0013-7227/01/$03.00/0
Printed in U.S.A.

Endocrinology 142(9):4040–4046

Ovarian Granulosa Cell Tumors Express a Functional Membrane Receptor for Anti-Müllerian Hormone in Transgenic Mice

MARTIN DUTERTRE, LUCILE GOUÉDARD, FRANÇOISE XAVIER, WEN-QING LONG, NATHALIE DI CLEMENTE, JEAN-YVES PICARD, AND RODOLFO REY

Unité de Recherches sur l'Endocrinologie du Développement (INSERM) (M.D., L.G., F.X., W.-Q.L., N.D.C., J.-Y.P., R.R.), Ecole Normale Supérieure, Département de Biologie, 92120 Montrouge, France; Centro de Investigaciones Endocrinológicas (R.R.), Hospital de Niños "R. Gutiérrez," C1425EFD Buenos Aires, Argentina

Anti-Müllerian hormone inhibits granulosa cell growth and function. Both anti-Müllerian hormone and its type II receptor are expressed in normal granulosa cells. We show by histologic and molecular analyses that ovarian tumors developing in transgenic mice, obtained by targeted oncogenesis using an anti-Müllerian hormone promoter-SV40 oncogene construct, are of granulosa-cell origin. Because tissue-specific, cell-surface molecules are of particular interest for the analysis and treatment of tumors, we examined the expression of anti-Müllerian hormone type II receptor in the ovaries of these transgenic mice. We demonstrate that the anti-Müllerian hormone type II receptor is expressed not only in normal ovarian follicles, but also in granulosa cell tumors. Using a cell line derived from one of these tumors, we show that the anti-Müllerian hormone type II receptor protein is present on the surface of tumor cells and binds anti-Müllerian hormone. Furthermore, we show that the anti-Müllerian hormone receptor is functional in the granulosa tumor cell line, with anti-Müllerian hormone treatment inducing selective activation of Smad1. In conclusion, in this study we present a new murine transgenic model of granulosa cell tumors of the ovary and, using this model, we demonstrate for the first time cell-surface expression of a highly tissue-specific molecule, anti-Müllerian hormone type II receptor, as well as the selective activation of Smad proteins by anti-Müllerian hormone, in granulosa tumor cells. (*Endocrinology* 142: 4040–4046, 2001)

POSTNATAL OVARIAN GRANULOSA cells express anti-Müllerian hormone (AMH) (1), a glycoprotein also known as Müllerian-inhibiting substance (2), and its type II receptor, AMHR-II (3–5). AMH inhibits cell growth (6, 7) and expression of aromatase and LH receptor (7) in cultured granulosa cells. Conversely, AMH gene knockout in mice results in faster recruitment of primordial follicles to the population of growing follicles (8). In addition to its effects on granulosa cells, recent studies show that AMH also inhibits the growth of human cancer cells derived from the ovarian surface epithelium (9–11).

AMH belongs to the TGF-β superfamily, including activins and bone morphogenetic proteins (BMPs). Most of these factors signal through specific binding to two distinct membrane receptors, type I and type II, and subsequent activation of intracellular proteins, such as Smads, which in turn modulate gene expression (12). The ligand binds to the type II receptor, which phosphorylates and activates a type I receptor. The latter phosphorylates receptor-regulated Smads (R-Smads), which then associate with a common partner, Smad4, and translocate to the nucleus. R-Smads 1, 5, and 8 participate in BMPs pathways, whereas R-Smads 2 and 3 transduce TGF-β and activin signals (13). AMH type II receptor (AMHR-II) has been identified (3, 4), and we recently showed that the bone morphogenetic protein type IB (BMPR-IB/ALK6) receptor can mediate an AMH-evoked response in testicular cell lines, involving Smad-1, but not Smad-2, activation (14).

Interestingly, both AMH and AMHR-II are expressed in a highly tissue-specific manner. AMH expression is restricted to Sertoli cells of the testis and granulosa cells of the ovary (15, 16). In women, AMH is a specific and sensitive marker of granulosa cell tumor progression (17–19). AMHR-II mRNA has been found in the Müllerian duct (the anlagen of the uterus in fetuses) (3, 5), in Sertoli (3, 5) and Leydig cells (20, 21) of the testis, and in ovarian granulosa cells (3, 5). More recently, AMHR-II has also been detected in cells of the ovarian surface epithelium in the human species (9), but not in rodents (3, 5).

We (22) and others (23) have previously shown that driving the expression of the simian virus 40 (SV40) oncogene by the AMH promoter induces gonadal tumorigenesis in transgenic mice. As expected from the AMH expression pattern, testicular tumors of these mice are Sertoli cell tumors, and we now show, using histologic and molecular analyses, that the ovarian tumors derive from granulosa cells. Using this model, we demonstrate for the first time the cell-surface expression of a highly tissue-specific molecule, AMHR-II, as well as the selective activation of Smad proteins by AMH, in granulosa tumor cells.

Materials and Methods

Generation of transgenic mice

The generation of the transgenic mice used in this study has been described previously (22). Basically, the transgene is a fusion construct,

Abbreviations: AMH, Anti-Müllerian hormone; AMHR-II, anti-Müllerian hormone type II receptor; BMPs, bone morphogenetic proteins.

named AT-t, containing 3.6 kb of the 5′ flanking region of the human AMH gene upstream of a mutant simian virus 40 (SV40) oncogene encoding the large T antigen. Female mice were killed at different ages. Gonads and other organs with apparent tumor development were weighed and either fixed for histologic studies or snap frozen for RNA preparation. All animal studies were conducted in compliance with European Community guidelines.

Cell culture

Ovarian tumors from a 9-month-old transgenic female were dilacerated in 1× trypsin-EDTA and 1 mg/ml collagenase. Cells were sedimented by centrifugation and plated onto tissue-culture dishes in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin, amino acids and 15% FCS. Culture was performed at 37 C in a humidified atmosphere of 94% air/6% CO_2. At the sixth passage, a subconfluent culture was trypsinized and cells diluted to one cell per 300 μl were plated (100 μl per well) in 96-well plates. The resulting three clones were amplified. The parental cell line and all three clones expressed AMHR-II, and the clone exhibiting the strongest expression of AMHR-II mRNA (named AT29C-U493) was selected for further study. AT29C-U493 cells were maintained in culture and underwent 20 additional passages. Northern blot analyses for granulosa cell markers were performed at the 10th, 15th and 20th passage. CHO and CHO-3W cells were cultured as previously described (24).

AMH binding in cell culture

Two different experimental protocols were used separately to test AMH binding to AT29C-U493 cells. In the first protocol, cells were plated on poly-D-lysine four-chamber Lab-Tek slides (Nunc, Naperville, IL) at 2–5 × 10^4 cells per chamber. Twenty-four hours later, cells were exposed for 3 h at 37 C to DMEM alone or containing 1 nM plasmin-cleaved AMH (3). After rinsing with DMEM, cells were incubated for 1 h in DMEM/0.1% FCS with 2.5 μg/ml of either L40, a rabbit polyclonal anti-AMH antibody (19), or nonimmune rabbit immunoglobulins. After rinsing, cells were incubated for 1 h with a peroxidase-conjugated goat antibody raised against rabbit IgG; as a negative control, a similar antibody raised against mouse IgG (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA) was used. The reaction was revealed by incubation with DAB Plus Reagent (DAKO Corp., Copenhagen, Denmark). In the second experimental protocol, iodinated AMH was used as previously described (3, 24). Briefly, cells were incubated for 3 h in 1 ml DMEM containing 1 nM iodinated plasmin-cleaved AMH or with a 200-fold excess of cold hormone. After rinsing, the slides were prepared for autoradiography as described (25). After 1 wk, the slides were developed, stained with toluidine blue, and examined under darkfield illumination.

Immunoprecipitation and Western blot analysis

Cells were seeded at 10^5 cells/ml and treated 1 d later with AMH 357 nM (24), TGF-β 1 nM (R&D Systems, Abingdon, UK) or bone morphogenetic protein 2 (BMP2) 10 nM (generous gift from Genetics Institute, Boston, MA) for 45 min. Immunoprecipitation and Western blot analyses were performed as previously described (14). In brief, cell lysates were immunoprecipitated for 2 h at 4 C with 1 μg/ml of anti-Smad1 or anti-Smad2 polyclonal antibodies (Upstate Biotechnology, Inc., Lake Placid, NY). Immunoprecipitates were washed and eluted, proteins were subjected to 7.5% SDS PAGE, Western blotting with anti-Smad4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 5 μg/ml and chemiluminescence detection (ECL Plus kit, Amersham Pharmacia Biotech). For Western blots, anti-phospho-Smad1 and anti-phospho-Smad2 antibodies (Upstate Biotechnology, Inc.) were used at 2 μg/ml.

Histology, immunocytochemistry/fluorescence and in situ hybridization

Classic histology was performed on 5-μm sections of paraffin-embedded tissue stained with hematoxylin and eosin. AMH immunohistochemistry was performed using the antigen retrieval technique by microwaves (19). For the detection of AMHR-II protein by immunofluorescence, a specific polyclonal antibody obtained in rabbit against the extracellular domain of AMHR-II purified under nondenaturing conditions was used as previously described (14). Immunofluorescence for the detection of Smad1, Smad4, β-catenin, cytokeratin, and vimentin in frozen tissues or in cultured cells was performed as previously described (14, 26). *In situ* hybridization in paraffin-embedded, paraformaldehyde-fixed tissue was performed as described (27), except that prehybridization was carried out for 2 h at 55 C and hybridization, overnight at 37 C. The digoxigenin-labeled AMHR-II riboprobe covered 312 bp of the second exon of the mouse AMHR-II (28).

RNA preparation and analysis

Total RNA of tissues and cells was prepared either by the method of Chirgwin *et al.* (29) or using the RNA plus kit (Bioprobe, Montreuil, France). Northern blots were performed as previously described (3, 22). Probes for AMH, rat sulfated glycoprotein-2 (SGP-2), rat FSH receptor (FSHR), steroidogenic factor-1 (SF-1) and human Wills' tumor-1 (WT-1) were obtained as previously described (22). Other probes (AMHR-II, P450aro, P450SCC and 3β-HSD) were synthesized from murine testes by RT-PCR (22). The AMHR-II probe covers an 890 bp cDNA fragment (exons 1–6). The oligonucleotide probe for rabbit 18S ribosomal RNA was GGGCGGTGTGTACAAAGGGCAGGGA. Probes were ^{32}P-labeled as described (22).

Measurement of serum AMH

Mouse AMH was measured by an enzyme-linked immunosorbent assay (ELISA) as described (30). Each sample was assayed in quadruplicate. Calibration curves were constructed using increasing concentrations (0.4–25 ng/ml) of recombinant mouse AMH. The lowest detectable concentration of mouse AMH was 10 ng/ml. Intra and interassay variability coefficients were 2.40% and 2.71%, respectively.

Results

Histologic and molecular characterization of ovarian granulosa cell tumors in AMH-SV40 transgenic mice

Female mice derived from 5 different AMH promoter-SV40 oncogene (AMH-SV40) transgenic founders were studied. The incidence of ovarian tumors was variable (Table 1). The ovarian tumors were frequently bilateral, though of variable size, and could occupy more than 25% of the abdominal cavity. When the gonads weighed up to 100 mg (approximately 6- to 10-fold increment compared with normal ovaries), normal follicles containing oocytes were still present, together with irregularly proliferating masses of small granulosa cells with intensely heterochromatic nuclei (Fig. 1A). Larger ovarian tumors (Fig. 1B) were characterized by the absence of normal tissue and the presence of serous cystic spaces and large areas of hemorrhage and necrosis, and also by local invasion of neighboring organs. In older animals,

TABLE 1. Incidence and size range of ovarian tumors in AMH-SV40 transgenic females

Mouse line	Age (months)	Females studied	Animals with ovarian tumors	Tumor weight (range)
AT-t 68	3–8	6	0 (0%)	
	> 8	6	1 (16.7%)	0.02 g
AT-t 83	3–8	34	6 (17.6%)	0.03–0.85 g
	> 8	15	10 (66.7%)	0.06–6.48 g
AT-t 84	3–8	28	26 (92.9%)	0.02–5.04 g
	>8	9	9 (100%)	0.07–12.02 g
AT-t 93	3–8	11	4 (36.4%)	0.01–0.13 g
	>8	10	6 (60.0%)	0.02–2.72 g
AT-t 94	3–8	6	0 (0%)	
	>8	10	6 (60.07%)	0.01–2.35 g

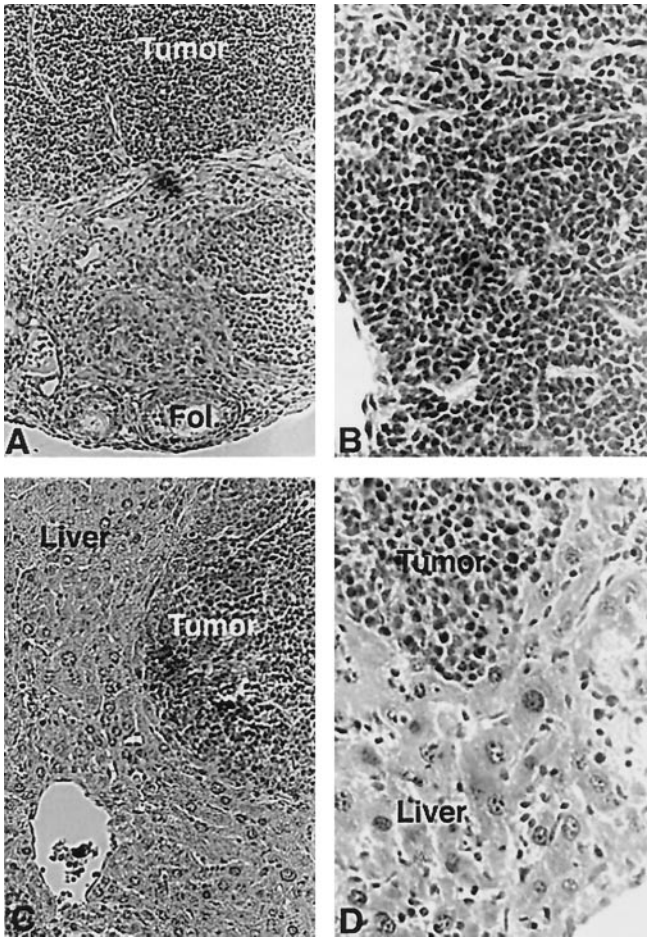

FIG. 1. Histological aspect of ovaries from AMH-SV40 transgenic mice, hematoxylin-eosin stain. A, Ovarian granulosa-cell tumor weighing 30 mg; few follicular structures (Fol.) are present in the periphery, whereas tumor mass cells occupy the center of the gonad. B, Ovarian granulosa-cell tumor weighing 125 mg; only tumor cells, arranged in a trabecular pattern can be seen. Magnifications: A, ×100; B, ×360. C–D, Liver from a 22-month-old transgenic female; tumor cells, of a similar aspect to those observed in the ovaries, invade the liver; both gonads weighed over 2 g and consisted of granulosa-cell tumors with big areas of necrosis and hemorrhage. Magnifications: C, ×200; D, ×360.

extragonadal tumors, localized to the liver or the lungs, were observed in approximately 10% of the cases. In all cases, the histologic features of these tumors confirmed they were metastases of ovarian tumors (Fig. 1, C and D). Vimentin and cytokeratin are useful markers for the classification of ovarian tumors. In coincidence with observations in human granulosa cell tumors (31), we found that while vimentin immunoreactivity was observed in the majority of tumor cells, only few cells expressed cytokeratin (results not shown).

AMH immunoreactivity both *in situ* (19, 32) and in the serum (17, 18) is a specific molecular marker for ovarian granulosa cell tumors in humans. In keeping with previous reports in other mammals (2, 33, 34), we found AMH protein expression in granulosa cells of primary, secondary and antral follicles of normal mouse ovaries (Fig. 2A). Ovarian tumors of less than 100 mg also exhibited AMH expression, confirming their granulosa cell origin. Within the tumor, AMH-positive and AMH-negative cells were intermingled (Fig. 2B). Serum AMH levels were increased in transgenic females with ovarian tumors weighing up to 100 mg, when compared with wild-type females (Fig. 3). AMH immunoreactivity *in situ* and serum AMH concentration dropped to undetectable levels in females with advanced tumors.

To obtain an *in vitro* counterpart of our new model of granulosa cell tumors, we established a clonal cell line from the ovarian tumors of a transgenic mouse. This cell line, named AT29C-U493, exhibited expression of several granulosa cell markers, including SF-1 (35), WT-1 (36) and AMHR-II (3, 5) (Fig. 4), as well as cytochrome P450scc (37) and SGP-2 (38) (data not shown). However, AT29C-U493 cells did not express AMH, the FSH receptor, aromatase (P450aro) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (data not shown). This expression profile did not change between the 10th and the 20th passages of AT29C-U493 cells culture *in vitro*. In coincidence with what was observed in the

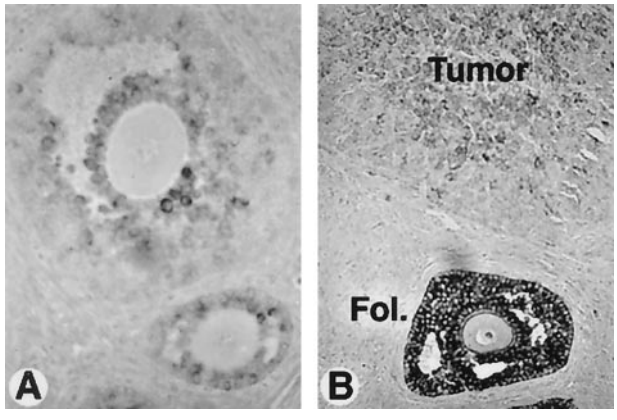

FIG. 2. Immunohistochemical localization of AMH in normal ovaries and ovarian tumors of AMH-SV40 transgenic mice, using a peroxidase-DAB revelation system. In normal ovaries, AMH immunoreaction faints progressively in the outer granulosa cell layers of antral follicles (A). In an ovary weighing 30 mg, obtained from a 5-month-old transgenic female, AMH reaction is positive in an apparently normal follicle (Fol.), whereas AMH-positive and AMH-negative cells can be seen in a small granulosa-cell tumor (B). Magnifications: A, ×150; B, ×100.

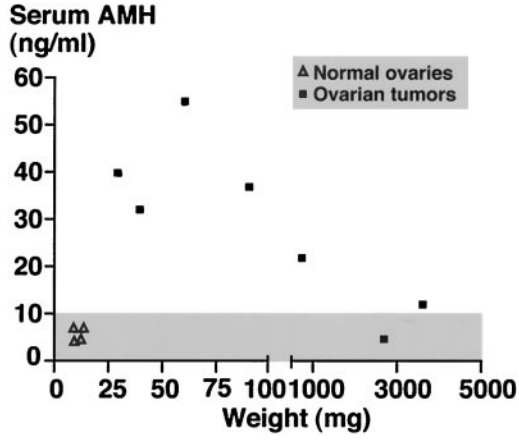

FIG. 3. Serum AMH levels in normal and AMH-SV40 transgenic female mice. The *shaded area* indicates levels below the lowest limit of detection of the assay. *Weight* reflects the weight of one gonad for each animal.

tumors, the majority of AT29C-U493 cells showed a strong expression of vimentin (results not shown).

AMHR-II is a cell surface marker for ovarian granulosa cell tumors

Because AMHR-II is a membrane protein expressed in a highly cell-specific manner in granulosa cells, we examined its expression in the tumors. AMHR-II mRNA expression was studied by *in situ* hybridization with a specific riboprobe in paraffin-embedded tissues, and AMHR-II protein expression was examined by immunofluorescence using a specific anti-AMHR-II polyclonal antibody, which required the use of frozen tissue not submitted to any prefixation procedure. AMHR-II expression was detected in granulosa cells of primary, secondary and antral follicles of normal ovaries (Fig. 5A), confirming previous observations in the rat (5) and rabbit (3). Tumors of less than 100 mg (10-fold the size of the normal ovary) were positive for both AMHR-II mRNA (Fig. 5B) and protein (Fig. 5C), but no expression was found in larger tumors (results not shown).

To test whether the AMHR-II protein was expressed at the cell surface of tumor cells, we used the AT29C-U493 cell line *in vitro*. In immunofluorescence experiments, the anti-AMHR-II antibody specifically stained AT29C-U493 cells not submitted to permeabilization (Fig. 6A), indicating that the AMHR-II is present on the cell surface.

Because AMHR-II is the only cell surface protein known to bind AMH, we then examined the ability of nonpermeabilized AT29C-U493 cells to bind AMH. A positive reaction was observed when AT29C-U493 cells were successively incubated with AMH, a rabbit anti-AMH antibody and a peroxidase antirabbit IgG secondary antibody (Fig. 6B). No reaction was seen when there was no AMH in the medium (Fig. 6C), when the anti-AMH antibody was replaced by preimmune serum, or when the antirabbit IgG secondary antibody was replaced by an antimouse IgG secondary antibody (results not shown). More importantly, no reaction was observed when CHO cells, devoid of AMHR-II expression (24), were incubated with AMH, the anti-AMH antibody and the secondary antibody (results not shown), *i.e.* the same conditions under which AT29C-U493 cells gave a positive reaction. Positive results were observed when CHO cells stably transfected with AMHR-II, called CHO-3W (24), were incubated under the same conditions (results not shown). Like in the case of AT29C-U493 cells, no reaction was seen in CHO-3W cells when AMH, L40 or the secondary antibody was replaced by its respective negative control. To validate these results, we also performed radioactive ligand binding experiments using iodinated AMH, which gave similar results, *i.e.* labeling of AT29C-U493 and CHO-3W cells and no labeling of untransfected CHO cells (results not shown).

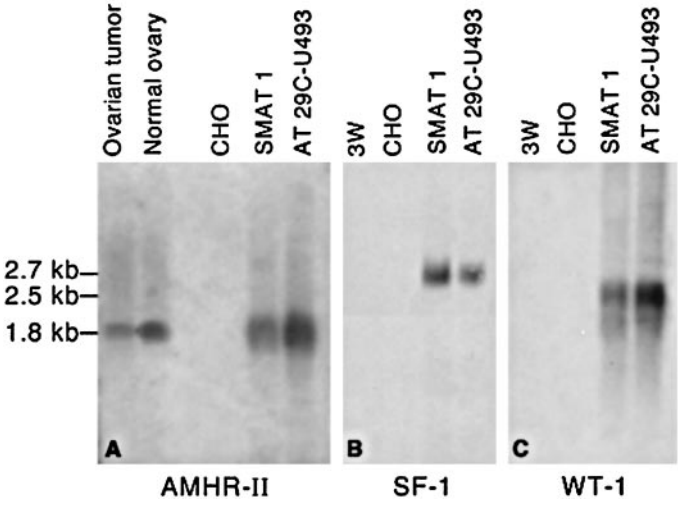

FIG. 4. Expression of AMHR-II, steroidogenic factor 1 (SF-1) and Wilms' tumor 1 (WT-1) in ovarian tumors from AMH-SV40 transgenic mice and in AT29C-U493 cells at the 20th passage, as revealed by Northern hybridization. SMAT1 is a clonal Sertoli cell line derived from the testicular tumors of a AMH-SV40 T antigen mouse (22). A, 1.8-kb band, corresponding to AMHR-II, is observed in normal ovarian tissue, in tissue from ovarian tumors of transgenic females, in AT29C-U493 cells, and in SMAT1 cells. No expression is seen in CHO cells. B and C, SF-1 and WT-1 are expressed in SMAT1 and AT29C-U493 cells but not in 3W and CHO cells.

AMHR-II is functional and activates specific Smads in granulosa tumor cells

Upon binding to their respective ligands, type II receptors for TGF-β superfamily members induce the phosphorylation and nuclear translocation of Smad proteins, with different ligands activating different Smads (13). We have recently shown that in testicular cells, AMH/AMHR-II specifically activate Smad1 but not Smad2, leading to its association with

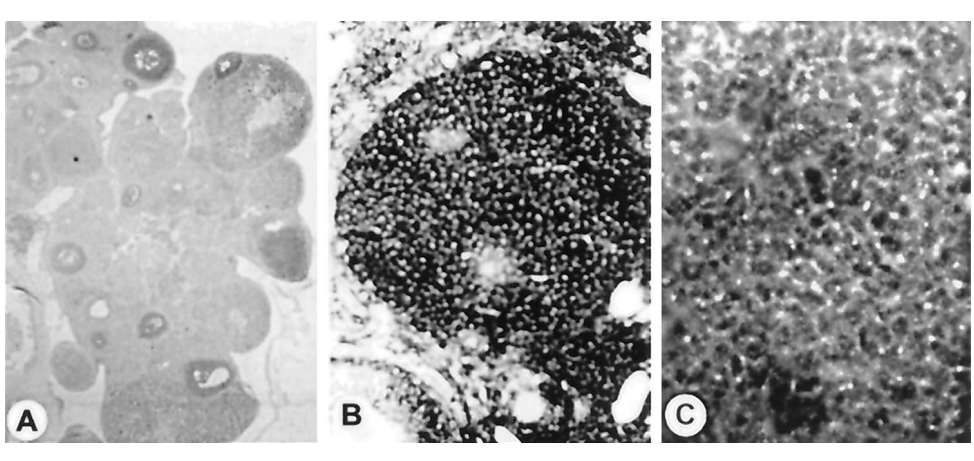

FIG. 5. AMHR-II expression in ovarian tumors of AMH-SV40 transgenic mice. A and B, Localization of AMHR-II mRNA by *in situ* hybridization using a digoxigenin-labeled probe and an alkaline phosphatase-NBT revelation system (*dark staining*) in a normal ovary and in an ovarian tumor. AMHR-II mRNA expression is detected in granulosa cells of primary and growing follicles (A) and in a small granulosa-cell tumor (B). C, Localization of AMHR-II protein by immunofluorescence in an ovarian tumor (a positive reaction is seen as *gray*/*white* in this black-and-white micrograph). Magnifications: A, ×15; B, ×100; C, ×400.

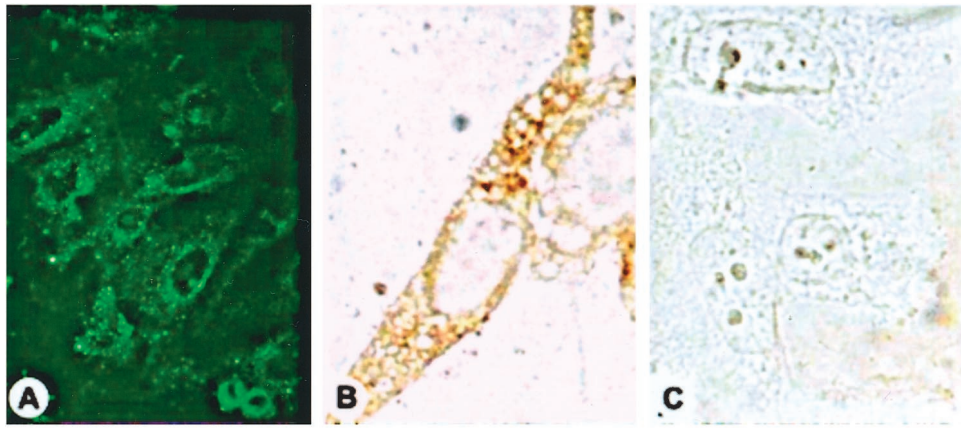

FIG. 6. Cell-surface expression of AMHR-II and AMH binding to AT29C-U493 cells. A, Localization of AMHR-II protein by immunofluorescence in nonpermeabilized AT29C-U493 cells using a specific anti-AMHR-II antibody. B and C, AMH binding to nonpermeabilized AT29C-U493 cells in culture. AT29C-U493 cells show a positive reaction when cultured successively in the presence of AMH, anti-AMH rabbit antibody L40, antirabbit Ig G coupled to peroxidase and DAB substrate (B); no reaction is observed when AMH is absent from the first incubation medium (C). Magnification: A, ×400; B and C, ×1000.

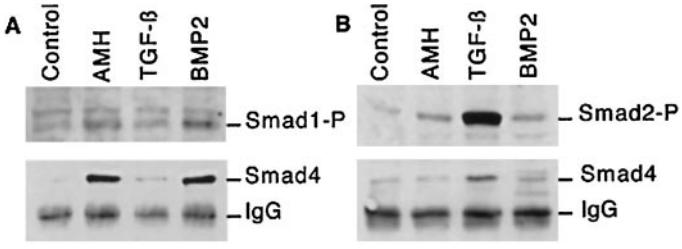

FIG. 7. Selective activation of Smad1 by AMH in the AT29C-U493 cell line. *Top*, Smad1 phosphorylation by AMH in AT29C-U493 cells. Cells were treated 45 min with AMH, TGF-β or BMP2, and Smad1 (A) or Smad2 (B) phosphorylation was analyzed by Western blotting with anti-Smad1-P or anti-Smad2-P antibodies. *Bottom*, AMH-dependent Smad1/Smad4 interaction: AT29C-U493 cells were treated 45 min with either AMH, BMP2 or TGF-β. Smad1/Smad4 complexes were detected by immunoprecipitation with an anti-Smad1 (A) or an anti-Smad2 (B) antibody followed by Western blotting with an anti-Smad4 antibody.

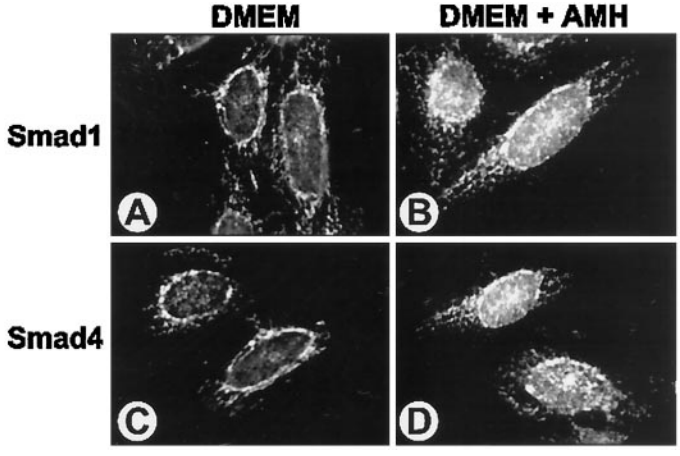

FIG. 8. Translocation of Smad1 and Smad4 proteins to the nucleus upon AMH treatment of AT29C-U493 cells: Cells were incubated 1 h in DMEM (A and C) or in DMEM with AMH (B and D), and Smad1 (A and B) or Smad4 (C and D) were detected by immunofluorescence using anti-Smad1 or anti-Smad4 antibodies and a fluorescein isothiocyanate-conjugated secondary antibody. Both Smads are present in the cytoplasm in basal conditions (A: Smad1; C: Smad4) and translocate to the nucleus after incubation with AMH (B: Smad1; D: Smad4).

Smad4 (14). To examine the functional significance of AMHR-II expression in AT29C-U493 cells, we investigated the effect of AMH on the phosphorylation, interactions and localization of Smads. First, a Western blot analysis using antibodies that specifically recognize the phosphorylated form of Smad1 and Smad2 showed that AMH induced the specific phosphorylation of Smad1 in AT29C-U493 cells (Fig. 7A, *top*). As expected, also treatment with BMP2, but not with TGF-β, resulted in Smad1 phosphorylation. On the contrary, only TGF-β phosphorylated Smad2 (Fig. 7B, *top*). Second, by immunoprecipitating Smad1 with a specific antibody and subjecting the precipitate to Western blot analysis using a Smad4 antibody, we showed that AMH and BMP2, but not TGF-β, induced Smad1/Smad4 interaction in AT29C-U493 cells (Fig. 7A, *bottom*). When immunoprecipitation was performed using an anti-Smad2 antibody, Western blotting with the Smad4 antibody showed that TGF-β but not AMH or BMP2 induced Smad2/Smad4 interaction (Fig. 7B, *bottom*). Finally, immunofluorescence performed in AT29C-U493 cells using anti-Smad1 and anti-Smad4 antibodies showed that both Smad proteins were localized in the cytoplasm before AMH treatment and translocated to the nucleus after incubation with AMH (Fig. 8). Taken together, these data indicate that the AMH receptor is functional and activates specific Smads in AT29C-U493 cells.

Discussion

We and others previously reported that transgenic mice expressing an AMH promoter-SV40 T antigen fusion gene develop ovarian tumors (22, 23). In agreement with the highly tissue-specific expression of AMH in the granulosa cells of the postnatal ovary, we now show, using histologic and expression studies of granulosa cell markers, that these ovarian tumors are of granulosa cell origin. AMH immunoreactivity both in the serum (17, 18) and *in situ* (19, 32) has been so far the most specific molecular marker for ovarian granulosa cell tumors in humans. In keeping with these observations, the granulosa cell tumors of our transgenic mice produce AMH and secrete it into the blood when they are relatively small. In advanced stages, the tumors invade neighboring organs and develop metastases in the liver and lungs, thus following a pattern that is very similar to that observed in humans (39, 40).

The clonal cell line AT29C-U493, derived from the ovarian tumors of a transgenic mouse, exhibited expression of several granulosa cell markers, including mRNAs for WT-1, SF-1,

and AMHR-II. Although none of the markers studied is exclusive to granulosa cells, their combined expression in the same clonal cell line strongly suggests a granulosa cell origin. We are aware that AT29C-U493 cells do not have all the features of normal granulosa cells because they do not express the FSH receptor and aromatase. Although the expression of these markers could be maintained in a few granulosa cell lines obtained in particular conditions, loss of or dramatic decrease in their expression has been most frequently observed in cell lines expressing the SV40 virus oncogene, which has been attributed to oncogenic transformation and culture conditions (Ref. 37 and references therein). Furthermore, it is possible that, by using the AMH gene promoter to drive SV40 oncogene expression, we selected a granulosa cell subpopulation. Specific markers of granulosa cells are also lost in human granulosa cell tumors (41–43). Nevertheless, the AT29C-U493 cell line retains several granulosa cell markers, and in the course of this study, it proved to be useful for the analysis of the AMH receptor transduction mechanisms, as discussed below. It is also interesting to note that expression of the transcription factor SF-1, which has been involved in the tissue-specific expression of AMH (44), AMHR-II (45) and aromatase (44), does not warrant expression of all of them in the cell line, in keeping with previous reports indicating the need for other cell-specific transcription factors (46, 47).

Granulosa cell tumors account for 6–10% of malignant neoplasms of the ovary (39). Although their malignant potential is relatively low in the first years of the disease (40), recurrences may appear up to 30 yr after surgical extirpation of the primary tumor. Prognosis of recurrences can be improved if the diagnosis is made early, when the recurrent tumor is still small. However, when tumor cells are spread over the peritoneum, complete surgical removal is impossible. In the last decade, cell surface antigens have been used as targets for the delivery of both diagnostic (48) and therapeutic (49) molecules to tumors, as an alternative to radio- or chemotherapy. Systemic injections are usually required in the case of spread out metastases and inaccessible tumors. However, one major difficulty has been the identification of tissue- or tumor-specific cell surface antigens, especially in the ovary (48–50). The AMHR-II gene encodes a cell surface protein and its mRNA was found to be expressed in ovarian granulosa cells in a highly tissue-specific manner (3, 5). In our murine model, the AMHR-II protein was detected not only in normal ovarian follicles, but also inside granulosa cell tumors, and was present at the cell surface of the derived granulosa tumor cell line. In humans, we have previously detected AMHR-II mRNA in granulosa cell tumors (24) and, more recently, AMHR-II has been detected also in cancers deriving from the ovarian surface epithelium (9). It should be noted that two properties of the AMHR-II make it a particularly interesting cell surface tumor marker. First, its expression is highly tissue specific. Second, it can be targeted not only by antibodies, but also by its ligand (AMH), which is much smaller. Indeed, in our model both an antibody and AMH bound to the surface of tumor cells.

In our murine model, AMH dropped to undetectable levels and AMHR-II expression was also lost when tumors are left to their natural progression and reach very large dimensions. This is a less probable finding in humans, because large tumors rarely escape diagnosis and treatment. Together with previous studies demonstrating growth-inhibitory effects of AMH on granulosa cells (6) and on ovarian surface epithelium cancer cells (9–11), the loss of AMH and AMHR-II expression in large granulosa cell tumors in our model raises the possibility that their expression is counter-selected to favor further tumor progression, and that AMH treatment of AMHR-II-positive tumors may inhibit tumor growth. Here we report the development of both *in vitro* and *in vivo* murine models that represent interesting tools to further test this hypothesis in the case of granulosa cell tumors. Recent data suggest that AMH effects on granulosa cell growth may depend on the follicular stage or environment (51), and clearly, AMH effects remain to be assessed in a granulosa cell tumor context as well.

The molecular mechanisms underlying the effects of AMH on ovarian cells are poorly understood. Receptors for TGF-β superfamily members signal in part through Smad proteins. Smad1 and Smad2 are activated by BMPs and TGF-βs, respectively, whereas Smad4 is activated by all (13). We have recently shown that in testicular cell lines, AMH/AMHR-II specifically activate Smad1, but not Smad2, leading to its association with Smad4 (14). Like in testicular cells, treatment of AT29C-U493 cells with AMH induced the phosphorylation of Smad1, its physical interaction with Smad4, and the translocation of both from the cytoplasm to the nucleus. Furthermore, AMH did not induce the phosphorylation of Smad2. These data indicate that the AMHR-II is functional in our granulosa tumor cell line and identify for the first time Smads activated by AMH in ovarian cells.

In conclusion, we have characterized a new model of granulosa cell tumors of the ovary obtained by cell type-specific, genetically targeted oncogenesis in transgenic mice. Using this model, we have identified the AMHR-II protein as a highly tissue-specific molecule expressed at the cell surface of ovarian granulosa tumor cells, and we show that the AMH receptor is functional and activates selective Smad proteins in these cells.

Acknowledgments

We are thankful to Peggy Adin for technical collaboration and to Dr. Héctor Chemes for helpful suggestions on binding experiments.

Received February 8, 2001. Accepted May 24, 2001.

Address all correspondence and requests for reprints to: Dr. Rodolfo Rey, Centro de Investigaciones Endocrinológicas, Hospital de Niños, Gallo 1330, C1425EFD Buenos Aires, Argentina. E-mail: rodolforey@infovia.com.ar.

This work was supported by grants of the Association pour la Recherche sur le Cancer, France, and of the Agencia Nacional de Promoción Científica y Tecnológica (PICT'97, No. 05-064) and the Ministerio de Salud (Beca Oñativia-Carrillo), Argentina. M.D. and L.G. were recipients of research fellowships of the Association pour la Recherche sur le Cancer. R.R. is an established researcher with the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) and an invited professor at the Ecole Normale Supérieure (supported by Procédure PAST of the Ministère de l'Education Nationale, de la Recherche et la Technologie, France).

References

1. **Vigier B, Picard JY, Tran D, Legeai L, Josso N** 1984 Production of anti-Müllerian hormone: another homology between Sertoli and granulosa cells. Endocrinology 114:1315–1320

2. **Takahashi M, Hayashi M, Manganaro TF, Donahoe PK** 1986 The ontogeny of Müllerian inhibiting substance in granulosa cells of the bovine ovarian follicle. Biol Reprod 35:447–454
3. **di Clemente N, Wilson CA, Faure E, et al.** 1994 Cloning, expression and alternative splicing of the receptor for anti-Müllerian hormone. Mol Endocrinol 8:1006–1020
4. **Baarends WM, van Helmond MJL, Post M, et al.** 1994 A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the Müllerian duct. Development 120:189–197
5. **Baarends WM, Uilenbroek JTJ, Kramer P, et al.** 1995 Anti-Mullerian hormone and anti-Mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951–4962
6. **Kim JH, Seibel MM, MacLaughlin DT, et al.** 1992 The inhibitory effects of Mullerian-inhibiting substance on epidermal growth factor induced proliferation and progesterone production of human granulosa-luteal cells. J Clin Endocrinol Metab 75:911–917
7. **di Clemente N, Goxe B, Remy JJ, et al.** 1994 Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine 2:553–558
8. **Durlinger ALL, Kramer P, Karels B, et al.** 1999 Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology 140:5789–5796
9. **Masiakos PT, MacLaughlin DT, Maheswaran S, et al.** 1999 Human ovarian cancer, cell lines, and primary ascites cells express the human Mullerian inhibiting substance (MIS) type II receptor, bind, and are responsive to MIS. Clin Cancer Res 5:3488–3499
10. **Ha TU, Segev DL, Barbie D, et al.** 2000 Müllerian inhibiting substance inhibits ovarian cell growth through an Rb-independent mechanism. J Biol Chem 275:37101–37109
11. **Stephen AE, Masiakos PT, Segev DL, Vacanti JP, Donahoe PK, MacLaughlin DT** 2001 Tissue-engineered cells producing complex recombinant proteins inhibit ovarian cancer in vivo. Proc Natl Acad Sci USA 98:3214–3219
12. **Josso N, di Clemente N** 1999 TGF-β family members and gonadal development. Trends Endocrinol Metab 10:216–222
13. **Zhang Y, Derynck R** 1999 Regulation of Smad signalling by protein associations and signalling crosstalk. Trends Cell Biol 9:274–279
14. **Gouédard L, Chen YG, Thevenet L, et al.** 2000 Engagement of bone morphogenetic protein type IB receptor and Smad1 signaling by anti-Müllerian hormone and its type II receptor. J Biol Chem 275:27973–27978
15. **Lee MM, Donahoe PK** 1993 Müllerian inhibiting substance - a gonadal hormone with multiple functions. Endocr Rev 14:152–164
16. **Josso N, Cate RL, Picard JY, et al.** 1993 Anti-Müllerian hormone, the Jost factor. Recent Prog Horm Res 48:1–59
17. **Gustafson ML, Lee MM, Scully RE, et al.** 1992 Müllerian inhibiting substance as a marker for ovarian sex-cord tumor. N Engl J Med 326:466–471
18. **Long WQ, Ranchin V, Pautier P, et al.** 2000 Detection of minimal levels of serum anti-Müllerian hormone during follow-up of patients with ovarian granulosa cell tumor by means of a highly sensitive enzyme-linked immunosorbent assay. J Clin Endocrinol Metab 85:540–544
19. **Rey R, Sabourin JC, Venara M, et al.** 2000 Anti-Müllerian hormone is a specific marker of Sertoli- and granulosa-cell origin in gonadal tumors. Hum Pathol 31:1202–1208
20. **Racine C, Rey R, Forest MG, et al.** 1998 Receptors for anti-müllerian hormone on Leydig cells are responsible for its effects on steroidogenesis and cell differentiation. Proc Natl Acad Sci USA 95:594–599
21. **Lee MM, Seah CC, Masiakos PT, et al.** 1999 Müllerian-inhibiting substance type II receptor expression and function in purified rat Leydig cells. Endocrinology 140:2819–2827
22. **Dutertre M, Rey R, Porteu A, Josso N, Picard JY** 1997 A mouse Sertoli cell line expressing anti-Müllerian hormone and its type II receptor. Mol Cell Endocrinol 136:57–65
23. **Peschon JJ, Behringer RR, Cate RL, et al.** 1992 Directed expression of an oncogene to Sertoli cells in transgenic mice using Müllerian inhibiting substance regulatory sequences. Mol Endocrinol 6:1403–1411
24. **Imbeaud S, Faure E, Lamarre I, et al.** 1995 Insensitivity to anti-Müllerian hormone due to a spontaneous mutation in the human anti-Müllerian hormone receptor. Nat Genet 11:382–388
25. **Mathews LS, Vale WW** 1991 Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65:973–982
26. **Allard S, Adin P, Gouédard L, et al.** 2000 Molecular mechanisms of hormone-mediated Mullerian duct regression: involvement of β-catenin. Development 127:3349–3360
27. **Carré-Eusèbe D, di Clemente N, Rey R, et al.** 1996 Cloning and expression of the chick anti-Müllerian hormone gene. J Biol Chem 271:4798–4804
28. **Mishina Y, Tizard R, Deng JM, et al.** 1997 Sequence, genomic organization, and chromosomal location of the mouse Müllerian-inhibiting substance type II receptor gene. Biochem Biophys Res Commun 237:741–746
29. **Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ** 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299
30. **Al-Attar L, Noël K, Dutertre M, et al.** 1997 Hormonal and cellular regulation of Sertoli cell anti-Müllerian hormone production in the postnatal mouse. J Clin Invest 100:1335–1343
31. **Costa MJ, Derose PB, Roth LM, Brescia RJ, Zaloudek CJ, Cohen C** 1994 Immunohistochemical phenotype of ovarian granulosa cell tumors—absence of epithelial membrane antigen has diagnostic value. Hum Pathol 25:60–66
32. **Matías Guiu X, Pons C, Prat J** 1998 Müllerian inhibiting substance, α-inhibin, and CD99 expression in sex cord-stromal tumors and endometrioid ovarian carcinomas resembling sex cord-stromal tumors. Hum Pathol 29:840–845
33. **Ueno S, Kuroda T, MacLaughlin DT, Ragin RC, Manganaro TF, Donahoe PK** 1989 Müllerian inhibiting substance in the adult rat ovary during various stages of the estrous cycle. Endocrinology 125:1060–1066
34. **Bézard J, Vigier B, Tran D, Mauléon P, Josso N** 1987 Immunocytochemical study of anti-Müllerian hormone in sheep ovarian follicles during fetal and postnatal development. J Reprod Fertil 80:509–516
35. **Hatano O, Takayama K, Imai T, et al.** 1994 Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 120:2787–2797
36. **Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA, Housman D** 1991 Expression of the Wilms' tumor gene WT1 in the murine urogenital system. Genes Dev 5:1345–1356
37. **Walther N, Einspanier A, Jansen M** 1999 Expression of granulosa cell-specific genes and induction of apoptosis in conditionally immortalized granulosa cell lines established from H-2K(B)- tsA58 transgenic mice. Biol Reprod 60:1078–1086
38. **Hurwitz A, Ruutiainen-Altman K, Marzella L, Botero L, Dushnik M, Adashi EY** 1996 Follicular atresia as an apoptotic process: atresia-associated increase in the ovarian expression of the putative apoptotic marker sulfated glycoprotein-2. J Soc Gynecol Invest 3:199–208
39. **Young RH, Scully RE** 1992 Endocrine tumors of the ovary. In: Sasano N, ed. Current Topics in Pathology. Berlin: Springer-Verlag; 85:114–164
40. **Stenwig JT, Hazenkamp JT, Beecham JB** 1979 Granulosa cell tumors of the ovary: a clinicopathological study of 118 cases with long-term follow-up. Gynecol Oncol 7:136–152
41. **Fox H** 1985 Sex cord-stromal tumors of the ovary. J Pathol 145:127–148
42. **van Niekerk C, Ramaekers F, Hanselaar A, Aldeweireldt J, Poels L** 1993 Changes in expression of differentiation markers between normal ovarian cells and derived tumors. Hum Pathol 142:157–177
43. **Takayama K, Sasano H, Fukaya T, et al.** 1995 Immunohistochemical localization of Ad4-binding protein with correlation to steroidogenic enzyme expression in cycling human ovaries and sex cord stromal tumors. J Clin Endocrinol Metab 80:2815–2821
44. **Parker KL, Schimmer BP** 1996 The roles of the nuclear receptor steroidogenic factor 1 in endocrine differentiation and development. Trends Endocrinol Metab 7:203–207
45. **de Santa Barbara P, Moniot B, Poulat F, Boizet B, Berta P** 1998 Steroidogenic factor-1 regulates transcription of the human anti-mullerian hormone receptor. J Biol Chem 273:29654–29660
46. **Watanabe K, Clarke TR, Lane AH, Wang X, Donahoe PK** 2000 Endogenous expression of Müllerian inhibiting substance in early postnatal rat Sertoli cells requires multiple steroidogenic factor-1 and GATA-4-binding sites. Proc Natl Acad Sci USA 97:1624–1629
47. **Arango NA, Lovell-Badge R, Behringer RR** 1999 Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 99:409–419
48. **Wasserman L, Neri A, Kaplan B, Manor Y, Galli M, Hardy B** 1995 Monoclonal antibodies against native ovarian tumor cells: Specificity and characterization of the antigen. A preliminary report. J Cancer Res Clin Oncol 121:387–392
49. **Maraveyas A, Epenetos AA** 1993 Targeted immunotherapy—an update with special emphasis on ovarian cancer. Acta Oncol 32:741–746
50. **Boerman OC, Tibben JG, Massuger LFAG, Claessens RAMJ, Corstens FHM** 1995 Tumour targeting of the anti-ovarian carcinoma X anti-CD3/TCR bispesific monoclonal antibody OC/TR and its parental MOv18 antibody in experimental ovarian cancer. Anticancer Res 15:2169–2174
51. **McGee EA, Smith R, Spears N, Nachtigal MW, Ingraham HA, Hsueh AJ** 2001 Müllerian inhibitory substance induces growth of preantral ovarian follicles. Biol Reprod 64:293–298