thyroid gland, kidney, and adrenal gland.

Finally, regulation of AP-2γ expression was studied *in vitro* in three cell lines. We showed by RT-PCR that AP-2γ was RA-regulated in NT2 cells in a biphasic manner. Strong expression was observed in cells without RA-induced differentiation, and after 3 days of RA treatment (which at that point induced cell proliferation) the expression was somewhat stronger and then declined after 15 days when the cells had differentiated (Fig. 1). A heterogeneous staining for AP-2γ protein was seen in NT2 cells by immunocytochemistry in all of the analyzed samples, regardless of the length of RA treatment. It was not possible to establish the pattern quantitatively because of differences in intensity from cell to cell. In contrast, in the 2102Ep cells, which do not differentiate with RA treatment, expression was unaffected by RA treatment at the RNA level, and immunocytochemical staining was uniformly strong in all of the analyzed cells. In MCF7 cells, expression was similar in cells exposed to estradiol, the antiestrogen ICI, or EtOH, both at the RNA level and at the protein level, where expression was seen in a subset of cells in all of the analyzed cells, regardless of treatment (Fig. 4). For examples of RT-PCR results, see Fig. 1.

DISCUSSION

In the present study we comprehensively investigated the pattern of expression of the transcription factor AP-2γ and established AP-2γ as a novel marker for neoplastic germ cells, including testicular CIS, the common precursor of testicular germ cell tumors. Furthermore, we discovered that AP-2γ was developmentally regulated in human gonads, with marked differences between the testes and ovaries.

Our investigations of the pattern of expression of AP-2γ in extragonadal normal and neoplastic tissues were largely in concert with previous studies, thus confirming the specificity of the antibody. The transcription factor was preferentially expressed in proliferating immature cells, which retained tissue-specific stem cell characteristics, suggesting a putative oncogenic function of AP-2γ.

In the testis, we found that the AP-2γ protein was expressed in early gonocytes but not in any cell type in the pre- or postpubertal testis. The expression rapidly declined from ~22 weeks of gestation and was gradually lost, whereas gonocytes differentiated into infantile spermatogonia up to ~4 months of postnatal age, when the final stage of this differentiation takes place, probably stimulated by the hormonal surge at the "mini-puberty" (40, 41). Interestingly, in a few prepubertal intersex cases, AP-2γ was observed in a subset of gonocytes outside the normal window of expression. This prolonged expression may reflect a delay in differentiation but may perhaps be a marker of the initiation of neoplastic transformation of these immature germ cells into "pre-CIS" cells, which then may additionally transform to a typical CIS pattern at the onset of puberty. In the ovary, AP-2γ protein was confined to oogonia and was not detectable after meiotic entry in oocytes. This pattern of expression suggests that the protein plays a role exclusively in undifferentiated germ cells. Analyses in cell lines confirmed that AP-2γ was most abundant in undifferentiated cells, as expression of the transcript was down-regulated after a transient induction in AP-2γ–positive NT-2 cells treated with RA for up to

15 days. In cells that do not differentiate (2102Ep), expression was constantly high. Oulad-Abdelghani *et al.* (9) reported a transient rise in AP-2γ levels with a peak after 12 hours of RA treatment in embryonal carcinoma-derived P19 cells but did not analyze levels beyond 24 hours. An association of AP-2γ with an immature state of germ cells was also observed in overt testicular germ cell tumors, where classical seminoma and embryonal carcinoma were strongly positive, whereas highly differentiated somatic elements of teratomas were negative. Spermatocytic seminoma, which is not derived from CIS but from mature spermatogonia (42), was also AP-2γ–negative. Overall, the pattern of expression of AP-2γ in testicular germ cell tumors resembles that of KIT (31) and OCT-4 (34, 43), which are also associated with an undifferentiated state and pluripotency, typical for cells retaining some stem cell-like features. The high expression of AP-2γ in CIS supports our hypothesis that this premalignant lesion is derived from an early fetal germ cell. In adult testes AP-2γ was only seen either in CIS or in overt testicular germ cell tumors or in rare specimens harboring dysgenetic features and in the latter only in gonadoblastoma and CIS cells. Thus, AP-2γ can be added to a list of markers for germ cell neoplasms that are also present in gonocytes but not detectable in the normal adult testis, such as placental alkaline phosphatase, KIT, or OCT-4 (4).

Besides an inverse association with cell differentiation, which was also noted by others in other cell systems (44, 45), very little is known concerning the biological function and the mechanism of action of AP-2γ, especially in germ cells. The most interesting question is the following: what are the gene targets for AP-2γ in germ cells and with which other proteins does this transcription factor interact? In other cell types AP-2γ has been shown previously to interact with a number of genes that are also highly expressed in CIS cells, *e.g.,* insulin-like growth factor-binding protein 5 (44, 46) or KIT (25, 31). KIT appears to be of particular interest, as it was reported to be down-regulated in several types of tumors derived from tissues that express AP-2γ, *e.g.,* breast cancer, colorectal cancer, or melanoma (25, 38, 47).

Estrogen signaling is another possible candidate pathway that could be regulated by AP-2γ, as is suggested by its expression in the epithelium of milk ducts and the association with some forms of breast cancer. Whereas the classic estrogen receptor (ERα) is not expressed in the testis (neither in the fetal life or adulthood), the ERβ is expressed in human fetal testis in the second trimester, and gonocytes are potential targets for estrogen action (48). This is of interest because it has been suggested that inappropriate exposure to estrogens during fetal life may have an impact on the development of CIS and testicular germ cell tumors (30). Furthermore, the progression from CIS into an overt testicular germ cell tumor occurs after the endocrinological surge of puberty, when the intratesticular levels of steroid hormones rise markedly. In the current study, we have analyzed the expression of AP-2γ in a breast tumor-derived cell line, MCF7, which is estrogen dependent. We have not observed any changes in expression, neither after treatment with estradiol nor with an antiestrogen. This is in disagreement with a recent study (20), which showed induction of AP-2γ by estrogens in several breast tumor cell lines. Whether AP-2γ is indeed involved in estrogen signaling remains an unresolved issue, because existing reports are partially contradictory and will require additional investigations. We need also to keep in mind that the function of AP-2γ may be cell-type specific, as was demonstrated recently for the transcriptional activation of the *ERBB2* gene (49), and, thus, may be different in germ cells compared with somatic cell types.

In summary, our investigations suggest a role for AP-2γ in pathways regulating cell differentiation, including fetal gonocytes, which was not known previously. Frequent expression of AP-2γ in various malignancies suggests that the pathways may be involved in oncogenesis. Finally, developmental expression of this transcription factor in the testis makes AP-2γ a very promising new marker for diagnosis of early stages of germ cell neoplasia.

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