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Anti-Müllerian hormone : a look back and ahead

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22 **Abstract**

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

31 **Introduction**

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

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

47 **Alfred Jost and the AMH concept**

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

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

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

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

81 **The basics**

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

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

100 **AMH is a glycoprotein**

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

106 But how is one expected to purify a testicular protein using a bioassay? Well, as we quickly found
107 out, not by adding a testicular homogenate to the culture medium. After three days in culture, the
108 target organ was dead as a doornail. Better results were obtained by incubating the bovine testicular
109 tissue and then using the incubation medium to culture the fetal reproductive tract (Josso, et al.
110 1975). The incubation medium exhibited clear signs of anti-Müllerian activity and became the
111 starting material for further purification. Most methods however require minimal information on the
112 characteristics of the substance of interest, at least an approximate molecular weight or isoelectric
113 point. We –myself and my scientific partner Jean-Yves Picard- were forced to proceed by trial and
114 error. We first determined the molecular weight of AMH using gel filtration and reported a mass of
115 approximately 215,000 daltons (Picard and Josso 1976). The medium with anti-Müllerian activity was
116 then submitted to density gradient sedimentation with surprising results: the AMH molecule had
117 shrunk to 124,000 daltons! The discrepancy suggested that AMH might be a glycoprotein. Evidence
118 for this hypothesis was obtained by incubating fetal bovine testicular tissue in the presence of
119 tritiated fucose: anti-Müllerian activity always co-purified with radioactivity (Picard, et al. 1978). Two
120 years later, a Boston team also led by a woman, Patricia Donahoe, confirmed the glycoprotein
121 nature of AMH using lectin-affinity chromatography (Budzik, et al. 1980). In our hands, analysis of
122 the incubation medium by polyacrylamide electrophoresis showed a single major radioactive peak of
123 140,000 Da, 70,000 if the electrophoresis was carried out in reducing conditions, suggesting that AMH
124 is a homodimer linked by disulfide bonds (Picard, et al. 1978).

125 **Monoclonal antibodies to the rescue**

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

142 **AMH belongs to the transforming growth factor β family**

143 Monoclonal antibodies raised against bovine AMH do not recognize human AMH and cannot be used
 144 to purify it. Two groups undertook to clone the AMH gene. Jean-Yves Picard, in Paris, cloned the
 145 cDNA for bovine AMH (Picard, et al. 1986) but before he could finish the job, Richard Cate, an
 146 investigator in a biotechnology company, in collaboration with Patricia Donahoe, cloned the human
 147 gene and produced recombinant human AMH (Cate, et al. 1986). The human gene measures only 2.8
 148 kpb and contains 5 exons. Richard Cate noticed that the 3' end of the 5th exon is extremely
 149 guanine/cytosine rich, a characteristic of the transforming growth factor β (TGF- β) family. Indeed, as
 150 shown in Cate's seminal paper, AMH is a distant member of this family with a 28% homology to TGF-
 151 β itself for the C-terminal domain of the protein. The AMH gene has been mapped to chromosome
 152 19 p13.3 (Cohen-Haguenaer, et al. 1987)

153 The AMH gene codes for a 70 kDa monomer of 560 amino acids. After elimination of the signal
 154 peptide, it dimerizes through disulfide bonds giving rise to a 140 kDa AMH full length proprotein. Like
 155 the other members of the TGF- β superfamily, the proprotein undergoes proteolytic cleavage at a
 156 dibasic site to yield a short 109 amino acid C-terminal domain and a 426 N-terminal one (Pepinsky, et
 157 al. 1988). The C-terminus, the only one with homology to the TGF- β family, carries the bioactivity,
 158 the N-terminus playing a stabilizing role (Wilson, et al. 1993). Cleavage is required for AMH
 159 bioactivity, the N and C fragments remain associated in a non-covalent complex (Pepinsky, et al.
 160 1988). The identity of the proteases responsible for AMH cleavage *in vivo* has not yet been
 161 determined. Possible candidates include plasmin, a serine protease (Pepinsky, et al. 1988) or
 162 proprotein convertases such as PCKS3 and PCSK5 (Nachtigal and Ingraham 1996)

163 **The AMH gene is tightly regulated**

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

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

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

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

191 **AMH signaling**

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

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

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

222 To bind AMHR2, the full length AMH proprotein must be cleaved, dissociation of the non-covalently
 223 bound fragments is not required. After binding of cleaved AMH to AMHR2, dissociation occurs, the
 224 N-terminal fragment is lost, the type 1 receptor is recruited and signaling begins (di Clemente, et al.
 225 2010) (**Fig 1**). Because full-length AMH is biologically inactive, it has been suggested that measuring
 226 it separately might provide an indication of AMH bioactivity in body fluids (Pankhurst and McLennan

227 2016, Pierre, et al. 2016). There are marked differences in the proportion of cleaved versus full-
228 length AMH according to sex, age and clinical status (Mamsen, et al. 2015) but interpretation is
229 difficult.

230 **Extra-Müllerian roles of AMH**

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

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

249 **Clinical relevance**

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

260 Patricia Donahoe has promoted AMH as a biotherapeutic agent for gynecological cancer (see Park, et
261 al. 2017). In mice co-administration of AMH protects the germline during chemotherapy (Kano, et al.
262 2017, Sonigo, et al. 2019) Clinical trials should be carried out to bear out these claims in women but
263 unfortunately AMH is not available for clinical use at the present time.

264 **Conclusion**

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

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

276

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278

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607 Legend of Figures

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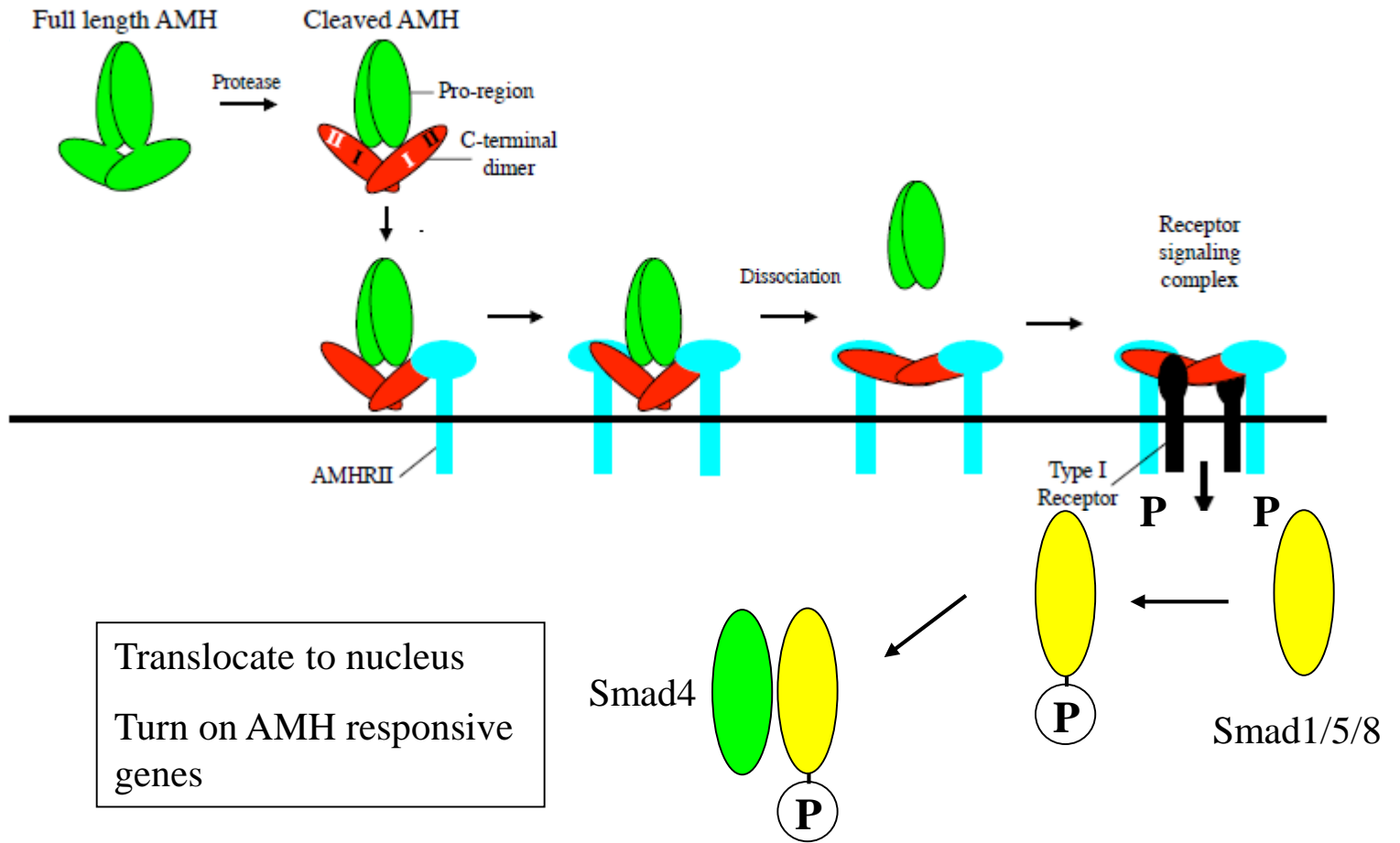
609 Fig 1

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

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619 Fig 2

620 Number of yearly publications retrieved from Pubmed (NLM) using the keyword AMH and its
621 synonyms. Vigier et al (1984) showed that AMH is produced by the adult ovary and van Rooij et al
622 (2002) showed the correlation between the level of circulating AMH and the state of ovarian reserve.
623 Only then did the number of AMH publications grow dramatically.



Translocate to nucleus
 Turn on AMH responsive genes

