Anti-Müllerian hormone : a look back and ahead

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Abstract

AMH is a member of the TGF-β family secreted by immature Sertoli cells and by granulosa cells of growing ovarian follicles. In males, it induces the regression of fetal Müllerian ducts and represses androgen synthesis through receptors located on the Leydig cell membrane. In female mice, AMH inhibits primary follicle recruitment and sensitivity to FSH. Measurement of circulating AMH is of value to pediatric endocrinologists allowing them to detect the presence and functional activity of testicular tissue without resorting to stimulation by human chorionic gonadotropin. In women, AMH levels are correlated with the size of the ovarian follicle pool and provide information on the likelihood of spontaneous or induced pregnancy.

Introduction

Anti-Müllerian hormone, AMH for friends, has an eventful history. Its existence was recognized in the middle of the 20th century (Jost 1953). Initially, AMH was thought to act exclusively in young male fetuses and as such did not appear particularly attractive to the medical community. The demonstration by Bernard Vigier et al. (1984) that AMH is produced by growing follicles in the adult ovary did nothing to change that opinion. However, when a team in Rotterdam built on Vigier's finding to show that the level of AMH in a woman's serum is correlated to the number of her ovarian follicles (de Vet, et al. 2002, van Rooij, et al. 2002), things started to change while biotechnology companies fought tooth and nail to win the market for AMH clinical assays.

I first fell in love with AMH in 1964 during Professor Jost's graduate course at the Paris Science Faculty. I had enrolled thinking that a brief introduction to basic science could be useful to an aspiring pediatric endocrinologist. Jost was a fascinating teacher. He made research sound so exciting that I upended my plans of a medical career and instead joined the National Institute for Health and Medical Research with the ambition of purifying the mysterious substance advertised by my mentor. Little did I imagine that it would take the efforts of three people during 20 years! The full story is told in the book "Le Sexe des Anges : une histoire d'hormones" (Josso 2017).

Alfred Jost and the AMH concept

At the beginning of fetal life, the reproductive tract is sexually undifferentiated. Both males and females possess Wolffian ducts, the primordia for male accessory organs and Müllerian ducts which in females develop into uterus and tubes. If testes are present, Wolffian ducts persist and Müllerian ducts disappear. The idea that a testicular product distinct from testosterone is responsible for Müllerian regression was born in 1953, following the seminal experiments of Alfred Jost (1953). Jost grafted a testosterone crystal near the ovary of a rabbit fetus and obtained a florid development of Wolffian ducts, but no regression of Müllerian ducts. These disappeared only if a fragment of testicular tissue was implanted instead. Jost concluded that a separate factor, different from testosterone, is responsible for Müllerian regression in mammals. He called it the Müllerian inhibitor, subsequently known as Müllerian inhibiting substance (MIS), factor (MIF) or anti-Müllerian hormone (AMH), the term generally in use today.

Régine Picon's contribution : the bioassay for anti-Müllerian activity

The AMH concept was not adopted without a struggle. « Professional » embryologists, for instance Professor Etienne Wolff in France, insisted that testosterone alone was responsible for male sex differentiation, Müllerian regression included. Their skepticism was understandable.The father of the AMH concept, Alfred Jost, was unable to suggest, let alone prove, its biochemical nature or its cellular origin. Fetal surgery, the method he had used to demonstrate the existence of AMH, was out of the reach of ordinary mortals. Thus, for 15 years, AMH remained a mystery. Then, in 1969, Régine Picon , a member of Jost's group at Paris University, set the ball rolling again (Picon 1969).

Since this issue is about women in Reproduction, I would like to take this opportunity to pay a tribute to Régine, whose contribution to AMH research does not get the recognition it deserves. Born in Sénégal, a former French colony in Africa, Régine Picon had studied zoology and botany in Dakar. She had published a paper on the development of the genital tract of sharks. After her marriage, she came to Paris and in 1960 joined the Jost group. There, her favorite species not being available, she switched to rats and undertook to study the effect of the rat fetal testis upon rat Müllerian ducts in organ culture. She dissected the reproductive tract of a sexually undifferentiated rat fetus and placed it on a metal grid in a dish containing a nutritive solution. Then, she placed a fetal rat testis it next to it. Three days later, she checked the result by histological examination. At the start of the culture period, the Müllerian duct was intact. At the end, it was gone ! Régine demonstrated that the Müllerian duct loses its responsiveness to AMH very early in fetal life, while in contrast the testis retains its anti-Müllerian activity until birth. Régine's findings represent a giant step forward because the bioassay she set up is accessible to any reasonably gifted researcher. Her work has been the key to all the early developments in AMH research.

The basics

Most hormones are either steroids, peptides or proteins. Steroids are small, so they are expected to cross obstacles impermeable to proteins. Insertion of a piece of dialysis membrane between the testis and the fetal Müllerian duct in the bioassay abolished testicular anti-Müllerian activity**,** proving that the size of the AMH molecule was greater than 15,000 daltons. Therefore, AMH was probably a protein, not a steroid or a small peptide (Josso 1972).

Which cells produce AMH ? To find out, the easiest way would have been to challenge a section of testicular tissue with a specific anti-AMH antibody but that was not available. Failing that, one could try to separate interstitial tissue from seminiferous tubules to test their anti-Müllerian activity separately. Rodents, let alone fetal ones, were too small but calf fetal testes, which could be obtained at a slaughter house proved suitable. After several months spent in trying to clean seminiferous tubules without harming them, it appeared that anti-Müllerian activity was carried by seminiferous tubules (Josso 1973). The question was not completely solved, however. Fetal seminiferous tubules contain a mixture of gonocytes and Sertoli cells. Mechanical separation was impossible without micro-manipulation instruments. But there was another way out. Germ cells are very sensitive to ionizing radiation. After exposure of fetal testicular tissue to X-rays, germ cells had disappeared but anti-Müllerian activity was not affected (Blanchard and Josso 1974). The Sertoli cell origin of AMH was confirmed later by immunocytochemistry applied to the calf (Hayashi, et al. 1984) and human (Tran, et al. 1987) fetal testis.

AMH is a glycoprotein

Establishing that AMH is a macromolecule secreted by fetal Sertoli cells was just the beginning. The next challenge was purification, using the only available tool, the AMH bioassay. Would the fetal rat Müllerian duct respond to AMH produced by other species ? If not, the perspective of purifying AMH from the testes of fetal rats was not appealing. Fortunately, AMH activity is interspecific, at least between mammals (Josso 1971) and a larger species, the bovine, was chosen for use in the bioassay.

But how is one expected to purify a testicular protein using a bioassay? Well, as we quickly found out, not by adding a testicular homogenate to the culture medium. After three days in culture, the target organ was dead as a doornail. Better results were obtained by incubating the bovine testicular tissue and then using the incubation medium to culture the fetal reproductive tract (Josso, et al. 1975). The incubation medium exhibited clear signs of anti-Müllerian activity and became the starting material for further purification. Most methods however require minimal information on the characteristics of the substance of interest,at least an approximate molecular weight or isoelectric point. We –myself and my scientific partner Jean-Yves Picard- were forced to proceed by trial and error. We first determined the molecular weight of AMH using gel filtration and reported a mass of approximately 215,000 daltons (Picard and Josso 1976). The medium with anti-Müllerian activity was then submitted to density gradient sedimentation with surprising results : the AMH molecule had shrunk to 124,000 daltons! The discrepancy suggested that AMH might be a glycoprotein. Evidence for this hypothesis was obtained by incubating fetal bovine testicular tissue in the presence of tritiated fucose: anti-Müllerian activity always co-purified with radioactivity (Picard, et al. 1978). Two years later, a Boston team also led by a woman, Patricia Donahoe, confirmed the glycoprotein nature of AMH using lectin-affinity chromatography (Budzik, et al. 1980). In our hands, analysis of the incubation medium by polyacrylamide electrophoresis showed a single major radioactive peak of 140,000 Da, 70,000 if the elecrophoresis was carried out in reducing conditions, suggesting that AMH is a homodimer linked by disulfide bonds (Picard, et al. 1978).

Monoclonal antibodies to the rescue

It then became obvious that AMH purification would never be achieved by standard biochemical methods alone, even in most purified fractions the AMH concentration was minimal compared to the contaminants. Staining of polyacrylamide gels failed to show a protein band at the site of the radioactive peak. Immunochromatography was a possibility but how to raise a specific antibody against an impure antigen ? Perhaps monoclonal antibody technology could help, provided we could come up with a suitable screening method. Bernard Vigier, a former student of Jost who had joined us, prepared monoclonal antibodies from mice immunized with partially purified AMH and added fucose-labelled semi-purified incubation medium to cultured hybridomas. Three out of a hundred secreted antibodies which precipitated the radioactivity. One hybridoma was cloned and grown in mice : the monoclonal antibody abolished anti-Müllerian activity of partially purified AMH (Vigier, et al. 1982b) and was successfully used to purify bovine AMH to homogeneity (Picard and Josso 1984). Vigier et al. (1982a) went on to devise a radioimmunoassay which replaced the tedious qualitative bioassay. Other monoclonal antibodies against bovine AMH were used in Boston for possible AMH purification (Budzik, et al. 1985). Bernard Vigier's initial antibodies have recently been used to set up an immunoassay for bovine AMH (Arouche, et al. 2015). The screening method was so crude that only antibodies with very high affinity were picked up!

AMH belongs to the transforming growth factor β family

Monoclonal antibodies raised against bovine AMH do not recognize human AMH and cannot be used to purify it. Two groups undertook to clone the AMH gene. Jean-Yves Picard, in Paris, cloned the cDNA for bovine AMH (Picard, et al. 1986) but before he could finish the job, Richard Cate, an investigator in a biotechnology company, in collaboration with Patricia Donahoe, cloned the human gene and produced recombinant human AMH (Cate, et al. 1986). The human gene measures only 2.8 kpb and contains 5 exons. Richard Cate noticed that the 3' end of the 5th exon is extremely guanine/cytosine rich, a characteristic of the transforming growth factor β (TGF-β) family. Indeed, as shown in Cate's seminal paper, AMH is a distant member of this family with a 28% homology to TGF-β itself for the C-terminal domain of the protein. The AMH gene has been mapped to chromosome 19 p13.3 (Cohen-Haguenauer, et al. 1987)

The AMH gene codes for a 70 kDa monomer of 560 amino acids. After elimination of the signal peptide, it dimerizes through disulfide bonds giving rise to a 140 kDa AMH full length proprotein. Like the other members of the TGF-β superfamily, the proprotein undergoes proteolytic cleavage at a dibasic site to yield a short 109 amino acid C-terminal domain and a 426 N-terminal one (Pepinsky, et al. 1988). The C-terminus , the only one with homology to the TGF-β family, carries the bioactivity, the N-terminus playing a stabilizing role (Wilson, et al. 1993). Cleavage is required for AMH bioactivity, the N and C fragments remain associated in a non-covalent complex (Pepinsky, et al. 1988). The identity of the proteases responsible for AMH cleavage *in vivo* has not yet been determined. Possible candidates include plasmin, a serine protease (Pepinsky, et al. 1988) or proprotein convertases such as PCKS3 and PCSK5 (Nachtigal and Ingraham 1996)

The AMH gene is tightly regulated

Sertoli cells produce AMH as early as the 7th post-natal week, production continues long after Müllerian ducts have disappeared. After birth, AMH levels remain high up to puberty and then decline rapidly falling to minimal levels in the adult. Comparably low amounts are produced by ovarian granulosa cells (Vigier, et al. 1984) from the perinatal period up to menopause (reviewed in Dewailly, et al. 2014, Visser, et al. 2006). The first immunoassays for AMH in human serum were adapted to the high concentrations seen in prepubertal boys (Baker, et al. 1990, Hudson, et al. 1990, Josso, et al. 1990), and were relatively insensitive. The realization that AMH levels in women are correlated with ovarian reserve and may be clinically useful led to a flurry of new assays with high sensitivity but not necessarily in agreement with one another (Nelson, et al. 2015, Pigny, et al. 2016). An international AMH standard has not yet been agreed upon.

The expression of AMH is regulated differently in males and females. In males, intratesticular testosterone concentration curtails AMH secretion provided the androgen receptor is present on the Sertoli cells membrane (Rey, et al. 1993). Androgen acts through the binding sites for steroidogenic factor 1 (SF-1) on the proximal promoter(Edelsztein, et al. 2018). FSH has the opposite effect, it stimulates AMH production but to a lesser degree (Al Attar, et al. 1997). Cyclic AMP mediated stimulation by FSH uses response elements located on both the proximal and distal promoter(Lasala, et al. 2011). Transcription factors SOX9, SF-1, GATA4, WT-1 or DAX-1 regulate AMH gene transcription either by direct binding to specific response elements in the AMH proximal promoter or by protein-protein interaction (reviewed in Lasala, et al. 2011).

In females, AMH production is maximal in small growing follicles becoming undetectable in large ones (reviewed in Taieb, et al. 2011) Estrogen inhibits (Grynberg, et al. 2012)bk ;:kl and

gonadotropins stimulate (Pierre, et al. 2013, Taieb, et al. 2011) AMH transcription in luteinized granulosa cells from women undergoing *in vitro* fertilization. ~~In contrast, FSH down-regulates AMH expression in the immature follicles of infantile mice~~. AMH transcription in granulosa cells is up-regulated by bone morphogenetic proteins (Shi, et al. 2009) and by co-culture with oocytes of growing follicles (Convissar, et al. 2017, Salmon, et al. 2004). Hormonal regulatory mechanisms are disrupted in women with polycystic ovaries (Pierre, et al. 2013, Pierre, et al. 2017)

AMH signaling

Like all members of the TGF-β superfamily, AMH uses two serine-protein kinases for signaling. The primary receptor, ~~inappropriately named~~ AMHR2, is AMH-specific, it was cloned in 1994 independently by a Dutch and by a French team. Neither used the classical AMH target organ to construct a cDNA library. In Rotterdam, Willy Baarends and Axel Themmen were interested in androgen-responsive Sertoli cell genes and stumbled upon a cDNA appearing to encode a novel receptor of the TGF-β superfamily (Baarends, et al. 1994). Based upon its expression in the mesenchymal cells surrounding the fetal Müllerian duct, they rightly suggested that the cDNA clone encoded an AMH receptor. Finally, it turned out that the receptor was not androgen-responsive after all ! In Paris, at the same time, Nathalie di Clemente, Richard Cate and their co-workers set out to clone the AMH receptor gene by more conservative methods. To construct a cDNA library, instead of the fetal Müllerian duct, they chose a lesser-known AMH target organ, the fetal ovary which is larger and easier to dissect. They screened the library with a consensus sequence of the TGF-β receptor superfamily and detected a clone which differed from the consensus sequence by a single amino acid but had the pattern of expression expected for an AMH receptor. By transiently expressing the transcript in COS cells, they were able to prove binding to AMH (di Clemente, et al. 1994b).

In the TGF-β superfamily, the primary receptor binds the ligand but another receptor, called type 1, is required to initiate signaling. Unlike the type 2 receptors, which are reasonably specific, type 1 receptors are common to subsets of TGF-β family members and it made sense to look for the AMH type 1 receptor among those already cloned. This approach was successful. The AMH signaling cascade borrows type 1 receptors, ACVR1 (ALK2) and BMPR1A (ALK3), and rSmads 1, 5 and 8, from the BMP ~~and activin~~ family (Clarke, et al. 2001, Jamin, et al. 2002, Visser, et al. 2001). BMPR1B (ALK6) acts as a negative regulator of intracellular signaling (Belville, et al. 2005, Gouédard, et al. 2000). In summary (Orvis, et al. 2008) expression of Wnt7a by the mesothelium together with SF-1 and WT-1 expression in the coelomic epithelium activates the expression of AMHR2 by coelomic epithelial cells which then migrate to the peri-Müllerian mesenchyme.

After binding to AMH, AMHR2 recruits ACVR1 and BMPR1A to phosphorylate Smads 1, 5 or 8. This initiates transcription of target genes mediating the regression of Müllerian duct epithelium. The identity of the target genes is not clear at the present time, a member of the matrix metalloproteinase gene family (Roberts, et al. 2002) or a member of the Wnt family (Hossain and Saunders 2003, Kobayashi, et al. 2011) are possible candidates.

To bind AMHR2, the full length AMH proprotein must be cleaved, dissociation of the non-covalently bound fragments is not required. After binding of cleaved AMH to AMHR2, dissociation occurs, the N-terminal fragment is lost, the type 1 receptor is recruited and signaling begins (di Clemente, et al. 2010) **(Fig 1)** . Because full-length AMH is biologically inactive, it has been suggested that measuring it separately might provide an indication of AMH bioactivity in body fluids (Pankhurst and McLennan

2016, Pierre, et al. 2016). There are marked differences in the proportion of cleaved versus full-length AMH according to sex, age and clinical status (Mamsen, et al. 2015) but interpretation is difficult.

Extra-Müllerian roles of AMH

In males, Sertoli cells continue to produce AMH long after the Müllerian ducts have completely disappeared, suggesting that AMH may play other roles. Some have been confirmed experimentally while others are hypotheses inferred from correlations with AMH ontogeny (Morgan, et al. 2017) or with the location of the AMH receptor. AMHR2 is present on the membrane of Leydig cells, where it mediates the repression of steroidogenic enzymes and downregulates testosterone secretion (Racine, et al. 1998). Since then, AMHR2 has been identified in the nervous system : motoneurons (Wang, et al. 2005), GnRH neurons (Cimino, et al. 2016) pituitary gonadotropes (Garrel, et al. 2016), the developing and adult brain (Lebeurrier, et al. 2008) and the cerebellum (Wittmann and McLennan 2011). In the ovaries, AMH inhibits estrogen production by granulosa cells (di Clemente, et al. 1994) and inhibits primordial follicle recruitment as well as the responsiveness of growing follicles to follicle-stimulating hormone (reviewed in Visser and Themmen 2014).

Teleost fishes have no Müllerian ducts but they do have *amh* orthologs, some species even have two!. The second copy is located on the Y chromosome and acts as a male determining factor (Hattori, et al. 2012, Yamamoto, et al. 2014). Teleost fishes display a bewildering diversity of all biological aspects including sex determination and differentiation. Not surprisingly, the variability extends to the structure, expression and function of *amh* (Pfennig, et al. 2015), nevertheless regulation of germ cell proliferation and follicular development appears to be a conserved function that preceded Müllerian duct evolution during phylogeny (Morinaga, et al. 2007).

Clinical relevance

Measurement of circulating AMH by immunoassay is increasingly used for diagnostic purposes. In prepubertal children, it detects the presence of testicular tissue and explores its functional activity (reviewed in Freire, et al. 2018). In the persistent Müllerian duct syndrome, AMH level distinguishes between mutations of the AMH or AMHR2 genes (Picard, et al. 2017). In women, AMH level provides indirect, non invasive information on the size of the follicle pool hence its use in assisted reproduction, however it does not predict the probability of implantation or live birth (Pilsgaard, et al. 2018). AMH blood concentration is extremely high in active granulosa cell tumors (Gustafson, et al. 1992, Rey, et al. 1996) and usually more than twice the normal level in the polycystic ovaries syndrome (Pellatt, et al. 2007). The role and regulation of ovarian AMH are targets of active ongoing investigation,deserving of a review in their own right.

Patricia Donahoe has promoted AMH as a biotherapeutic agent for gynecological cancer (see Park, et al. 2017). In mice co-administration of AMH protects the germline during chemotherapy (Kano, et al. 2017, Sonigo, et al. 2019) Clinical trials should be carried out to bear out these claims in women but unfortunately AMH is not available for clinical use at the present time.

Conclusion

At a time when medical research is funded only if it holds promise for medical applications, the AMH story among many others shows that this approach can be terribly wrong. AMH started out as a

mysterious and perhaps imaginary fetal testicular hormone. As such, it could not be of the slightest practical use and if the present funding rules had applied seventy years ago, AMH would never have been discovered, let alone purified. Figure 2 shows the number of papers referenced over time by the National Library of Medicine, USA, with the keyword “AMH” or its synonyms. Initially, there were only a handful each year but in 2002, the year the Rotterdam group reported correlation with ovarian reserve, the numbers exploded and now surpass 400 per year and counting !(Fig 2). AMH has even been detected in the central nervous system, an unexpected promotion for a sex hormone ! So please judge scientific projects according to their scientific merits and they may eventually lead to medical progress.

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Legend of Figures

Fig 1

A suggested model for the assembly of the AMH signaling complex. Cleavage of full-length AMH results in a conformational change in the C-terminus dimer (indicated by the color change from green to red), allowing binding of 2 molecules of AMHR2. Binding induces dissociation of the N-terminal dimer via an negative interaction between the receptor and the N-terminal binding sites on the C-terminal dimer (indicated by the shape change).Finally, the type 1 receptor is recruited, receptor Smads 1, 5 or 8 are phosphorylated, bind to Smad 4 and enter the nucleus to turn on AMH-responsive genes. The type 1 and 2 receptor-binding sites on the C-terminal dimer are indicated by either a 1 or a 2.

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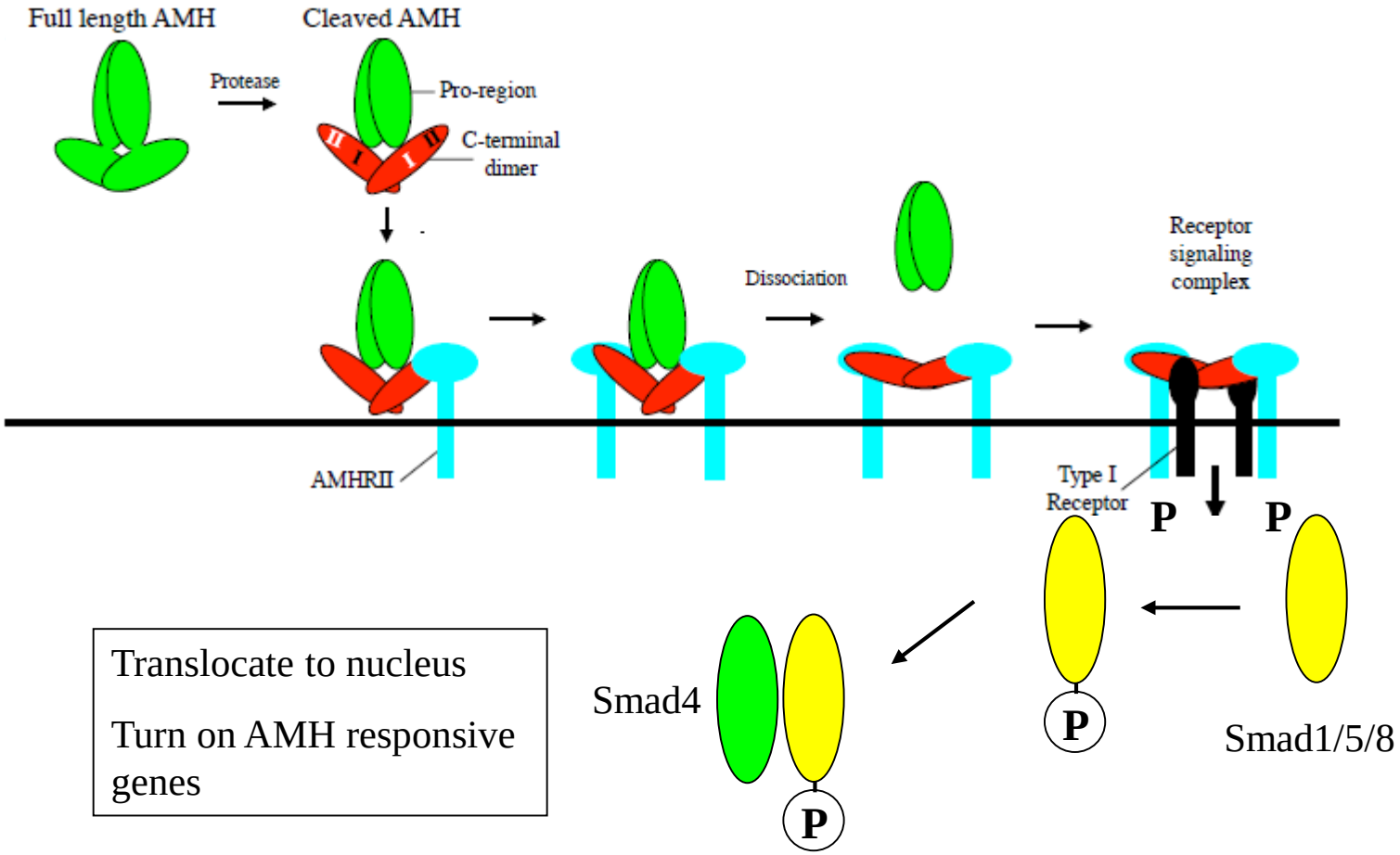
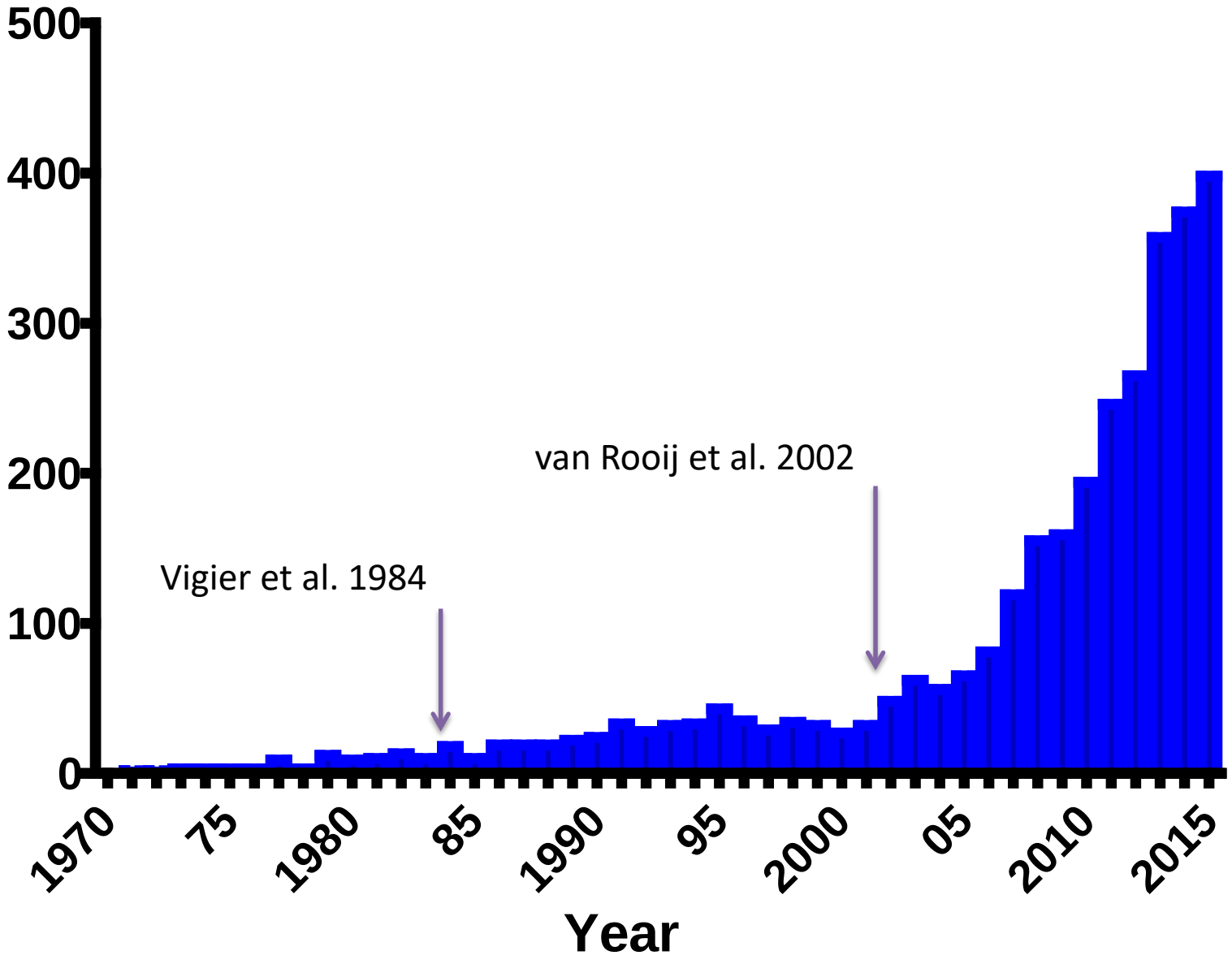
Fig 2

Number of yearly publications retrieved from Pubmed (NLM) using the keyword AMH and its synonyms. Vigier et al (1984) showed that AMH is produced by the adult ovary and van Rooij et al (2002) showed the correlation between the level of circulating AMH and the state of ovarian reserve. Only then did the number of AMH publications grow dramatically.



Translocate to nucleus

Turn on AMH responsive genes


500
400
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Vigier et al. 1984
van Rooij et al. 2002
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