Expression and function of ErbB receptors and ligands in the pituitary

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Abstract

The role of ErbB family in discrete pituitary functions is reviewed. Several ErbB receptor ligands, EGF, TGF α , and heregulin are differentially expressed in normal gonadotroph and lactosomatotroph lineages, and other elements of the anterior pituitary. ErbB receptors, i.e. EGFR and ErbB2, are also localized to the anterior pituitary with preferential EGFR lactosomatotroph expression. EGF regulates CRH and ACTH secretion and corticotroph proliferation as well as exhibiting autocrine and paracrine effects on gonadotrophs and on lactosomatotroph proliferation, gene and protein expression, and hormonal secretion. EGF and EGFR are expressed in both functioning and non-functioning pituitary adenomas, with higher expression in more aggressive tumor subtypes. ErbB2 receptor is detected in all tumor subtypes, particularly in invasive tumors. ErbB tyrosine kinase inhibitors regulate hormonal secretion, cell morphology, and proliferation in lacto-somatotroph tumors, reflecting the emerging application of targeted pituitary therapeutics.

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Introduction

Pituitary tumors are monoclonal adenomas accounting for ~15% of primary intracranial neoplasms. Although usually benign, excess hormone secretion may lead to distinct endocrine syndromes such as acromegaly, Cushing's disease, and hyperprolactinemia, while non-functional tumors lead primarily to hypogonadism and critical compressive symptoms (Melmed 2003). Dopamine agonist and/or somatostatin analog therapy of tumors arising from the lactosomatotroph cell lineage is effective in most tumors though less effective in tumors manifesting drug resistance. No effective pituitary targeted drug therapies are currently available for ACTH-secreting or non-functional adenomas (Melmed 2003, 2009). Alternative treatment options are required for recurring invasive macroadenomas and for infrequently encountered but aggressive pituitary carcinomas resistant to

most currently available treatments (Kaltsas *et al.* 2005, Scheithauer *et al.* 2005).

Over the last two decades, the EGFR pathway has been explored in both pituitary physiology and pathology. The human epidermal growth factor receptor (EGFR, ErbB, and HER) family comprises four known members EGFR (ErbB1 and HER1), p185^{Her2/neu} (ErbB2 and HER2), ErbB3 (HER3), and ErbB4 (HER4), which are transmembrane glycoprotein receptors containing an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (Fig. 1). Aberrant receptor expression, mutations, or overexpression lead to receptor homo- or heterodimerization, activation of the intrinsic tyrosine kinase and subsequent induction of specific intracellular signaling cascades (Zhang *et al.* 2007). Activating ligands include epidermal growth factor (EGF), transforming growth factor (TGF)- α ,

Figure 1 ErbB receptor family. ErbB receptor family consists of four closely related type 1 transmembrane tyrosine kinase receptors, EGFR, ErbB2, ErbB3, and erbB4. EGFR and erbB4 have active tyrosine kinase domains and known ligands, ErbB3 can bind to several heregulins as ligands but lacks tyrosine kinase activity. By contrast, ErbB2 possesses an active tyrosine kinase domain but no ligand has been identified. This receptor exists in the active conformation state, so this receptor is constitutively available for dimerization. Ligand binding to ErbB receptors induces the formation of receptor homo- and heterodimers and activation of the intrinsic kinase domain, resulting in phosphorylation on specific tyrosine residues. These phosphorylated residues serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signaling pathways. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-11-0066>.

amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin, epiregulin (ER), and heregulins (HRG, neuregulins (NR); Zhang *et al.* 2007). Increased ErbB-receptor kinase activity has been implicated in the pathogenesis of several human cancers. Selective therapeutic targeting particularly of EGFR and p185^{her2/neu} with monoclonal antibodies and small compound tyrosine kinase inhibitors (TKIs; Zhang *et al.* 2007) has exhibited therapeutic efficacy.

This review critically appraises the role of the ErbB family in pituitary development and tumorigenesis to heighten awareness of this growth factor family that may lead to novel medical therapy for pituitary tumors.

Anterior pituitary tissue

EGFR ligands and receptor

Initial studies examined whole pituitaries for expression of ErbB ligands and distinguished that EGF localizes to the anterior and not posterior pituitary (Kasselberg *et al.* 1985), present at all stages of development from fetus to adulthood (Kasselberg *et al.* 1985) and confirmed by PCR (LeRiche *et al.* 1996),

immunohistochemistry (IHC; Kajikawa *et al.* 1991), radio-receptor-binding assay, and western blot (WB) of autopsy-derived human pituitaries (Halper *et al.* 1992). Examination of male mouse pituitaries by *in situ* hybridization (ISH) revealed *EGF* mRNA in the anterior and intermediate lobes (Honda *et al.* 2000); *EGF* mRNA-expressing cells were medium-sized and round, making up to 40% of the total number of secretory cells (Honda *et al.* 2000). These cells functionally secrete EGF, confirmed on reverse hemolytic plaque assays and IHC of rat anterior pituitary plurihormonal cells (Mouihate & Lestage 1995a,b, Mouihate *et al.* 1996b).

TGFα mRNA has been demonstrated in both animal and human pituitary tissue. It is expressed in the rat anterior pituitary as detected by ribonuclease protection assay, RT-PCR (Fan *et al.* 1995) and ISH immunocytochemistry (ICC; Fan & Childs 1995), in intact bovine anterior pituitary gland and cell cultures (Mueller *et al.* 1989), and in all adenohypophysial cell types in human normal pituitary tissue (Ezzat *et al.* 1995). *TGFα* expression increases in response to 12-*O*-tetradecanoylphorbol-13-acetate and

EGF (Mueller *et al.* 1989) and decreases in response to treatment with TGF β 1.

Concordant with ligand expression, EGF receptors are distributed throughout the anterior pituitary. In pituitaries derived from 2-month-old male mice, *EGFR* mRNA was detected in 47% of anterior pituitary cells by ISH, while no expression was found in intermediate and posterior pituitary cells (Honda *et al.* 2000). About half of all rat anterior pituitary cells express EGFR as detected by IHC (Mouihate & Lestage 1995a) though when using an antibody directed against the intracellular domain of the rat EGFR, positive staining is found in only ~15% of all pituitary cells (Fan & Childs 1995). In normal human pituitary glands, EGFR protein expression is demonstrated in all cell types with both antibodies directed against the intra- and extracellular receptor domains (Theodoropoulou *et al.* 2004). EGFR protein expression was also detected by WB (170 kDa), *EGFR* mRNA by RT-PCR in normal pituitaries, and weak EGFR positivity by ISH using microarrays (Onguru *et al.* 2004).

Approximately 64% of pituitary adenomas express EGFR mRNA and/or protein, in 66% of all non-functioning adenomas and 62% of functioning adenomas with higher expression levels observed in tumors with a more aggressive phenotype (LeRiche *et al.* 1996, Jaffrain-Rea *et al.* 1998).

ErbB2 receptor

Positive pituitary ErbB2 immunostaining by ICC with the use of a monoclonal antibody to the cytoplasmic domain of ErbB2 was observed in normal rat and human pituitary cells (Chaidarun *et al.* 1994a, Nose-Alberti *et al.* 1998, Martin-Lacave & Utrilla 2000). IHC with an antibody to the internal ErbB2 domain shows a granular and cytoplasmic ErbB2 staining pattern in only a few scattered cells, though mRNA transcript signals are detected in the normal pituitaries tested (Ezzat *et al.* 1997).

ErbB2 receptor expression is found overall in 31% of all pituitary tumors, in 43% of non-functioning pituitary adenomas (Birman *et al.* 1987, Chaidarun *et al.* 1994a, Kontogeorgos *et al.* 1996, LeRiche *et al.* 1996, Jaffrain-Rea *et al.* 1998, Onguru *et al.* 2004, Theodoropoulou *et al.* 2004) and in 24% of functioning adenomas (Chaidarun *et al.* 1994a, Ezzat *et al.* 1997, Nose-Alberti *et al.* 1998, Ferreira *et al.* 2005, Botelho *et al.* 2006, Vlotides *et al.* 2009). Positive cytoplasmic ErbB2 staining was observed in a variable number of cells in 40% of invasive pituitary adenomas ($n=103$), while only 1.2% of the

non-invasive tumors ($n=241$) expressed this protein ($P<0.001$; Nose-Alberti *et al.* 1998).

ErbB3/4 ligands and receptors

HRG, also called neuregulin (NRG), neu differentiation factor, glial growth factor (GGF), and acetylcholine receptor-inducing activity, is a soluble secreted growth factor which serves as a ligand for ErbB3 and ErbB4 (Breuleux 2007). *HRG-1* type II encodes GGF (Breuleux 2007), which was originally identified from bovine pituitary extracts (Brockes *et al.* 1980, Lemke & Brockes 1984). However, the cellular source and function of HRG, as well as that of other ErbB receptor ligands such as HB-EGF, AR, ER, and betacellulin in the normal anterior pituitary are not known.

ErbB3 normal pituitary gland expression has not been examined. However, the fourth member of the EGFR family ErbB4 is expressed at relatively high levels in the normal pituitary gland, as determined by RT-PCR (Plowman *et al.* 1993); the cellular source and function of pituitary ErbB4 expression is not known.

Corticotroph cells

EGFR ligands and receptor

EGFR ligand expression has been detected in normal pituitary corticotroph cells by some (Kontogeorgos *et al.* 1996) though not confirmed by all (Kasselberg *et al.* 1985, Chaidarun *et al.* 1994a). Though *TGF α* mRNA expression has not been found in corticotroph cells (Fan & Childs 1995), TGF α induces proliferation of corticotrophs via enhanced BrdU uptake (Oomizu *et al.* 2000, Sharma *et al.* 2003).

EGFR expression localizes to normal human corticotrophs on autopsy specimens (Kontogeorgos *et al.* 1996) using IHC with antibodies directed against both the intra- and extracellular domains of EGFR (Theodoropoulou *et al.* 2004).

EGFR ligands have a functional role in corticotrophs and may regulate HPA-axis activation at several levels as observed in animal models. EGF may regulate ACTH release through hypothalamic CRH induction (Luger *et al.* 1988) shown through EGF infusions in near-term ovine fetuses that led to increased circulating ACTH concentrations (with a similar potency to synthetic CRF) without affecting circulating CRF, AVP, or catecholamine levels (Polk *et al.* 1987). EGF may also have a direct effect on corticotrophs or participate in paracrine-pituitary interactions, as seen when EGF induced *POMC* mRNA expression, ACTH secretion and corticotroph cell proliferation in

populations of mixed and enriched rat corticotrophs obtained by counterflow centrifugation (Childs *et al.* 1991, 1995). Treatment of mouse pituitary primary cultures with EGF for 5 days induced corticotroph replication (Honda *et al.* 2000). Furthermore, TGF α also exerts mitogenic effects on mouse corticotrophs and appears to be involved in EGFR-dependent estrogen-mediated induction of corticotroph cell proliferation (Oomizu *et al.* 2000). TGF α mRNA and protein is also expressed in corticotroph adenomas (Ezzat *et al.* 1995).

EGFR ligands and receptor are observed in normal corticotrophs and also in adenomatous tissue. In one series, EGF expression by IHC was reported in 38% (7/19) of ACTH-secreting tumors adenomas, with higher expression in invasive adenomas, and in 2/2 carcinomas, while metastases exhibited a higher EGF content compared with the carcinoma itself (Lubke *et al.* 1995). Other groups reported positive EGF expression rates as high as 80% in corticotroph adenomas (Kontogeorgos *et al.* 1996) and detected *EGF* mRNA by RT-PCR and protein in 4/5 corticotroph adenomas (LeRiche *et al.* 1996). However, measurable quantities of EGF were not detected by ELISA of the medium of cultured adenomas (LeRiche *et al.* 1996).

With the exception of one series which used an antibody targeting only the extracellular domain (Chaidarun *et al.* 1994a), EGFR expression in corticotroph adenomas has consistently been demonstrated, with a reported overall positive rate of 75% of 77 corticotroph tumors tested, by both IHC and ISH techniques and variable antibodies (Kontogeorgos *et al.* 1996, Jaffrain-Rea *et al.* 1998, Onguru *et al.* 2004, Theodoropoulou *et al.* 2004). Using an antibody targeting the intracellular EGFR domain in 102 pituitary adenomas, strongly positive EGFR immunoreactivity was detected in corticotroph adenomas with weaker staining noted in other functioning and non-functioning adenomas (Theodoropoulou *et al.* 2004). Overall, ACTH-secreting adenomas exhibit significantly higher number of EGFR immunoreactive cells, and a higher rate of phospho-EGFR (Tyr 922), the active form of EGFR (Theodoropoulou *et al.* 2004).

In contrast to the known functional effects of EGFR and its ligands on normal corticotrophs, little is known of the functional role of this pathway in corticotroph tumors. In AtT20 cells, a mouse corticotroph tumor cell line, EGF-stimulated cell proliferation, but this did not translate to increased ACTH secretion (van Wijk *et al.* 1995). Furthermore, neither EGFR nor ErbB2 receptor expression were detected in AtT20 cells, nor were inhibitory effects on AtT20 cell proliferation

observed by treatment with the EGFR inhibitor gefitinib (Vlotides *et al.* 2008).

ErbB2 receptor

ErbB2 receptors localize to normal corticotrophs on ICC (Chaidarun *et al.* 1994a) but are not as abundantly detected as EGFR in corticotroph adenomas, with overall rate of 13% tumors positive in several series (Chaidarun *et al.* 1994a, Ezzat *et al.* 1997, Nose-Alberti *et al.* 1998). However, a report of one pituitary ACTH-producing carcinoma exhibited granular cytoplasmic and membrane staining in 20% of the cells of the surgery and 30% of autopsy material, while liver metastases showed positive ErbB2 staining in the cytoplasm and membrane in 80% of cells (Nose-Alberti *et al.* 1998).

ErbB3/4 ligands and receptors

To date, ErbB3 and ErbB4 have not been explored in normal or tumoral corticotrophs.

Discussion

Of the ErbB receptor family, EGFR and its ligands play the dominant role in both normal and adenomatous corticotrophs. Expression studies confirm localization of EGFR and its ligands, and functional studies demonstrate a role of EGF and EGFR in corticotroph proliferation and hormone secretion both in a direct and paracrine fashion. Further studies remain to determine the role of ErbB receptors in corticotroph tumor development and behavior.

Gonadotroph cells

EGFR ligands and receptor

Double immunostaining for EGF protein with pituitary hormones performed by two different groups identified EGF-positive cells in gonadotrophs and thyrotrophs (Kasselberg *et al.* 1985, Chaidarun *et al.* 1994b) while ISHIC coupled with immunostaining for pituitary hormones revealed *EGF* mRNA localization only in LH and FSH cells (Fan & Childs 1995). In pituitary specimens derived from immature female rats, most EGF-secreting cells were identified as LH-positive gonadotrophs (~72%), and EGF secretion was enhanced by LHRH treatment (Mouihate *et al.* 1996a). Secreted TGF α from untransformed bovine anterior pituitary cells in culture (Kobrin *et al.* 1986, Samsoondar *et al.* 1986) and subsequent expression analysis by IHC did not reveal TGF α protein expression in gonadotrophs (Kobrin *et al.* 1986) though

TGF α mRNA was positive in rat gonadotrophs (Fan & Childs 1995). EGF receptor expression is abundant in normal gonadotrophs with both extra- and intracellular targeted antibodies on ICC (Fan & Childs 1995, Theodoropoulou *et al.* 2004). Approximately 45% of pituitary cells from metestrous rats demonstrated positive EGFR labeling, though expression levels gradually declined in later stages (to 25% by proestrus; Armstrong & Childs 1997a).

A number of studies have investigated the functional effects of the EGFR pathways in normal gonadotrophs both at the level of the hypothalamus and pituitary. In primary pituitary cultures, EGF enhances LH release (Przylipiak *et al.* 1988) while in turn LHRH stimulation further increases EGF secretion, in a positive feedback mechanism. Moreover, EGF stimulates gonadotroph proliferation, increases the percentage of gonadotroph cells in S phase, as well as stimulates immediate early genes (i.e. *c-fos*; Childs & Unabia 2001).

Further evidence of the direct effects of EGFR and its ligands on gonadotrophs is demonstrated by studies in menstrual changes in animal models. At the initial proestrous stage, EGFR expression is at its highest level in gonadotrophs, concurrent with increasing *LH β* mRNA in LH cells as well as higher levels in FSH β antigen-bearing cells (Armstrong & Childs 1997a, Childs & Armstrong 2001). EGF further stimulates the GnRH receptor as well as its own cognate receptor in gonadotroph cells, which is under negative feedback from the elevated estrogen levels in proestrous stage (Armstrong & Childs 1997b, Childs & Unabia 2001). In the estrous stage, GnRH then stimulates EGFR expression in FSH cells (Childs & Armstrong 2001). Next, in metestrous, pituitary EGFR expression undergoes cyclic change with most EGF responsive cells evident during metestrous, leading to the lowest levels of EGFR in the cycle (Armstrong & Childs 1997a). However, estradiol treatment of metestrous cultured anterior pituitary cells can increase the size and percentage of EGF plaque-forming cells to levels seen in proestrous cultures (Mouihate & Lestage 1995b). EGF can also stimulate GnRH receptors during this stage (Childs & Armstrong 2001). Finally, in diestrous, EGFR again increases together with *LH β* mRNA in developing LH gonadotroph cells (Armstrong & Childs 1997a). In summary, these studies suggest that EGF acts as an autocrine or paracrine factor to maintain and develop gonadotrophs and functions in preparation for the LH surge (Childs & Armstrong 2001).

EGF also has an indirect regulatory effect on gonadotrophs through the hypothalamus. In cultured

adenohypophyseal cells, EGF stimulated GnRH binding and potentiated LH response to GnRH but did not directly increase LH secretion (Leblanc *et al.* 1997). Early study perfused pituitaries of cycling female rats with EGF that led to LH release from hypothalamo-pituitary pairs but not from the pituitary itself. On the other hand, infusion with both EGF and estradiol did lead to significant LH release, suggesting that EGF regulated pituitary gonadotropin secretion by a direct effect on the hypothalamus and indirectly at the pituitary by increasing pituitary responsiveness to estradiol (Miyake *et al.* 1985). Furthermore, *TGF α* is a physiological ligand for LHRH via the EGF/*TGF α* receptor in the developing female rat hypothalamus, as shown by the dose-related increase in LHRH when stimulated with both EGF and *TGF α* while blockade of EGF alone did not block LHRH release (Ojeda *et al.* 1990).

EGF receptors are modulated at the level of the hypothalamus as well. Src and Pyk2 potentiate transactivation required for GnRH-induced ERK1/2 phosphorylation in hypothalamic GnRH neurons (GT1-7; Shah *et al.* 2003b). GnRH-induced ERK1/2 phosphorylation caused by EGFR transactivation limited to GT1-7 neuronal cells and attenuated by an EGFR kinase inhibitor (Shah *et al.* 2003a). Looking at the GnRH development through menstrual cyclic changes, *EGFR* mRNA increases in medial basal hypothalamus at the initiation of puberty, decreases in the first morning of proestrous, and increases during the afternoon, at the time of the gonadotropin surge, concurrent with changes in EGFR protein levels (Ma *et al.* 1994). EGFR-induced signaling in hypothalamic astroglia has been shown to be crucial for production of glial-derived molecules that stimulate GnRH neurons to release GnRH (Ma *et al.* 1997). Disruption of EGFR signaling in astroglia of female mice resulted in irregular estrous cycles and decreased LH secretion (Li *et al.* 2003).

In vivo models have further confirmed that EGF inhibits the hypothalamic pulse generator for LH secretion while inhibiting follicular estradiol production. In rams treated with subcutaneous EGF, mean plasma LH, FSH, and testosterone were significantly reduced for 48 h compared with a control group. When both EGF-treated rams and the controls were then injected with LHRH, LH, and testosterone levels increased though the levels did not differ between the groups (Brown *et al.* 1989). On the other hand, EGF infusion into merino ovariectomized ewes did lead to reduced frequency of pulsatile LH secretion by inhibiting LHRH release from the hypothalamus (Radford *et al.* 1987). Infusion of EGF during luteal

phase of adult merino ewes had no effect on progesterone levels while infusion during the follicular phase led to suppression of estradiol rise. LH pulse amplitude was increased while pulse frequency decreased (Shaw *et al.* 1985).

Together, these studies suggest that EGFR and its ligands are involved in development and maintenance of gonadotroph function at different levels of the hypothalamic–pituitary–gonadal axis.

Fewer data are available on EGFR expression and function in gonadotroph adenomas. Overall, 75% of the 56 non-functioning adenomas tested express EGF either by IHC or by RT-PCR with rates varying between 10 and 100% in different series (Chaidarun *et al.* 1994a, Kontogeorgos *et al.* 1996, LeRiche *et al.* 1996, Otsuka *et al.* 1999). In addition, TGF α mRNA and protein are detected in non-functioning adenomas (Driman *et al.* 1992, Ezzat *et al.* 1995). EGFR mRNA and protein expression has been characterized in 379 non-functioning adenomas, with overall positive rate of 66% though each study had rates ranging from 0 to 100% of tumors tested, depending on the technique (Birman *et al.* 1987, Chaidarun *et al.* 1994a, Kontogeorgos *et al.* 1996, LeRiche *et al.* 1996, Jaffrain-Rea *et al.* 1998, Onguru *et al.* 2004, Theodoropoulou *et al.* 2004). EGF binding sites are detected in gonadotroph macroadenomas, with binding higher in invasive adenomas and especially in those invading the sphenoid sinus, independent of other markers (Jaffrain-Rea *et al.* 1998), suggesting that EGF binding may be an additional marker of pituitary tumor aggression.

The functional role of EGFR in non-functioning adenomas has been explored in a limited basis. An early study tested growth factors derived from tumor-conditioned media obtained from 23 cultured human NFAs. Neutralizing antibodies directed against EGF reduced the growth promoting activity of tumor-conditioned media (Renner *et al.* 1993). In primary cultures of NFAs expressing α -subunit mRNA, addition of EGF led to increased ^3H -thymidine uptake and cell number. *EGFR* mRNA level was enhanced fourfold by EGF while α -subunit mRNA was reduced by EGF (Chaidarun *et al.* 1994a).

In alphaT3-1 cells, a gonadotroph tumor line, GnRH challenge led to ERK activation, an effect that was abrogated by an EGFR TKI (Grosse *et al.* 2000). Crosstalk between GnRH and EGFR may occur through gelatinases of the MMP family. Administration of MMP inhibitors in alphaT3-1 cells abolished transactivation of EGFR by GnRH (Roelle *et al.* 2003).

ErbB2 receptor

In contrast to the extensive research on the EGFR pathways, little is known on the expression and function of ErbB2 receptors in normal gonadotrophs, with only one study reporting positive ErbB2 expression in gonadotroph cells (Chaidarun *et al.* 1994a). ErbB2 expression in non-functioning adenomas ranges from 0 to 100%, with overall positive rate of 43% in 174 tumors tested (Chaidarun *et al.* 1994a, Ezzat *et al.* 1997, Nose-Alberti *et al.* 1998, Ferreira *et al.* 2005). However, direct sequencing of codon 659 within a set of tumors revealed no point mutations, and differential PCR did not show the presence of DNA amplification (Ezzat *et al.* 1997). Positive cytoplasmic ErbB2 staining was observed in a variable number of cells in 40% of invasive pituitary adenomas ($n=103$), while only 1.2% of the non-invasive tumors ($n=241$) expressed this protein ($P<0.001$); however, no particular immunohistological type preferentially expressed the protein in this study (Nose-Alberti *et al.* 1998). In two pituitary gonadotroph cell carcinomas, ErbB2 immunoreactivity and low level ErbB2 gene amplification were observed in the metastases of the first case while the second case did not display ErbB2 overexpression or gene amplification in the sellar component or the metastases of the tumor (Roncaroli *et al.* 2003).

Discussion

Similar to corticotroph cells, EGFR and its ligands are the dominant ErbB receptor in normal and adenomatous gonadotrophs. It is evident that EGFR is a regulator of gonadotroph development, confirmed in the menstrual cycle studies, both at the hypothalamic and pituitary level. In non-functioning adenomas, EGFR expression may be associated with more invasive phenotypes, but it remains to be determined how this is mediated and whether it is of clinical import.

Lacto-somatotroph cells

EGFR ligands and receptor

EGFR and its ligands are detected in normal lacto-somatotroph cells in multiple animal models. In rat anterior pituitary cells, EGF localized to 27% of PRL-positive and 20% of GH-positive cells, by both reverse hemolytic plaque assay and IHC techniques (Mouihate & Lestage 1995b) while others did not confirm these findings on routine immunostaining (Kasselberg *et al.* 1985, Chaidarun *et al.* 1994a). However, *EGF* mRNA

is detected using ISH (Fan & Childs 1995). *TGF α* mRNA expression has been detected in mouse and rat anterior pituitary cell cultures by RT-PCR, ISH, and IHC, particularly in somatotrophs as well as some lactotrophs (Fan & Childs 1995, Sharma *et al.* 2003). Identification of secreted *TGF α* from untransformed bovine anterior pituitary cells in culture (Kobrin *et al.* 1986, Samsoondar *et al.* 1986) and subsequent expression analysis by IHC revealed *TGF α* expression in lactotrophs and somatotrophs (Kobrin *et al.* 1987). In human normal pituitary tissue, immunoreactive *TGF α* is present in all adenohypophyseal cell types, with predominant reactivity in the lateral wings (Driman *et al.* 1992), and one group observed *TGF α* co-localization in GH- but not PRL-immunopositive cells (Finley *et al.* 1994). Finally, receptors to EGF have been detected on binding assays and IHC in somatotrophs and lactotrophs (Chabot *et al.* 1986, Fan & Childs 1995, Theodoropoulou *et al.* 2004).

EGFR and its ligands are expressed in lacto-somatotroph cells and also have a functional role in PRL regulation at both gene and protein levels, leading to changes in prolactin transcription and synthesis (Murdoch *et al.* 1982), pituitary proliferation, and hormonal secretion (Mouihate & Lestage 1995a,b, Mouihate *et al.* 1996a,b); thus far, EGF has been shown to have little effect on GH regulation (Ikeda *et al.* 1984, Lewis *et al.* 2002).

Evidence for stimulation of lactotroph proliferation by EGFR ligands is demonstrated from mouse models. Treatment of mouse pituitary primary cultures (serum-free medium) with EGF (1 and 10 ng/ml) for 5 days stimulated BrdU labeling approximately threefold in lactotrophs, while no effect was observed in other subsets of pituitary cells (Honda *et al.* 2000). Treatment with *TGF α* (0.1 and 1 ng/ml) also induced BrdU labeling in anterior pituitary mouse cells (Oomizu *et al.* 2000, Takahashi *et al.* 2002, Sharma *et al.* 2003). Interestingly, estrogen (10^{-9} M; 5 days)-induced lactotroph proliferation (threefold) as well as expression of *TGF α* and *EGFR* mRNA, while estrogen-mediated induction of cell proliferation was blocked by the EGFR inhibitor RG-13022 or the *TGF α* antisense oligodeoxynucleotide, suggesting that *TGF α* mediates stimulatory effects of estrogen on lactotroph cell proliferation (Oomizu *et al.* 2000, Takahashi *et al.* 2002, Sharma *et al.* 2003).

Furthermore, EGF enhances PRL secretion, up to 240% in neonatal lactotrophs (Felix *et al.* 1995). In cultured pituitaries derived from vehicle- and estrogen-treated rats, EGF increased PRL response to TRH in vehicle-treated rats while decreasing PRL response in estrogen-treated rats. EGF treatment also

increased the dopaminergic inhibition in estrogen-treated rats and blocked the post-dopamine prolactin rebound seen in vehicle-treated rats (Spuch *et al.* 2006). These reports suggest that EGF effects on lactotrophs are estrogen dependent.

The effects of EGFR ligands can be blocked at the level of the receptor in normal lacto-somatotrophs. Using a mutant EGFR lacking the intracellular tyrosine kinase domain (EGFR-tr), EGFR signaling was blocked in cultured cells. EGFR-tr was expressed in transgenic mice, under control of the PRL or GH promoters respectively. EGFR-tr overexpression in GH-producing cells during embryogenesis resulted in dwarf mice with pituitary hypoplasia with blockade of lactotroph and somatotroph development. Overexpression during the postnatal period did not lead to a distinct phenotype which may implicate an EGFR role in differentiation of lacto-somatotrophs in early pituitary organogenesis but not in postembryotic periods (Roh *et al.* 2001).

In contrast to other pituitary tumors as described above, much work has been done to understand the EGFR pathway in GH- and PRL-secreting adenomas. First, EGFR and its ligands have been shown to be expressed in the majority of studies. By either IHC or RT-PCR techniques, EGF has been detected in 50% of GH-secreting adenomas, 74% of prolactinomas, and 41% of mixed GH/PRL adenomas tested (Chaidarun *et al.* 1994a, Kontogeorgos *et al.* 1996, LeRiche *et al.* 1996, Muller *et al.* 1999, Otsuka *et al.* 1999). Level of invasion does not seem to affect EGF expression levels in bi- and plurihormonal adenomas of patients with acromegaly, though expression levels were higher in non-invasive GH–PRL-mixed cell adenomas compared with non-invasive plurihormonal adenomas (Muller *et al.* 1999). In addition, *TGF α* expression has been confirmed with both IHC and RT-PCR in GH- and PRL-secreting adenomas (Driman *et al.* 1992, Ezzat *et al.* 1995). Finally, over half of GH, PRL, and GH/PRL adenomas express EGFR, as confirmed on multiple techniques including ICC, IHC, binding assays, RT-PCR, and ISH (Kontogeorgos *et al.* 1996, Jaffrain-Rea *et al.* 1998, Onguru *et al.* 2004, Theodoropoulou *et al.* 2004).

The availability of lacto-somatotroph tumor cell lines (GH3 and GH4 cell lines) has made it possible to study the function of EGFR and its ligands in pituitary tumorigenesis. EGF has a number of effects on cell proliferation and morphology in lacto-somatotroph tumor cells. While some studies demonstrate that EGF decreased cell proliferation in GH3/GH4 cells (Schonbrunn *et al.* 1980, Hapgood *et al.* 1983), others found that cell proliferation increased with EGF

treatment (Murdoch *et al.* 1982, Chen *et al.* 2009) and is further enhanced by estrogen (Chen *et al.* 2009). Gefitinib, an EGFR kinase inhibitor, dose-dependently decreased cell proliferation in GH3 cells (Vlotides *et al.* 2008). However, treatment of GH3 cells with another EGFR kinase inhibitor, AG1478, did not block estrogen effects on cell proliferation, suggesting that estrogen receptor function is not dependent on EGFR (Chen *et al.* 2009).

EGF signaling alters lacto-somatotroph morphology, leading to elongation of cells (Johnson *et al.* 1980, Hapgood *et al.* 1983). A monoclonal antibody to EGF-induced synthesis of PRL and morphological changes in GH3 cells (Hapgood *et al.* 1983). Similar to effects of TRH, chronic EGF treatment (>24 h) of GH4 cells decreases cell proliferation by 30–40% with change in cellular morphology from spherical shape to elongated flattened shape and 40–60% increase in cell volume (Schonbrunn *et al.* 1980). These effects may be blocked by a MEK1 inhibitor, implicating the MAPK pathway (Lewis *et al.* 2002).

In addition to affecting cell proliferation and morphology in GH3/4 cells, EGF upregulates PRL gene expression (Murdoch *et al.* 1982, Chen *et al.* 2009) though Pit1 and D2 receptor mRNA are not increased (Zhang *et al.* 1993). EGF regulation of PRL gene transcription may instead be mediated by protein kinase C (Pickett *et al.* 2002). EGF and TRH both induce transcription of the PRL promoter via similar *cis* elements (Berwaer *et al.* 1993) while the distal enhancer sequence of the PRL gene contains elements conferring EGF responsiveness (Day & Maurer 1989). EGF regulates TRH receptors on pituitary GH4C1 cells and TRH responsiveness of these cells (Hinkle *et al.* 1991). Together, these changes lead to increased proportion of lactotrophs in GH3 cultures without affecting the proportion of GH-positive cells (Felix *et al.* 1995).

GH3 cells lack D2 dopamine receptors, but treatment with EGF induces expression of the endogenous D2 receptor gene, leading to a tenfold increase in G_{i3}α subunit (Missale *et al.* 1991). These induced D2 receptors are functional and coupled to delayed outward K⁺ current (Gardette *et al.* 1994). In GH4C1 cells transfected with D2 receptor, EGF treatment leads to decreased coupling of D2, inhibiting cAMP-dependent responses while increasing membrane content of G_{i3} protein (Missale *et al.* 1994). These effects are independent of the Ras pathway (Pickett & Gutierrez-Hartmann 1994).

The effects of EGFR and its ligands on gene expression translate to changes in hormone secretion.

Initially, EGF inhibits GH synthesis in GH3 cells while long-term incubation results in enhanced PRL secretion (Johnson *et al.* 1980, Schonbrunn *et al.* 1980, Hapgood *et al.* 1983). In GH4C1 cells, EGF dose-dependently stimulates acute PRL release with peak secretion achieved after 60 s (Aanestad *et al.* 1993). GH3 cells transfected with a PRL/luciferase reporter gene and treated with EGF had a dose-dependent increase in PRL gene expression that positively correlated with PRL release. When GH3 cells were treated with EGF for 48 h with/without EGFR inhibitor AG1478 or ErbB2 inhibitor AG825, AG1478 blocked EGF action, confirmed by blockade of ERK1/2 that was not seen with AG825. The antiestrogen ICI 182 780 and an ERα-specific antagonist blocked EGF-induced PRL release and gene expression while an ERβ antagonist had no effect as confirmed by siRNA directed against ERα. ERK1/2 was not affected by antiestrogens, indicating that they do not block proximal EGFR signaling events. These results demonstrate that EGF signals through EGFR activation in lactotrophs and that PRL stimulation by EGF is dependent on ERα and involves ERK1/2 phosphorylation (Ben-Jonathan *et al.* 2009).

EGF induces tyrosine phosphorylation of EGFR and ErbB2 in GH3 cells, and these effects can be blocked by gefitinib (Vlotides *et al.* 2008). While EGF enhances baseline and serum-induced PRL mRNA and attenuates GH mRNA expression in GH3 cells, treatment with gefitinib, an EGFR TKI, dose-dependently attenuates serum-induced S phase entry while stimulating GH and inhibiting PRL mRNA expression and decreasing cell proliferation (Vlotides *et al.* 2008). To examine *in vivo* effects of EGFR of lacto-somatotroph tumors, GH3 cells were implanted subcutaneously in female athymic mice. Gefitinib-treated mice had a 50% decrease in tumor volume compared with 26-fold increase in tumor volume in placebo as well as a decrease in PRL levels. Tumor ERK 1/2 phosphorylation was decreased in gefitinib-treated mice, and PRL but not GH gene expression was decreased (Vlotides *et al.* 2008). Together, these results indicate a potential role of EGFR blockade in lacto-somatotroph tumors.

ErbB2 receptor

ErbB2 expression has been more extensively investigated in lacto-somatotroph cells than in other pituitary cell lines. In addition to detection of ErbB2 in normal lacto-somatotrophs (Chaidarun *et al.* 1994a), IHC with antibodies targeting intra- and extracellular domains and RT-PCR confirms ErbB2 expression in 24% of

GH-secreting adenomas, 26% of PRL-secreting, and 32% of GH/PRL-adenomas tested (Chaidarun *et al.* 1994a, Ezzat *et al.* 1997, Nose-Alberti *et al.* 1998, Botelho *et al.* 2006, Vlotides *et al.* 2009). In one study, immunofluorescence staining for ErbB2 was performed in eight human PRL-secreting tumors and positive ErbB2 staining with varying intensities was observed in 7/8 tumor specimens (Vlotides *et al.* 2009). In a patient who initially underwent surgery for invasive but histologically benign prolactinoma (specimen from 1997) which exhibited progressive dopamine agonist resistance and recurred after four consecutive transsphenoidal pituitary surgeries (specimen from 2008, Ki-67 index of 20%), *ErbB2* mRNA expression compared by quantitative PCR analysis showed approximately fivefold increased *ErbB2* mRNA expression levels in the more aggressive

second specimen (Fig. 2; Vlotides *et al.* 2009). A more recent observation in two prolactinomas demonstrated positive nuclear and membranous EGFR staining in one tumor, and in the nucleus in a second tumor (Fig. 3; Fukuoka *et al.* 2011).

When GH3 cells were stably transfected with an expression vector containing a constitutively active form of ErbB2cDNA (HER2CA) which express tenfold higher HER2 protein, higher levels of phosphorylated EGFR, higher EGF-induced levels of EGFR and MAPK, and higher HRG-induced phosphorylated ErbB3 and AKT levels were observed. HER2CA cells exhibited 250-fold induction of *PRL* mRNA and 100-fold PRL secretion with no effects on *GH* mRNA expression and secretion. Wistar–Furth rats implanted with HER2CA transfectants also had larger tumors elevated PRL levels (Fukuoka *et al.* 2011).

Figure 2 ErbB2 and ErbB3 expression in human prolactinomas. (A) ErbB2 and ErbB3 immunostaining in a benign human prolactinoma: fluorescent confocal microscopy images of ErbB2 (left panel) and ErbB3 (right panel) in a human PRL-secreting adenoma. ErbB receptor expression in green (Alexa 488) and nucleic acid staining (TO-PRO-3) in blue. The field size is 375 and 75 μ m for the insert. (B & C) *ErbB2* and *ErbB3* mRNA expression in malignantly transformed prolactinoma: quantitative PCR analysis for *ErbB2* and *ErbB3* mRNA expression in tumor specimens derived from the same patient who initially presented with a benign prolactinoma (1997) which underwent malignant transformation (2008). (B) Internal normalization was performed with two housekeeping genes (GAPDH and TFRC) which were unchanged between the two specimens. (C) Internal normalization was performed with ten housekeeping genes (GUSB, ACTB, GAPDH, TFRC, PGK1, HPRT, PP1A, RPL13A, TBP, and B2M) (reproduced with permission from Vlotides G, Cooper O, Chen YH, Ren SG, Greenman Y & Melmed S 2009 Heregulin regulates prolactinoma gene expression. *Cancer Research* 69 4209–4216 Copyright 2009, American Association for Cancer Research). Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-11-0066>.

Figure 3 Lapatinib attenuates PRL secretion and mRNA expression in human prolactinoma cell cultures. (A, B, D, and E) After transsphenoidal surgery of human prolactinomas, tumor cells were cultured. Prolactinoma cells (Tumor A) were treated with lapatinib (0.1–10 μ M) or gefitinib (10 μ M) for 24 h, and real-time PCR of PRL performed (A). PRL levels in culture media were measured using RIA (B). H&E and PRL staining of tumor and confocal immunocytochemistry of EGFR and HER2 (Tumor A) (C), or for Tumor B (F). Prolactinoma cells (Tumor B) were treated with lapatinib (0.01–10 μ M) or gefitinib (0.01–10 μ M) for 24 h, and real-time PCR performed (D). PRL levels in culture media were measured using RIA (E). Prolactinoma cells (Tumor B) were treated with U0126 (0.1–5 μ M) for 24 h, and real-time PCR of PRL performed (F). Values are mean $\pm$ S.E.M. * P < 0.05, ** P < 0.01 vs control. *** P < 0.001 vs control (Fukuoka H, Cooper O, Mizutani J, Tong Y, Ren SG, Bannykh S & Melmed S 2011 HER2/ErbB2 receptor signaling in rat and human prolactinoma cells: strategy for targeted prolactinoma therapy. *Molecular Endocrinology* **25** 92–103) Copyright 2011, Endocrine Society. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-11-0066>.

ErbB3/4 ligands and receptor

Staining for ErbB3 receptor was reported in 4/8 human PRL-secreting tumors (three adenomas and one carcinoma) with a Ki-67 index of $\geq 4\%$. *ErbB3* mRNA expression compared by quantitative PCR analysis of two tumor specimens derived from a patient with progressive aggressive features showed ~ 41 -fold increased *ErbB3* mRNA expression levels in the more aggressive specimen (Fig. 2), suggesting a role for ErbB3 in malignant transformation of pituitary prolactinomas (Vlotides et al. 2009).

HRG, the ligand for ErbB3 and erbB4, did not stimulate PRL secretion in non-tumorous mixed primary rat pituitary cultures from female Wistar-Furth rats. However, HRG induced rapid ErbB2 and ErbB3 tyrosine phosphorylation in GH4 cells, leading to increasing *PRL* mRNA expression and secretion while failing to induce GH secretion and cell proliferation. This was associated with formation of ErbB2/ErbB3 heterodimers, confirmed by immunoprecipitation. Pretreatment with gefitinib dose-dependently suppressed receptor activation and

signaling as well as prevented HRG-induced ErbB2/ErbB3 heterodimerization. Using siRNA to downregulate ErbB receptor members, there was a 60% reduction of ErbB2 and ErbB3 expression in GH4 cells, associated with decrease of total tyrosine phosphorylation and *PRL* mRNA and secretion in response to HRG, with no effect on GH. Suppression of MAPK1 by siRNA attenuated HRG-induced ERK phosphorylation, *PRL* mRNA, and PRL secretion, indicating that HRG mediates PRL through ERK signaling. These findings demonstrate the specificity and requirement of ErbB2 and ErbB3 for HRG-mediated PRL induction (Vlotides et al. 2009).

Discussion

ErbB receptors and ligands are expressed in normal lacto-somatotroph cells and participate in PRL regulation at both gene and protein levels, leading to changes in prolactin transcription and synthesis. Lacto-somatotroph derived tumors express ErbB receptors and ligands, and manipulation of this system

has multiple effects at the levels of gene, protein, and structure of these tumors.

Therapeutic implications

Knowledge of the role of ErbB family in malignant transformation has led to development of a class of therapeutics designed to interfere with this mechanism, including monoclonal antibodies targeting the extra-cellular domains, and TKIs. Application of these treatments has begun in cellular and animal models of pituitary tumors.

For instance, treatment of GH3 cells with increasing concentrations of gefitinib decreased cell proliferation and *PRL* mRNA expression. When GH3 cells were pretreated with gefitinib before EGF induction, the lactotroph phenotype was reversed and EGF-induced tyrosine phosphorylation of EGFR and EGF induced p185^{cneu} activation blocked along with the ERK downstream signaling pathways. Gefitinib treatment of mice with GH3 induced lacto-somatotroph tumors decreased tumor volume and PRL levels by 50% compared with a 26-fold increase in vehicle-treated mice (Vlotides *et al.* 2008). Pretreatment of GH4C1 cells with gefitinib suppressed HRG-induced ErbB receptor activation and signaling and prevented p185^{cneu} and ErbB3 heterodimerization and PRL secretion (Vlotides *et al.* 2009).

In stable HER2CA GH3 transfectants, lapatinib, a dual EGFR/Her2 kinase inhibitor, suppressed EGF-induced Her2 and MAPK phosphorylation as well as intracellular PRL levels to lower levels than gefitinib while *GH* mRNA were unaffected. Lapatinib-treated cells had 40% lower PRL secretion, cell proliferation, and colony formation. Rats implanted with HER2CA cells and treated with lapatinib exhibited smaller tumor volumes and suppressed PRL levels. In another model, Fischer rats treated with 17- β -estradiol developed prolactinomas and subsequently treated with lapatinib that suppressed tumor weight and PRL levels (Fukuoka *et al.* 2011). Finally, tumor tissue from surgically resected human prolactinomas in primary culture were treated with lapatinib which suppressed *PRL* mRNA $\sim$ 90% and PRL secretion $\sim$ 70% (Fig. 3; Fukuoka *et al.* 2011). These experiments show the efficacy of TKIs in reducing experimental pituitary tumor size and functionality.

Members of the ErbB family play an increasingly recognized role in pituitary development and tumorigenesis. Targeted ErbB therapeutics may prove effective as alternative or adjunctive medical therapy for pituitary tumors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. M I Greene is the inventor of patents dealing with therapy of ErbB tumors, and these are owned by the University of Pennsylvania.

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