

# Alpha-Fetoprotein: From a Diagnostic Biomarker to a Key Role in Female Fertility

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**Abstract:** Alpha-fetoprotein (AFP) is a well-known diagnostic biomarker used in medicine to detect fetal developmental anomalies such as neural tube defects or Down's syndrome, or to follow up the development of tumors such as hepatocellular carcinomas. However, and despite the fact that the protein was discovered almost half a century ago, little was known about its physiological function. The study of *Afp* knock-out mice uncovered a surprising function of AFP: it is essential for female fertility and for expression of normal female behaviors, and this action is mediated through its estrogen binding capacity. AFP sequesters estrogens and by so doing protects the female developing brain from deleterious (defeminizing/masculinizing) effects of these hormones.

**Keywords:** Alpha-fetoprotein, Estrogens, Anovulation, Female brain differentiation.

Alpha-fetoprotein (AFP), discovered about half a century ago (Bergstrand and Czar, 1956; Abelev et al. 1963), is the major serum fetal protein in mammals. AFP is actively produced and secreted during the fetal life by the liver hepatocytes, the visceral endoderm of the yolk sac and, to a lesser extent, by the intestine and the kidneys (Sell and Becker, 1978; Andrews et al. 1982; Belayew and Tilghman, 1982). The concentration of this protein in the fetal serum reaches the order of several mg/ml, and its synthesis decreases dramatically in the first weeks after birth to reach only trace amounts in adulthood (Sell and Becker, 1978; Belayew and Tilghman, 1982). It is then essentially produced by the liver.

AFP produced by the embryo is secreted in the amniotic fluid and is also able to cross the placental barrier to reach the maternal blood circulation, where its titer is used as a diagnostic marker to reveal developmental anomalies of the fetus (Haddow et al. 1979; Brownbill et al. 1995; Newby et al. 2005). Abnormally high levels of AFP in the maternal serum indicates elevated risk for neural tube defects of the fetus such as spina bifida or anencephaly (Leighton et al. 1975), whereas abnormally low levels indicates elevated risk for a Down's syndrome (Cuckle et al. 1984). Measurements of the AFP levels in the maternal serum are undertaken at 14–22 weeks of each pregnancy and are part, along with unconjugated estradiol, human chorionic gonadotropin and inhibin A, of the quadruple test for antenatal Down's syndrome screening (Wald et al. 2003). Abnormal AFP levels can also be indicative of other fetal pathologies (for review see Mizejewski, 2004).

Synthesis of AFP is dramatically reduced in adulthood but can resume in case of liver pathologies (cirrhosis, hepatitis...) or of tumors such as hepatocellular carcinoma, germ cell tumors (embryonic carcinoma and teratocarcinoma) and some pancreatic and renal tumors (Masopust et al. 1968; Chiu et al. 1983; Abelev and Eraiser, 1999; Labdenne and Heikinheimo, 2002; Yuen and Lai, 2005; Ishigami et al. 2006). Little is known about the exact mechanisms that control the AFP gene expression or silencing (for reviews see Lazarevich, 2000; Spear, 1999). The regulatory region of the AFP gene contains distal enhancers, a promoter element, and silencer elements (Godbout et al. 1986; Watanabe et al. 1988; Poliard et al. 1990; Vacher and Tilghman, 1990; Henriette et al. 1997). The promoter element is regulated by numerous transcriptional factors such as HNF1 and HNF3 (Feuerman et al. 1989; Zhang et al. 1990; Crowe et al. 1999), Nkx2.8 (Apergis et al. 1998), FTF (Galerneau et al. 1996), NF1 (Bernier et al. 1993), Zfx2 (Perincheri et al. 2005), FOXA (Huang et al. 2002), the thyroid hormone receptor (Van Reeth

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et al. 2002) and Ku (Lienard et al. 2006). The thyroid hormone T3 probably contributes to the post-natal shut off of the gene (Van Reeth et al. 2002).

Until recently, the precise function of AFP was unknown. In order to identify this function, *Afp* knock-out mice were generated (Gabant et al. 2002) by replacing an *Afp* genomic fragment extending from exon 1, to intron 3 (*Afp*<sup>tm11bmm</sup> allele), or extending from exon 2 to intron 3 (*Afp*<sup>tm21bmm</sup> allele), by a IRES-*LacZ-neo* selection cassette. Both invalidations gave rise to viable homozygous animals. These AFP KO mice are apparently normal, but females are sterile, while males are fertile.

AFP KO female mice suffer from anovulation. Reciprocal ovary transplantation experiments demonstrated that AFP KO ovaries are functional: AFP KO ovaries transplanted in normal mice were able to ovulate and the transplanted females generated pups from the mutated parental oocytes. AFP KO ovaries contain follicles at different stages of maturation, including the last Graafian follicle stage. However no corpora lutea, indicative of ovulation, could be detected, which is in accordance with the smooth exterior aspect of the ovaries and the abnormally low levels of progesterone in the serum. Ovulation in AFP KO mice can be induced by injection of gonadotropins. In that case, ova are released, fertilized, but the blastocysts are unable to implant in the uterine horns which are non-receptive because of overstimulation by estrogens, as a result of the absence of corpora lutea.

Since the AFP KO female mice defect does not lie within the ovaries, it must implicate the HPG axis (hypothalamic-pituitary-gonadal axis), which provides the adequate hormonal environment necessary for ovulation. In the proestrus phase of the sexual cycle, the HPG axis responds to a stimulatory signal of the estrogens by the secretion of the GnRH decapeptide which binds to its receptor in the pituitary and triggers the release of the LH and FSH hormones responsible, in fine, for ovulation. In order to define the defect of the HPG axis in the AFP KO females, we used the microarray technique to compare the gene expression profile of these mice with that of their wild type littermates. We found that in the pituitary, several genes previously implicated in female fertility are down-regulated in the AFP KO female mice (De Mees et al. 2006). These genes are *Egr1*, *Cish2*, *Ptprf*, *Psa*, and *Tkt*. Furthermore, we also found that genes participating in the GnRH pathway are downregulated in the AFP KO female

mice. These genes are the GnRH receptor gene, and several genes activated by the GnRH receptor (*cFos*, *Egr2*, *Tgfbli4*, *Ptp4a1*). In the hypothalamus, the gene encoding the hypothalamic GnRH decapeptide is itself down-regulated. In the context of an anovulation phenotype, dysfunction of the GnRH pathway seems extremely relevant.

In addition to being sterile, female AFP KO mice are defeminized (they show a diminution of female behavior) and masculinized (they exhibit some male characteristics): in the presence of a sexually active male, they do not exhibit the female typical behavior of lordosis (posture with raised head and rump, and deflected tail, to facilitate copulation) and they show a male pattern of distribution of tyrosine hydroxylase expressing neurons in sexually dimorphic areas of the hypothalamus (Bakker et al. 2006).

## What Can be the Link Between AFP and Female Fertility?

The answer lies in the capacity of AFP to inhibit estrogen responsiveness. AFP is able, at least in rodents, to bind estrogens, but not androgens, at its C-terminal extremity, with a  $K_d$  of  $10^{-9}$  M<sup>-1</sup>, indicating that it can act as an estrogen carrier in the blood (Uriel et al. 1976; Savu et al. 1981; Nishi et al. 1991). Estrogens are known to exert deleterious, masculinizing effects on the female developing brain in the perinatal period. Perinatal exposure to estrogens in rodent females results in anovulatory sterility in adulthood, associated with altered gonadotropin production and absence of female typical behaviors (Gorski, 1963; Whaler and Nadler, 1963). Based on the observation, it is classically assumed that the function of AFP is to shield the female brain from estrogens, by sequestering them in the fetal serum (Mc Ewen et al. 1975). As AFP is unable to bind androgens, testosterone produced by the male embryo's testes is free to reach the developing brain where it can locally be converted to estrogens by an enzyme called aromatase, and according to this classic hypothesis, estrogens could thus exert their masculinizing effects. An alternative hypothesis, based on the fact that AFP is found inside neurons without being produced locally, is that AFP has more than a neuroprotective role and specifically delivers estrogens to targeted brain cells in order to ensure correct female differentiation (Dohler et al. 1984; Toran-Allerand, 1984). The AFP KO mice allowed us to test these hypotheses.

We reasoned that if AFP was essentially a passive estrogen carrier, the fertility of the AFP KO female mice should be restored provided their embryonic development took place in an environment strongly reduced in oestrogens; alternatively, if AFP was an active estrogen carrier, then the lack of both estrogens and AFP should be as deleterious as the lack of AFP alone. Gestation is compatible with very low level of estrogens, which can be reached by treating gestating females with an aromatase inhibitor. We found that the fertility of the so-treated AFP KO female mice was restored. No treatment was needed in adulthood to sustain this fertility, thereby proving that the cause of anovulation in AFP KO mice is indeed estrogen overexposure. Furthermore, normal gene expression in the HPG, normal lordosis behavior, and normal distribution of the tyrosine hydroxylase expressing neurons were also regained in the treated females (Bakker et al. 2006; De Mees et al. 2006). These results demonstrate that AFP has merely a passive, neuroprotective role, and protects the female developing brain from estrogen deleterious effects.

However, these findings do not explain why AFP is found inside neurons without being locally produced. AFP could have other neurological functions, independent from fertility control. Our results do not exclude the possibility of an active post natal role of estrogen in female differentiation. Indeed, females mice ovariectomized at birth display less feminine behaviors than intact females in adulthood (Gerall et al. 1973). This hypothesis is supported by the finding that aromatase knock-out females are sterile and remain so even after adult estradiol treatment (Toda et al. 2001). Lastly, very low quantities of estrogens could indeed be needed for correct female brain sexual differentiation, but in that case, they would be transported by another carrier than AFP.

Finally, it should be pointed out that the anti-estrogenic effects of AFP could go beyond its estrogen binding and sequestering properties, as deduced from experiments which have shown an AFP induced hypo responsiveness to estrogens in the presence of molar excess of estrogens over AFP (Mizejewski et al. 1983).

Translation of the observed results to human still needs to be tested. There are diverging views in the literature as to whether human AFP can or cannot bind estrogens. In either case, it appears that human AFP-derived peptides are able to

display some anti-estrogenic activity (Vakharia and Mizejewski, 2000; Bennett et al. 2002; Mizejewski et al. 2004). AFP-derived peptides are under investigation as chemopreventive agents for estrogen-dependent breast cancers and other tumors (Bennett et al. 2006, Mizejewski et al. 2006). Androgens could also, in the human, play a more important role than estrogens in sexual brain differentiation. In that case the sex hormone binding globulin, able to bind both estrogens and androgens, could then play a significant role.

In conclusion, our work demonstrates that the function of AFP extends well beyond its traditional marker role for developmental anomalies of the fetus or liver tumors. AFP plays a crucial role (at least in rodents) in the control of female fertility through its anti-estrogenic action.

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