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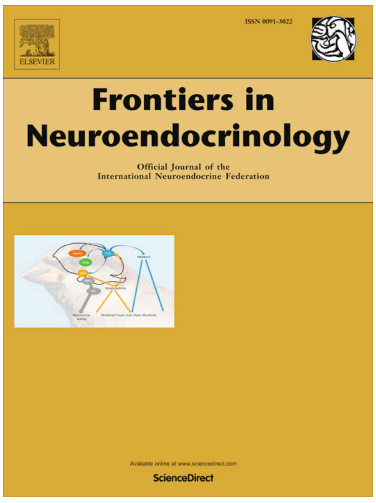

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Sex differences in the pituitary TGFβ1 system; the role of TGFβ1 in prolactinoma development.

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List of abbreviations

TGFβ1: Transforming growth factor beta 1

Drd2: Dopamine type 2 receptors

DAs: Dopamine agonists

DARPs: Dopamine agonist resistant prolactinomas

LAP: Latency-associated peptide

LTBP: Latent TGFβ binding protein

TSP-1: Thrombospondin-1

hCGβ: human chorionic gonadotrophin β subunit

PRL: Prolactin

Drd2-/-: Drd2 knockout mouse

FGFR4: Fibroblast growth factor 4

PRLR-/-: Prolactin receptor deficient mouse

TβRII: TGFβ receptor type II

ECM: Extracellular matrix

TβRII: TGFβ receptor type II

TβRII+/-: TβRII heterozygous knockout mice

ABSTRACT

Prolactinomas are the most frequent functioning pituitary adenomas, and sex differences in tumor size, behavior and incidence have been described. These differences have been associated with earlier diagnosis in woman, as well as with serum estradiol levels. Experimental models of prolactinomas in rodents also show a higher incidence in females, and recent findings suggest that gender differences in the transforming growth factor beta 1 (TGFβ1) system might be involved in the sex-specific development of prolactinomas in these models.

The aim of this review is to summarize the literature supporting the important role of TGFβ1 as a local modulator of pituitary lactotroph function and to provide recent evidence for TGFβ1 involvement in the sex differences found in prolactinoma development in animal models.

Key words: Prolactinoma; sex differences; TGFβ1; estradiol; dopamine

1. Pituitary tumors are slow growing adenomas accounting for 15% of all intracranial neoplasms (Farrell 2006; Melmed 2015), with relatively high prevalence (77 cases per 100.000) (Daly *et al.* 2009; Fernandez *et al.* 2010), which increases up to 20% when considering tumors found unexpectedly in autopsies (Ezzat *et al.* 2015). Despite usually presenting as benign tumors, pituitary adenomas can cause several clinical disorders due to both hypersecretion of pituitary trophic hormones and excessive tumor growth with a compressive mass effect in surrounding tissues, inducing visual impairment, headaches, neurological disorders and also hypopituitarism caused by compression and disruption of the hypothalamic-pituitary axis (Arafah & Nasrallah 2001; Melmed 2011).

Among hormone-secreting pituitary tumors, prolactinomas (prolactin-secreting tumors originated from lactotroph cells) are the most frequent (40%) (Ciccarelli *et al.* 2005).The clinical disorders observed in patients are related to hyperprolactinemia, which mainly affects gonadal/reproductive function, inducing amenorrhea (cause of early medical consultation in women), hypogonadism, galactorrhea, decreased libido and infertility. Large tumors can also cause symptoms associated to the mass effect.

1.1 Gender differences in human pituitary adenomas

Sexual differences in prolactinoma size, incidence and behavior have been described. The prevalence is higher in women during the fertile period (between 20-50 years, when the tumor ratio between sexes is estimated to be 10:1), but the frequency balances after the fifth decade of life (Colao *et al.* 2003; Gillam *et al.* 2006). The hormonal alterations caused by hyperprolactinemia in women (menstrual cycle disorders) lead to earlier clinical

evaluation and therefore, women usually present microprolactinomas at the time of diagnosis. On the contrary, the clinical manifestation of increased serum prolactin levels in men is more polymorphic, the length of the asymptomatic phase is higher than in women, and symptoms such as sexual dysfunction or decreased libido are usually underestimated which causes a delay in diagnosis (Colao *et al.* 2003; Pinzone *et al.* 2000). Thus, men generally present symptoms of mass effects due to the presence of macroprolactinomas at the time of diagnosis (Delgrange *et al.* 1997; Khare *et al.* 2016; Nishioka *et al.* 2003). Nevertheless, delayed diagnosis in men may not be the only explanation for the differences in tumor size, since prolactinomas in men tend to be more aggressive, with lower rates of surgical cure and higher proliferative indexes, suggesting a sex-specific behavior for these tumors (Delgrange *et al.* 1997; Gillam MP & Molitch ME 2015).

Gender differences in the prevalence, severity, age of onset, and clinical presentation have been also described in other types of pituitary adenomas. For instance, Cushing's disease presents a remarkable preponderance in females, with a female-to-male ratio of 3-8:1. However, more severe clinical presentation, with larger adenomas, higher invasion rate, and higher recurrence rate have been found in men (Liu *et al.* 2015; Zilio *et al.* 2014). On the other hand, no gender differences were described in human GH-pituitary adenomas, neither in the prevalence nor in the maximal tumor size (Colao *et al.* 2002). Regarding the clinically non-functioning pituitary adenomas (NFs), which are mostly of gonadotroph cell origin, even though the prevalence is similar in both sexes, macroadenomas are more commonly diagnosed in men and at an older age, compared to women. Gender differences in nature and biological behavior in NFs have been suggested

because the higher proliferative activity and tumor aggressiveness found in men (Iglesias *et al.* 2017).

Sex differences have also been found in animal models of pituitary adenomas, particularly prolactinomas, which represent a great tool to study the sex-specific biology of these tumors.

2. Animal models of prolactinomas

Several animal models of prolactinomas are used to study factors involved in tumor development. Although these models do not completely recapitúlate the human disease, they provide important tools to study pituitary tumor pathogenesis. All of these models share the same characteristics: increased pituitary weight, hyperprolactinemia and lactotroph hyperplasia.

It is well known that dopamine, acting through dopamine type 2 receptors (Drd2), is the main inhibitor of lactotroph function (Wong *et al.* 2015). On the other hand, estradiol is the main stimulus of lactotroph function acting in both, the hypothalamus inhibiting dopamine release, and at the pituitary increasing proliferation and secretion and also reducing the inhibitory action of dopamine (Ben Jonathan & Hnasko 2001). Based on this regulation, the increase in estradiol stimulus or the decrease in dopamine inhibition were the first tools used to induce prolactinomas in animal models: the estrogen-treated rat (Heaney *et al.*

1999; Heaney *et al.* 2002) and the dopamine receptor type 2 (Drd2) knockout mouse ($Drd2^{-/-}$) (Asa *et al.* 1999; Kelly *et al.* 1997).

In addition to the control exerted by dopamine and estradiol on lactotroph function, there are a myriad of peptides and growth factors participating in intra-pituitary regulation (reviewed in (Freeman *et al.* 2000)). Based on this fact, additional transgenic mice models of prolactinomas were developed (reviewed in (Lines *et al.* 2016)), such as the overexpression of several factors under the control of the PRL promoter, e.g.i) galanin (Cai *et al.* 1999; Perumal & Vrontakis 2003), ii) TGFα (McAndrew *et al.* 1995a), iii) fibroblast growth factor receptor 4 (FGFR4) (Ezzat *et al.* 2015), iv) nerve growth factor (NGF) (Borrelli *et al.* 1992); the PRL receptor-deficient mouse ($PRLR^{-/-}$) (Schuff *et al.* 2002)), and overexpression of human chorionic gonadotropin β subunit (hCGβ+ mice) (Faraoni *et al.* 2017; Rulli *et al.* 2002). All these models have helped to clarify, at least in part, the mechanisms involved in prolactinoma development.

2.1 Gender differences in animal models of prolactinomas

Sexual differences in prolactinoma size, incidence and behavior have been described not only in humans, but also in animal models of prolactinoma. In the~~transgenicmouse~~ hCGβ+ mice, females develop strong hyperprolactinemia and large prolactinomas, while hCGβ+ males do not (Faraoni *et al.* 2017; Rulli *et al.* 2002). In the trasgenic mouse model with overexpression of human TGFα directed to the pituitary lactotrophs, females develop pituitary adenomas by the age of 12 months while males developed neither hyperplasia nor adenomas (McAndrew *et al.* 1995b). Similar findings were observed in transgenic mice

overexpressing galanin in lactotrophs (Cai *et al.* 1999); while females develop hyperprolactinemia and prolactinomas from 10 months onwards, no differences were detected in PRL contentor release between normal and transgenic male mice. In the mouse models with overexpression of FGFR4 (Ezzat *et al.* 2015), overexpression of NGF (Borrelli *et al.* 1992), $PRLR^{-/-}$ mice (Lines *et al.* 2016) and $Drd2^{-/-}$mice (Asa *et al.* 1999; Kelly *et al.* 1997), transgenic mice of both sexes develop lactotroph hyperplasia, however the tumor enlargement and the hyperprolactinemia are more pronounced in females.

The participation of ovarian steroids in the sex differences observed in the development of prolactinoma is well established. Ovariectomized $Drd2^{-/-}$ female mice, as well as hCGβ+ female mice, do not develop prolactinomas. However, hormonal replacement treatment is not sufficient to achieved the same tumoral size observed in the intact transgenic females (Ahtiainen *et al.* 2010; Hentges & Low 2002), suggesting the participation of other factors, that might be under sex steroid control and deserves furher studies.

On the other hand, studies performed in the four core genotype model demonstrated that some sex differences occur due to sex steroids while others depend on chromosomal sex (hormonal vs genetic effects) (Arnold 2009; Arnold & Chen 2009; Carruth *et al.* 2002), hence, we cannot rule out the possibility that chromosomal sex factors could be also involved in the sex differences found in prolactinoma development.

2.2 Differences among animal models and human prolactinomas

As exposed, mice models of prolactinoma share features of exacerbated phenotype (hyperpolactinemia and prolactinoma development) in females compared to males. These

observations are contrary to those found in humans where men usually present with larger and more aggressive adenomas. Even though these differences could be attributed, at least partially, to the delayed diagnosis in men vs. women, as it was discussed before, species-specific mechanisms accounting for the higher propensity of female mice to develop prolactinomas may be involved. One possibility is that the effect of sex hormones, as estradiol, plays a more important role in mice vs. humans. In fact, it was demonstrated that contrary to what is observed in rats, estrogens have little effect on PRL release in humans (Ben Jonathan & Hnasko 2001; Sarkar *et al.* 1998a). Moreover, estradiol exerted distinctive and strain-specific effects on lactotroph function in mice and rats (Sinha & Gilligan 1982). In addition, estradiol could be exerting different permissive effects for the action of other growth factors in the development of prolactinomas in mice vs. humans.

Mechanisms underlying strain-specific and gender-specific responses to estradiol remain largely unknown, and other intra-pituitary growth factors could be also involved in strain- and gender differences found prolactinoma development.

3. Pituitary TGFβ1 system

Among the growth factors participating in the intra-pituitary regulation of lactotroph function, TGFβ1 has received special attention, as it was demonstrated that TGFβ1 mediates dopamine and estradiol effect on lactotroph physiology (Recouvreux *et al.* 2011; Sarkar *et al.* 2005). Furthermore, the pituitary TGFβ1 system has been proposed as a novel target for the development of new therapies in resistant prolactinomas (Faraoni *et al.* 2017; Recouvreux *et al.* 2012). In addition, sex differences found in the expression and activity of pituitary TGFβ1 system were associated with gender differences found in the development of prolactinomas (Faraoni *et al.* 2017; Recouvreux *et al.* 2012).

TGFβ1 is a well-known inhibitor of lactotroph proliferation and prolactin secretion, and both TGFβ1 and its receptor TβRII are expressed in lactotrophs (Sarkar *et al.* 1992; Sarkar *et al.* 1998b). Moreover, the main physiological modulators of lactotroph function, dopamine and estradiol, also regulate TGFβ1 and TβRII expression in the pituitary. Whereas estradiol decreases the expression of TGFβ1 and stimulates prolactin secretion, dopamine up-regulates local TGFβ1 expression and secretion, with a concomitant reduction in prolactin release and proliferation rate of lactotrophs (Recouvreux *et al.* 2013).

The TGFβ biology is complex, and, due to the potent biological activity of the cytokine, its synthesis, secretion, storage and activation are tightly regulated. The three TGFβ isoforms are synthesized as precursor molecules containing a pro-peptide called the latency-associated peptide (LAP). Within the trans-Golgi, LAP is processed by furin-like enzymes, but remains associated with TGFβ by non-covalent interactions (Annes *et al.* 2003). In addition, within the endoplasmic reticulum, LAP is linked to a latent TGFβ binding protein (LTBP) by disulfide bonds. LTBPs belong to a family of large secretory extracellular matrix (ECM) proteins. LTBPs are glycoproteins that facilitate the secretion, storage, and activation of the TGFβ–LAP complex. The large latent complex (LTBP-LAP-TGFβ) is incorporated as component of the extracellular matrix, which acts as a cytokine reservoir. Trapped in the ECM, latent TGFβ must undergo a highly regulated process of activation by which the mature cytokine is released from its latent complex to enable the active form to bind its receptor and signal through the SMAD2/3 signaling pathway (Annes *et al.* 2003; Rifkin 2005).

Even though several latent TGFβ1 activators have been described (including proteases, integrins αvβ6 and αvβ8, thrombospondin-1 (TSP-1), and reactive oxygen species), their individual contribution in releasing TGFβ1 from its latent complex in each tissue, and their local regulation are still not fully understood (Annes *et al.* 2003; Annes *et al.* 2004; Yoshinaga *et al.* 2008).

4. TGFβ1 alterations during prolactinoma development:

TGFβ1 expression and activity are reduced in different animal models of prolactinomas, and TGFβ1 has been postulated to be involved in tumor development (Faraoni *et al.* 2017; Pastorcic *et al.* 1995; Recouvreux *et al.* 2011; Recouvreux *et al.* 2012; Recouvreux *et al.* 2016). In the estrogen-treated rat model ~~for~~of prolactinoma (Heaney *et al.* 1999; Heaney *et al.* 2002), chronic estradiol treatment decreases pituitary TGFβ1 and TβRII mRNA and protein, together with an increase in prolactin levels (De *et al.* 1996; Hentges & Sarkar 2001; Pastorcic *et al.* 1995; Sarkar *et al.* 1992). In agreement, pituitary tumorigenesis induced by estrogen treatment is greatly accelerated in TβRII heterozygous knockout mice ($T\beta RII^{+/-}$) where the expression of TβRII is markedly reduced (Shida *et al.* 1998). On the other hand, active and total pituitary TGFβ1 levels, as well as the expression of several components of the TGFβ1 system, were also reduced in female pituitaries from $Drd2^{-/-}$ mice compared to wild type counterparts (Recouvreux *et al.* 2011; Recouvreux *et al.* 2013).

Moreover, we recently described a reduced pituitary TGFβ1 system in another model of prolactinoma: mice that overexpress the human chorionic gonadotropin β subunit (hCGβ+ mice) (Faraoni *et al.* 2017; Recouvreux *et al.* 2011).

Alterations in the pituitary TGFβ1 system have been also observed in human pituitary adenomas. It has been described that TGFβ1 and p-Smad3 protein levels gradually decreased, while the inhibitory Smad7 protein gradually increased when normal anterior pituitaries were compared to noninvasive and invasive pituitary adenomas (Zhenye *et al.* 2014), suggesting that the activity of TGFβ signaling would be limited during tumor development. A recent report also described a significant down-regulation of the TGFβ1/Smad signaling in 12 cases of dopamine resistant prolactinomas compared to normal human pituitaries (Li *et al.* 2015).

These findings support the contention that decreased pituitary TGFβ1 activity is involved in the development of prolactinomas. Moreover, this hypothesis was corroborated when a pharmacological treatment that restore pituitary TGFβ1 activity was successful in recovering the intrapituitary pSMAD2/3 expression, decreasing pituitary tumor size and reducing hyperprolactinemia, in two different animal models of prolactinoma (Faraoni *et al.* 2017; Recouvreux *et al.* 2012). Thus, improvement of the local TGFβ1 biological activity is associated with the inhibition of prolactinoma growth.

5. Sex differences in the pituitary TGFβ1 system in animal models of prolactinoma.

The presence of sex differences in the pituitary TGFβ1 system was described for the first time in the Drd2$^{-/-}$ mice model of prolactinoma (Recouvreux et al. 2013). Male mice express more robust levels of pituitary TGFβ1 system components than females, including active TGFβ1 levels, and the expression of TβRII, LTBPs and many TGFβ1 activators (TSP1, KLK1, MMP2, MT1-MMP, integrins αvβ6 and αvβ8). These results suggest that TGFβ1 could be protective in males by restraining excessive lactotroph proliferation and prolactin secretion (Figure 1).

The expression of higher levels of several components of the TGFβ1 system in male pituitaries could be attributed to the lower serum estradiol levels present in males, as it was demonstrated that estradiol negatively controls most of the components of the system (Recouvreux *et al.* 2013).

Gender differences in dopaminergic and estradiol effects at the pituitary level were described several years ago. It is well known that the concentration of dopamine in the median eminence of female rats in diestrus is 7 times higher than in males (Gudelsky & Porter 1981). On the other hand, basal tuberoinfundibular dopaminergic system (TIDA) activity is five times higher in females than in males (Freeman *et al.* 2000). In agreement with these sex divergences in the dopaminergic regulation of lactotroph function, the loss of dopamine action through Drd2 disruption (Drd2$^{-/-}$ mice model) has a stronger effect in female than in male mice (Diaz-Torga *et al.* 2002; Saiardi *et al.* 1997). Although in Drd2$^{-/-}$ mice both female and male pituitaries are devoid of dopamine inhibitory control, females develop higher hyperprolactinemia than males from 2 months onwards, and while in females lactotroph hyperplasia is observed at 6 months, Drd2$^{-/-}$males only develop pituitary lactotroph adenomas at 17 to 20 months of age (Asa *et al.* 1999).

Interestingly, the loss of dopamine action also has a stronger effect in the pituitary TGFβ1 system in females than in males. Active and total TGFβ1 levels are reduced in Drd2$^{-/-}$ female pituitaries compared to wild-type, highlighting the stimulatory role of dopamine on pituitary TGFβ1 system (Recouvreux *et al.* 2011; Recouvreux *et al.* 2013). Concomitantly, downregulation of several putative TGFβ1 activators (as TSP1, MMP2, MMP9, MT1-MMP, and tissue kallikrein), as well as the decreased expression of TGFβ1 target genes, TGFβ receptor and LTBPs, was observed in Drd2$^{-/-}$ females vs. their wild-type counterparts. However, the disruption of dopaminergic inhibition did not affect these parameters in male pituitaries. These results demonstrated, for the first time, a gender difference in the dopaminergic regulation of the pituitary TGFβ1 system (Figure 2).

Similar observations of the link between sex differences in the TGFβ1 system and prolactinoma development were recently described in the mice model of hCGβ overexpression (Faraoni *et al.* 2017), in which only hCGβ+ females develop prolactinomas. The pituitary hyperplasia becomes evident in females from 2 months of age onwards, and progresses to adenoma by the age of 8-10 months, concomitant with severe hyperprolactinemia (Rulli *et al.* 2002). Because of the high levels of circulating hCG, the ovaries of hCGβ+ females present massive luteinization, and progesterone becomes the main steroid hormone produced; however, serum estradiol remains at physiological levels.

In this model TGFβ1 levels (active and total cytokine), as well as TGFβ1 target genes, TGFβ receptor, LTBPs, Smad4 and Smad7 expression, were found decreased in hCGβ+ female pituitaries compared to their wild-type counterparts. However, no differences in the pituitary TGFβ1 system were found between the transgenic and wild-type male pituitaries.

On the other hand, a stronger TGFβ1 system was observed in male pituitaries and this fact could protect them from excessive lactotroph proliferation (Faraoni *et al.* 2017) (Figure 1). Once more, the sex differences in the regulation of the pituitary TGFβ1 system correlate with gender differences in the incidence of prolactinoma.

It is worth to mention that the sexual differences in pituitary TGFβ1 described here have been only studied in animal models of prolactinoma. Whether these differences are also occurring in human pituitary tumors and whether they can account for sexual differences in tumor incidence and/or behavior in humans remain to be studied.

There is a critical relationship between sex steroids and dopamine tone in regulating lactotroph function. The balance between estradiol and dopamine is an important factor in regulating pituitary TGFβ1 function. The recent findings on sex divergences in the control of the pituitary TGFβ1 system by dopamine and estradiol suggest the involvement of local TGFβ1 in gender-related differences found in the control of lactotroph function in animal models.

Summarizing, sex differences were observed in the regulation of the pituitary TGFβ1 system in animal models of prolactinoma. In males, the increased cytokine activity and increased levels of most of the pituitary TGFβ1 system components could be protective from excessive lactotroph proliferation and prolactinoma development. On the other hand, when dopaminergic regulation is lost, the pituitary TGFβ1 system is deeply affected in

females, but not in males, and this could also account for the gender divergences in prolactinoma incidence in the animal models (Figure 2).

It would be worth determining whether sexual differences in pituitary TGFβ1 system are also found in humans, and if they are involved in the differences found in prolactinoma incidence during fertile period.

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Figure Legends

Figure 1. Sex differences in pituitary TGFβ1 system in mice models of prolactinomas. TGFβ1 is a potent suppressor of lactotroph proliferation and prolactin secretion. In the Drd2 $^{-/-}$ and hCGβ+ mice models of prolactinoma, males present higher levels of active TGFβ1, and higher expression of TβRII receptor, LTBPs, and TGFβ1 activators compared to females. Increased levels the pituitary TGFβ1 system components, could be protective from excessive lactotroph proliferation and prolactinoma development. Modified from Recouvreux et al, J Endocrinol. 2016; 228(3):R73-83. doi: 10.1530/JOE-15-0451 .

Figure 2. Gender differences in the pituitary TGFβ1 system, and its regulation by estradiol (E2) and dopamine (DA). Males present a more robust pituitary TGFβ1 system compared to females (see Figure 1), which is proposed to have a protective effect, restraining

excessive lactotroph proliferation and prolactin secretion. Estradiol exerts a negative control on the expression of several components of the pituitary TGFβ1 system, possibly accounting for the observed gender differences. On the other hand, dopamine positively regulates pituitary TGFβ1 in females, therefore, loss of dopamine regulation in Drd2$^{-/-}$ mice deeply affects pituitary TGFβ1 system in females but not in males, also accounting for gender differences in prolactinoma development in this model.

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Figure 1

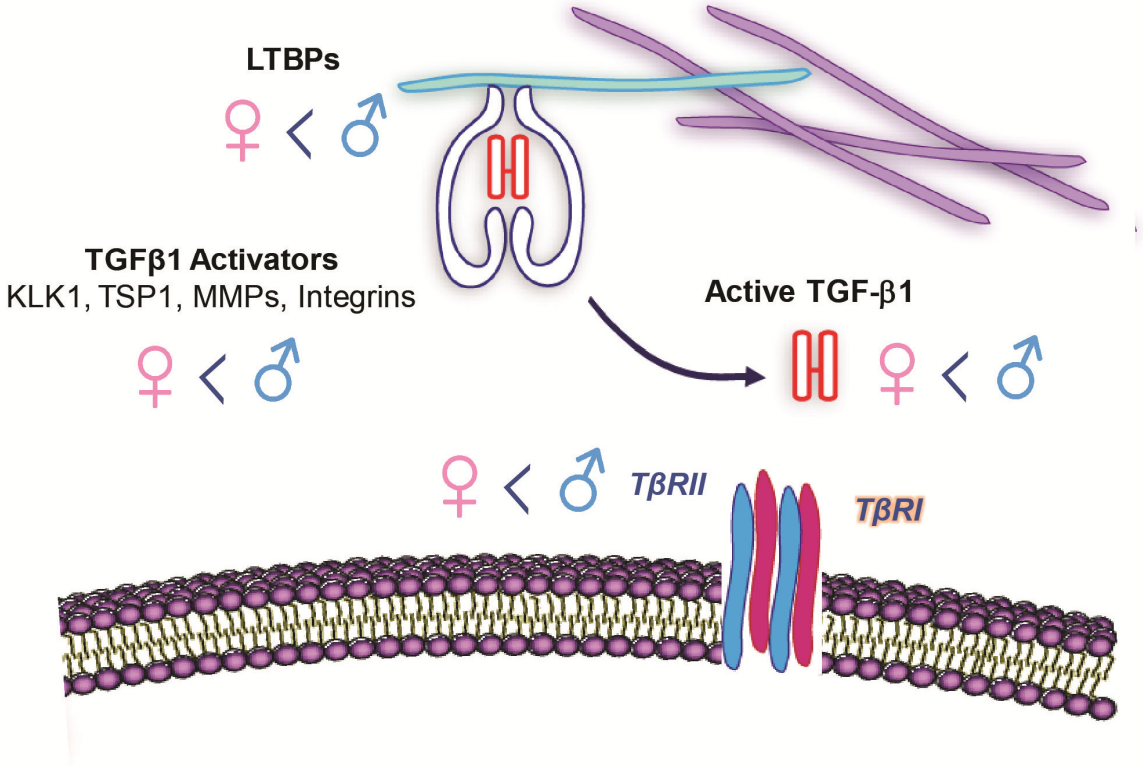
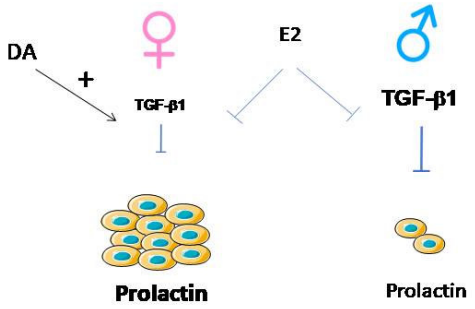
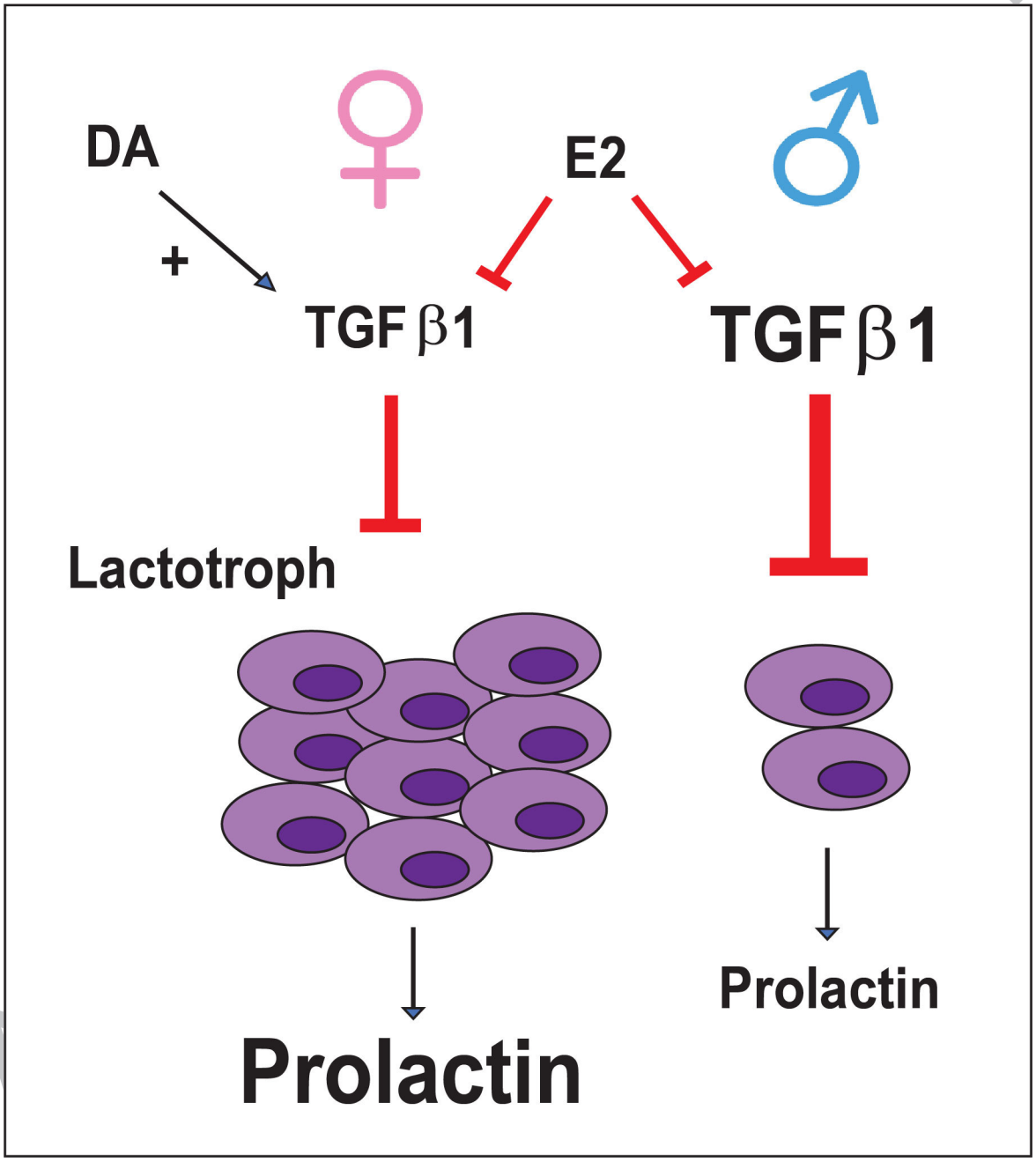


Figure 2



Graphical abstract



Highlights

- Sex differences in prolactinomas size, behavior and incidence is described.
- TGFβ1 is an important inhibitor of lactotroph function
- TGFβ1 activity is reduced in prolactinomas in a sexually dimorphic manner.
- TGFβ1 involvement in sex differences found in prolactinomas is suggested.