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Impaired Fertility in Transgenic Mice Overexpressing Betacellulin

Thesis for the attainment of the title of Doctor in Veterinary Medicine from the Faculty of Veterinary Medicine of the Ludwig-Maximilians University, Munich

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ABBREVIATIONS

ADAM	**A d**isintegrin **a**nd **m**etalloprotease
AREG	Amphiregulin
BMP-15	Bone morphogenetic protein 15
BSA	Bovine serum albumin
BTC	Betacellulin, protein, human
BTC	Betacellulin, gene, human
Btc	Betacellulin, protein, mouse
Btc	Betacellulin, gene, mouse
CCOs	Complexes Cumulus-Oocytes
DAB	diaminobenzidine
DNA	Deoxyribonucleic acid
DN–erbB4	Dominant-negative erbB4 receptor
dNTPs	deoxyribonucleoside triphosphates
E	Estrogen
E_2	Estradiol
EDTA	Ethylene diamine tetraacetic
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPGN	Epigen
ErbB1-4	Receptor tyrosine kinases 1-4
EREG	Epiregulin
Erk	Extracellular signal-regulated protein kinase
ERα	Estrogen receptor
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
HBEGF	Heparin-binding EGF-like
hCG	Human chorionic gonadotropin
HGF	Hepatocyte Growth Factor
hTGFA	Human transforming growth factor-α
icv	Intracerebroventricular

IGF-I and II	Insulin-like growth factors I and II
ILs	Interleukins
IU	International unit
LH	Luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
ml	Milliliter
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NDFs	Heregulins/neu-differentiating factors
NGF	Nerve growth factor
NRG	Neuregulin
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PGR	Progesterone receptor
PGs	Prostaglandins
PMSG	Pregnant mare serum gonadotropin
PI3K/Akt	Phosphatidylinositol 3-kinase
pro-BTC	BTC precursor
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse Polymerase chain reaction
TAE	Tris-acetate-EDTA
TGFA	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
µl	microliter

1. INTRODUCTION AND OBJECTIVES

Cells exchange growth and differentiation signals through a repertoire of several diffusible polypeptides known generically as growth factors. Peptide growth factors are omnipresent molecules that coordinate every conceivable aspect of mammalian development, growth, physiology, and pathology. Peptide growth factors regulate critical cellular functions including stimulatory or inhibitory effects but also immune response or tumor growth. These molecules can regulate the cell function by autocrine, paracrine, juxtacrine or endocrine mechanisms (Sporn & Roberts 1988).

Many peptide growth factors have been isolated and characterized, including: Nerve Growth Factor (NGF), Insulin-Like Growth Factors (IGF-I and II), Platelet-Derived Growth Factors (PDGFs), Fibroblast Growth Factor (FGF), Interleukins (ILs), Hepatocyte Growth Factor (HGF), Transforming Growth Factor-β (TGF-β) and Epidermal Growth Factor (EGF) (James & Bradshaw 1984).

The epidermal growth factor (EGF)-like peptides are emerging as major players in regulating different aspects of animal and human physiology and pathology. The EGF family includes Epidermal Growth Factor (EGF) itself, Transforming Growth Factor–α (TGFA), Heparin-binding Epidermal Growth Factor (HBEGF), Amphiregulin (AREG), Betacellulin (BTC), Epiregulin, (EREG) and Epigen (EPGN) (COHEN 1962; Derynck et al. 1984; Higashiyama et al. 1991; Shoyab et al. 1988; Shing et al. 1993; Toyoda et al. 1995; Strachan et al. 2001). The members of the EGF family bind and activate extracellular domains of a family of receptor called transmembrane receptor tyrosine kinase (RTKs) (Ullrich et al. 1984; Yamamoto et al. 1986; Kraus et al. 1989; Plowman et al. 1993; Massague & Pandiella 1993; van der et al. 1994). RTKs play a fundamental role in the regulation and differentiation of cell growth, and one of the most researched RTK's family comprises the four receptors called ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 (Schlessinger & Ullrich 1992; Yarden & Sliwkowski 2001; Holbro & Hynes 2004).

The EGFR and its ligands elicit essential actions in reproduction. For instance, different Egfr ligands have been shown to be involved in oocyte maturation and ovulation (Park et al. 2004; Ashkenazi et al. 2005; Shimada et al. 2006; Yoshino et al. 2006), preimplantational embryonic development (Hardy & Spanos 2002), and implantation (Das et al. 1995; Raab et al. 1996; Das et al. 1997a; Das et al. 1997b; Das et al. 1997c; Paria et al. 1999).

Btc, a rather poorly characterized Egfr ligand, was initially isolated from the conditioned medium of a mouse pancreatic β-cell carcinoma cell line (Shing et al. 1993). Although, in many aspects, Btc reproduces the actions of other Egfr ligands, it has some unique structural and functional properties (reviewed in (Dunbar & Goddard 2000). Concerning reproduction, Btc was identified as one of the Egfr ligands expressed in the mouse uterus exclusively at the sites of blastocyst apposition at the time of attachment reaction (day 4) and through the early phase of implantation (day 5) (Das et al. 1997a). In addition, it was identified as a mediator of luteinizing hormone (LH) (Park et al. 2004; Ashkenazi et al. 2005; Hernandez-Gonzalez et al. 2006) and prostaglandins (PGs) and progesterone receptor PGR) (Shimada et al. 2006) actions in the ovulatory follicle. Finally, Btc was recently identified as a possible ovarian mediator of bone morphogenetic protein 15 (BMP-15) actions, an oocyte-specific growth factor that plays a major role in determining ovulation quota in mammals (Yoshino et al. 2006).

Although mice lacking Btc expression show no overt phenotype and appear to reproduce normally (Jackson et al. 2003), transgenic mice overexpressing the growth factor show a whole array of phenotypical alterations (Schneider et al. 2005). During the routine breeding of these mice in our animal facility we observed that Btc transgenic female and male mice, as compared with wild-type littermates, appeared to produce fewer pups per litter and had often non-productive matings. This observation led us to investigate the different stages of the reproductive process.

In the present investigation, we systematically studied different aspects of Btc transgenic female's and male's reproduction, including puberty initiation, ovulation, *in vivo* and *in vitro* oocyte maturation, sperm parameters, ovulation, *in vivo* and *in vitro* fertilization, and implantation in order to uncover the reason for their reduced fertility.

2. REVIEW OF THE LITERATURE

2.1 Growth Factors

Peptides with potent stimulatory or strong inhibitory effects on cell proliferation have been historically termed growth factors. Moreover, peptide growth factors regulate many other critical cellular functions that have little to do with growth itself, such as signal transduction, cell survival, differentiation, cell adhesion, cell migration, immune response, hematopoiesis, inflammation, tissue repair, atherosclerosis and cancer. Peptide growth factors provide an essential way for a cell to communicate with its immediate environment and to ensure that there is a proper local homeostatic balance between the numerous cells that comprise a tissue. The mechanism of local cellular regulation by classical endocrine molecules involves their interface with autocrine and paracrine mechanisms of action of peptide growth factors (Sporn & Roberts 1988).

Many peptide growth factor have been isolated and characterized, including: Nerve Growth Factor (NGF), Insulin-Like Growth Factors (IGF-I and II), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Interleukins (ILs), Hepatocyte Growth Factor (HGF), Transforming Growth Factor-β (TGF-β) and Epidermal Growth Factor (EGF) (James & Bradshaw 1984).

Many growth factors derive from soluble precursors that mature through proteolytic cleavage within the cell. Another class of growth factors is called membrane-anchored growth factors and the biological effects of these growth factors are exerted on the cell surface by interacting with the extracellular domains of transmembrane receptor tyrosine kinase (RTKs) (Massague & Pandiella 1993; van der et al. 1994). RTKs play a fundamental role in the regulation of cell growth and differentiation. Ligand binding induces dimerization, activation of the intracellular kinase domain, and autophosphorylation by an intermolecular mechanism (COHEN 1962; Derynck et al. 1984; Holmes et al. 1992; Shing et al. 1993; Shoyab et al. 1988; Toyoda et al. 1995; Schlessinger & Ullrich 1992).

Peptide growth factor effects also depend on the stage of development or differentiation of its target cells. For example, the growth of fibroblasts from very early human embryos is stimulated by TGF-β, while the growth of fibroblasts from older embryos is inhibited by this peptide (Hill et al. 1986). Furthermore, it is clear that the

same growth factors that play a key role in the malignant process in cancer cells are expressed physiologically by cells that mediate inflammation and repair, namely platelets, macrophages, and lymphocytes (Sporn & Roberts 1986).

2.1.1 The Epidermal Growth Factor Receptor and its Ligands

In 1962, Cohen reported the isolation of a polypeptide from the submaxillary gland of male mice that accelerated eyelid opening and incisor eruption in the newborn animal. This polypeptide, EGF, was subsequently isolated from extracts of this tissue as a high molecular weight form. Its effects on the cell include mitogenesis, nutrient transport, glycolysis and morphological changes (COHEN 1962; Taylor et al. 1970).

EGF turned out to be the founding member of a family of seven Epidermal Growth Factor Receptor (EGFR) ligands (Harris et al. 2003). The common structural domain shared by these family members is a 40–60 amino acid domain characterized by six cysteine residues forming three disulphide bonds (Savage, Jr. et al. 1973). EGF family members are transmembrane proteins that undergo proteolytic cleavage to release their mature form into extracellular space. Major sheddases are members of the ADAM (**a** **d**isintegrin **a**nd **m**etalloprotease) family (Sahin et al. 2004).

The epidermal growth factor family includes Epidermal Growth Factor (EGF), Transforming Growth Factor–α (TGFA), Heparin-binding Epidermal Growth Factor (HBEGF), Amphiregulin (AREG), Betacellulin (BTC), Epiregulin, (EREG) and Epigen (EPGN) (COHEN 1962; Derynck et al. 1984; Holmes et al. 1992; Shing et al. 1993; Shoyab et al. 1988; Toyoda et al. 1995).

In addition to the EGFR, these growth factors also bind and activate the related tyrosine kinase receptors ErbB2 (neu), ErbB3 and ErbB4 (Ullrich et al. 1984; Yamamoto et al. 1986; Kraus et al. 1989; Plowman et al. 1993), (Figure 2.1). As typical RTKs, the ErbBs consist of an extracellular domain where ligand binding takes place, a short transmembrane domain and a cytoplasmic region containing the catalytic protein tyrosine kinase (Schlessinger 2000). Ligand binding and subsequent dimerization stimulates the receptor enzymatic activity, resulting in phosphorylation of cytoplasmatic domains. These phosphorylated residues serve as docking sites for a variety of signaling molecules whose recruitment initiate a cascade of intracellular

signaling. Subsequently, the ligand-receptor complex is internalized and degraded within lysosomes (Yarden & Sliwkowski 2001).

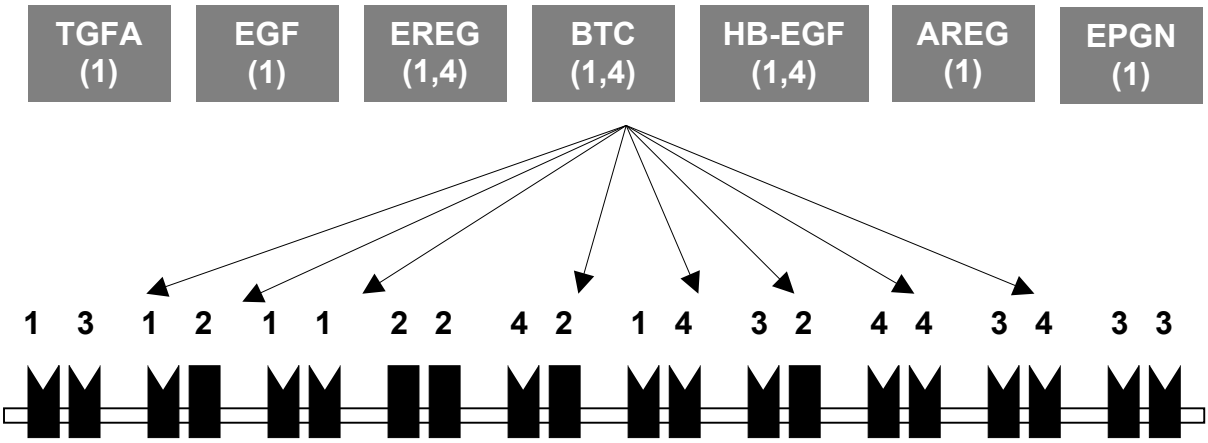

Figure 2.1 EGFR ligands and the ErbB network. Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors. For simplicity, specificities of receptor binding are shown only for BTC that possess the apparently unique property of activating the ErbB2/3 combination. ErbB2 binds no ligand with high affinity, and ErbB3 homodimers are catalytically inactive.

2.1.2 Betacellulin

Btc was initially described, purified and cloned from a mouse insulinoma cell line (Shing et al. 1993). In the same year, the human BTC cDNA was identified and characterized (Sasada et al. 1993). Subsequently it was characterized in the bovine species and in the rat (Dunbar et al. 1999; Tada et al. 2000).

The human BTC precursor (pro-BTC, Figure 2.2) encodes a 178 amino acid primary translation product and consists of:

- aa $^{13-26}$, presumptive signal peptide;
- aa $^{27-31}$, short propeptide;
- aa $^{32-111}$, mature BTC containing the EFG motif;
- aa $^{112-124}$, short juxtamembrane domain;
- aa $^{125-138}$, hydrophobic transmembrane domain;
- aa $^{139-178}$, Cytoplasmic tail domain.

The single copy gene for mouse *Btc* maps to chromosome 5 and for human *BTC* to chromosome 4q13-q21 (Harris et al. 2003; Dunbar & Goddard 2000; Pathak et al. 1995). The structure of genes encoding *Btc* and others members of EGF family (*Tgfa, Areg, and Hbegf*) are highly conserved:

- exon 1, encodes the 5´UTR and signal peptide;
- exon 2, encodes the N-terminal precursor;
- exon 3, comprises the mature EGF, including the first two disulfide loops of the EGF motif;
- exon 4, comprises the third loop of the EGF-like motif and the transmembrane domain;
- exon 5, comprises the cytoplasmic region;
- exon 6, encodes the 3´UTR.

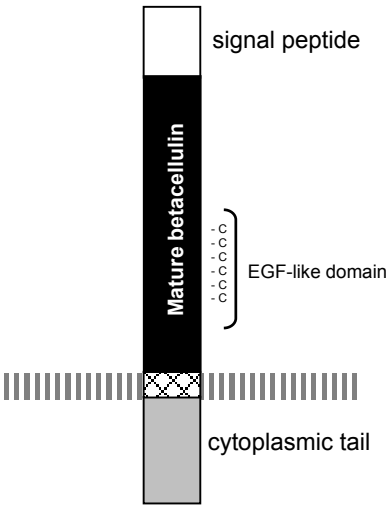

Figure 2.2 The domain structure of the pro-Btc.

The mature soluble form of mouse Btc consists of 80 amino acid residues and its molecular mass is 32 kDa. Btc is synthesized as a transmembrane precursor and its ectodomain is located in the extracellular domain. Due to this location, it is subject to proteolytic cleavage to produce a soluble mature growth factor (Figure 2.3). Reports suggest the role of metalloproteinases, MAP kinases and disintegrin in the processing of the ectodomain (Peschon et al. 1998; Izumi et al. 1998; Fan & Derynck 1999; Gechtman et al. 1999). Members of the ADAM family of enzymes have been identified as the main EGFR ligand sheddases (Sahin et al. 2004); (Tousseyn et al.

2006). Especially ADAM10 and ADAM17 are involved in the shedding of Egfr ligands. ADAM17 emerged as a constitutive sheddase of Tgfa, Areg, Hbegf, and Ereg, which is consistent with the essential role for ADAM17 in activation of the EGFR during development (Holbro & Hynes 2004). ADAM10 was found to be the major sheddase for Btc and Egf (Sahin et al. 2004).

Interestingly, BTC is a pan-ErbB ligand, capable of binding to ErbB1 and ErbB4 homodimers and all heterodimeric combinations including ErbB2/3 (Yarden & Sliwkowski 2001).

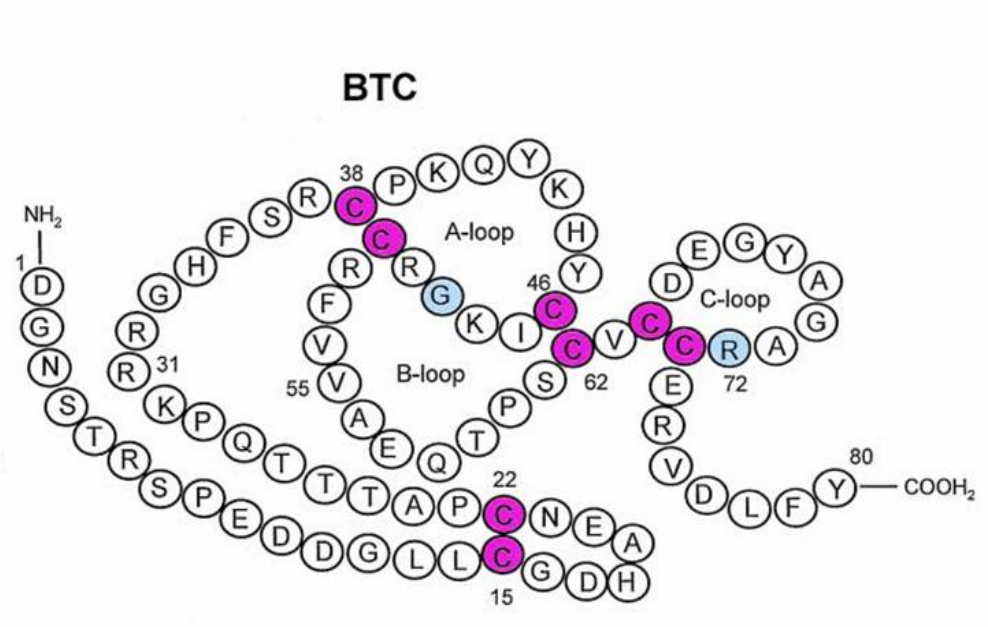

Figure 2.3 Schematic illustration of the structure of mature Btc (Dunbar & Goddard 2000).

Btc is expressed in a wide range of tissues in the mouse, with particularly high levels in the liver, kidney, pancreas, small intestine and uterus (Shing et al. 1993; Seno et al. 1996; Kojima et al. 2003). Btc has also been shown to be expressed in heart, lung, colon, testis and ovary (Seno et al. 1996). Btc expression in the gastric surface, jejunum and colon suggests an important role also in the gastrointestinal tract (Kallincos et al. 2000).

Btc is believed to play a major role in the physiology of the endocrine pancreas. For instance, Btc could convert pancreatic tumor cells into insulin-secreting cells and is required for the induction of insulin and glucokinase gene expression in glucagonoma cells (Mashima et al. 1996; Watada et al. 1996; Li et al. 2005).

Furthermore, Btc has been localized in primitive duct cells in fetal pancreas and some islet cell populations demonstrating a close association with insulin production (Tada et al. 1999). Evidence linking Btc and pancreas development was revealed by the administration of recombinant Btc resulting in improved glucose tolerance in mice with diabetes (Yamamoto et al. 2000). These findings can be associated with β-cell differentiation and regeneration from ductal or acinal cells.

Reflecting its wide expression *in vivo*, BTC expression has been detected in a variety of cultured cell lines. In many cases the addition of BTC causes altered cell proliferation, differentiation, survival and migration. Table 2.1 provides an overview of the reports describing these actions.

Btc was identified as one of the Egfr ligands expressed in the mouse uterus exclusively at the time of implantation (Das et al. 1997a). In addition, it was identified as a mediator of luteinizing hormone (LH) (Park et al. 2004; Ashkenazi et al. 2005; Hernandez-Gonzalez et al. 2006) prostaglandins (PGs) and progesterone receptor (PGR) (Shimada et al. 2006) actions in the ovulatory follicle and as a possible oocyte-specific growth factor (Yoshino et al. 2006).

Mice lacking *Btc* are viable, fertile and show no overt phenotype (Jackson et al. 2003). This is believed to be a consequence of functional redundancy within the family of Egfr ligands.

Transgenic mice overexpressing Btc ubiquitously exhibit a whole range of phenotypical alterations including high early postnatal mortality, impaired growth and reduced adult body weight, bone alterations, cataract and retinal problems, and pulmonary pathology (Schneider et al. 2005).

Table 2.1 Expression and effects of BTC on different cells culture lines.

in vitro *Culture*	**BTC expression and effects**	**Reference**
Retinal pigment epithelial, canine kidney epithelial, and vascular smooth muscle cells	Stimulation of the cell proliferation	Shing *et al.* 1993
BALB/c 3T3 fibroblasts	Stimulation of the cell proliferation	Watanabe *et al*. 1994*
Keratinocytes	Transcriptionally up-regulated BTC, indicating a further role in the development of the neoplastic phenotype.	Dlugosz *et al*. 1995
Pancreatic AR42J cells	Conversion of the amylase-secreting cells to insulin-secreting cells	Mashima *et al*. 1996*
Breast tumor cell lines	Tyrosine phosphorylation of erB1 and erbB4; Growth stimulation of the mammary epithelial cells, 3 fold.	Beerli &Hynes 1996
PDX(+)αTC1.6	Induction of insulin gene expression	Watada *et al*. 1996
Pharyngeal carcinoma cells	Up-regulation of MMP-9 and increased tumor cell invasion.	O-Charoenrat *et al*. 2000a
Pharyngeal carcinoma cells	Expression of mRNAs and protein production	O-Charoenrat *et al*. 2000b
Pharyngeal carcinoma cells	Up-regulation of VEGF-A and VEGF-C transcript levels; Down-regulation of VEGF-D m RNA.	O-Charoenrat *et al*. 2000c
Human aortic vascular smooth muscle cells	Mitogenic activity	Tamura *et al*. 2001*
Sertoli cells	Increase DNA synthesis by the Sertoli cells.	Petersen *et al*. 2001
Middle ear epithelium	Hyperplasia of the middle ear mucosal epithelium	Palacios *et al*. 2001
Keratinocytes	Involvement in epidermal morphogenesis and/or in maintenance of the differentiated phenotype of psoriatic epidermis.	Piepkorn *et al*. 2003
Seminiferous tubule	Dose-dependent stimulatory effects on DNA synthesis.	Wahab-Wahlgren *et al*. 2003
Pharyngeal carcinoma cells	Induction of MMP-9 production and invasion primarily through activation of EGFR, MAPK and PI3K/Akt.	O-Charoenrat *et al*. 2004
Cumulus-oocyte complexes	Cumulus expansion and maturation	Park *et al*. 2004
Cumulus-oocyte complexes	Partial stimulation of the resumption of meiosis, activation of genes involved in cumulus expansion, and follicle rupture *in vitro*	Ashkenazi *et al*. 2005
Pancreatic ducts cells	Proliferation (spreading and monolayer formation) and phosphorylation of MEK1/2 and ERK1/2.	Rescan *et al*. 2005
Pancreatic β-cells	Differentiation and conversion of the β-cells to insulin-producing cells	Ogata *et al*. 2005**
Transdifferentiated β-cells	Increase insulin mRNA expression	Li *et al*. 2005

*Recombinant human BTC, **BTC-δ4: splice isoform of BTC lacking a transmembrane domain

2.2 Female Reproduction

EGFR ligands have been shown to play a significant role in female reproductive functions in humans and animals, such as puberty onset, ovulation and implantation processes (Leach et al. 1999; Chobotova et al. 2002; Dey et al. 2004); (Hourvitz et al. 2006). Interestingly, they have also been implied in the development of cervical and endometrial cancer (Pfeiffer et al. 1997; Srinivasan et al. 1999).

Female sexual maturation requires the coordinated and timely activation of luteinizing hormone-releasing hormone (LHRH) neurons. LHRH binds to specific receptors to stimulate the secretion of the gonadotropins LH and FSH and these hormones promote gonadal development and support reproductive physiology. One of these pathways uses the EGFR ligands and receptors. The functional integrity of both Egfr and erbB-4 signaling is critical for the time of puberty, while the suppression of Egfr activity during sexual differentiation in mice changes the time of puberty initiation (Apostolakis et al. 2000; Ojeda et al. 2003; Prevot et al. 2005).

Oocyte maturation and ovulation require several sequential events involving a timed secretion of hormones that can induce expression of EGF-like growth factors. Areg, Ereg, and Btc can also recapitulate the oocyte maturation *in vitro* (Richards 1994; Richards et al. 1995; Richards et al. 2002; Park et al. 2004).

In humans, due to the interest in infertility studies, the expression of growth factors in human endometrium has been extensively investigated. EGF, TGFA, AREG, BTC and the ErbBs were found to be expressed with a variation during the menstrual cycle, with maximal levels in the late secretory phase, when the endometrium becomes receptive, demonstrating an association with the implantation process (Imai et al. 1995; Srinivasan et al. 1999; Ejskjaer et al. 2005). In mice, multiple EGFR ligands are expressed around the time of blastocyst implantation, revealing a mechanism to assure implantation (Carson et al. 2000; Das et al. 1994b; Das et al. 1995; Das et al. 1997a; Lim et al. 1998).

The Table 2.2 gives an overview of the expression and actions of EGFR ligands in the female reproductive organs.

Table 2.2 Expression and actions of the EGFR-ligands in the female reproductive tract.

EGFR-ligand	Expression	Effects	References
EGF	Hypothalamus	Stimulation of the release of LHRH; Onset of puberty	(Plata-Salaman 1991; Kaser et al. 1992; Apostolakis et al. 2000)
	Endometrial epithelium	Growth differentiation	(Nelson et al. 1991)
	Trophoblast	Implantation	(Hofmann et al. 1992)
	Endometrial epithelium	Endometrial proliferation, regulated by E_2	(Huet-Hudson et al. 1990; Imai et al. 1995)
	Endometrium	Regulation of angiogenesis	(Moller et al. 2001)
HBEGF	Luminal Epithelium	Uterine receptivity; Implantation (*)	(Das et al. 1994b; Birdsall et al. 1996; Raab et al. 1996; Paria et al. 1999; Paria et al. 2001; Lessey et al. 2002; Hamatani et al. 2004; Klonisch et al. 2001)
	Stroma und glandular epithelium cells	Proliferation of stromal cells; Decidualization	(Chobotova et al. 2002; Chobotova et al. 2005)
	Deciduum and extravillous trophoblast	Implantation and trophoblast invasion	(Leach et al. 1999)
	Ovary	Mitogen for granulosa cells; maturation of the follicles	(Pan et al. 2004)
TGFA	Hypothalamus	Onset of puberty	(Ma et al. 1994)
	Luminal epithelium and peripheral stromal cells	Implantation	(Das et al. 1997b)
	Luminal, glandular and stromal cells	embryonic development, preparation of the uterus for implantation, and decidualization	(Tamada et al. 1991)
	Epithelial lining in human endometrium	Growth of endometrium	(Ejskjaer et al. 2005)
	Endometrial epithelium	Endometrial proliferation, regulated by E_2	(Imai et al. 1995)
AREG	Preovulatory follicles	Meiosis and Cumulus expansion	(Park et al. 2004)
	Luminal epithelium	Uterine receptivity, regulated by P4	(Das et al. 1995; Das et al. 1997b)
	Leucocytes and stromal cells in human endometrium	Growth of endometrium	(Ejskjaer et al. 2005)

BTC	Preovulatory follicles	Meiosis and Cumulus expansion	(Park et al. 2004)
	Luminal epithelium and underlying stroma	Implantation (*)	(Das et al. 1997a; Paria et al. 2000)
	Placenta	Placental growth	(Maruo et al. 1995; Birdsall et al. 1996; Watanabe et al. 1994)
	Epithelial lining in human endometrium	Growth of endometrium	(Ejskjaer et al. 2005)
	Ovary	Involvement in ovulatory process	(Hourvitz et al. 2006; Yoshino et al. 2006; Woods & Johnson 2006)
EREG	Preovulatory follicles	Meiosis and cumulus expansion	(Park et al. 2004)
	Luminal epithelium and Stroma	Implantation (*)	(Das et al. 1997a)
	Stromal cells in human endometrium	Growth of endometrium	(Ejskjaer et al. 2005)
	Placenta	Maintenance/development of normal cell growth	(Toyoda et al. 1997)
	Ovary	Involvement in ovulatory process	(Hourvitz et al. 2006)

(*) proposed

2.2.1 Sexual maturity

The initiation of mammalian puberty requires the activation of hypothalamic neurons secreting the neuropeptide LHRH. LHRH stimulates the secretion of pituitary gonadotropins. Astrocytes affect LHRH neuronal function via cell-cell signaling mechanisms involving several growth factors acting via receptors with tyrosine kinase activity (Ojeda et al. 2000). Egf and Tgfa and their receptors are key players in the glial-neuronal interactive process that regulates LHRH secretion. Tgfa and Neuregulin (NRG) are produced in hypothalamic astrocytes and stimulate LHRH release indirectly via activation of their respective receptors, located on astrocytes. Activation of Egfr by Tgfa, and/or the erbB2/erbB4 receptor complex by Nrg, leads to E_2, which then acts directly on LHRH neurons to stimulate LHRH release. A central blockade of *Tgfa* or *Nrg* action delays puberty, and focal overexpression of Tgfa advances it (Ojeda & Ma 1998).

The increase in pulsatile release of gonadotropin releasing hormone (GnRH) is very important in the puberty. Glial cells facilitate GnRH secretion via cell-cell

signaling loops mainly initiated by members of the EGF family. The control of these mechanism may involve the transcriptional regulation of subordinate genes that, by contributing to neuroendocrine maturation, are required for the initiation of the pubertal process (Ojeda et al. 2003).

Transgenic mice expressing a dominant-negative erbB4 receptor (DN–erbB4) were created to evaluate the function of the astrocytic ErbB receptors in the timely advent of puberty. The transgenic mice exhibit delayed sexual maturation and a diminished reproductive capacity in early adulthood. These abnormalities are related to a deficiency in pituitary gonadotropin hormone secretion, caused by impaired release of LHRH. Mice carrying both the *Wa-2* mutation (a defective Egfr) and a DN-erbB-4 mutant receptor exhibit a more pronounced delay in the onset of puberty and a dramatically impaired adult reproductive function in comparison to single-mutant mice and wild-type animals. These defects appear to be caused by loss of ErbB receptor-mediated astrocyte-to-neuron communication (Prevot et al. 2003; Prevot et al. 2005).

Tgfa is widely distributed in the nervous system, both glial and neuronal cells contributing to its synthesis, and have a neuronal participation in the control of female puberty. Tgfa contributes to the acceleration of puberty induced by anterior hypothalamic lesions. They also indicate that activation of *Tgfa* gene expression in glial cells is a component of the hypothalamic response to injury (Junier et al. 1991; Junier 2000).

Increased circulating levels of growth factors precede intracerebral aromatization of androgens to estrogen (E) during sexual differentiation and development (MacLusky & Naftolin 1981) and changes in E at the time of initiation of puberty (Ma et al. 1992). Indeed, the initiation of the puberty in females is dependent, in part, on activation of hypothalamic membrane-bound growth factor receptors (Ma et al. 1994). The absence of Egfr activity in mice delays the initiation of puberty and estrus cyclicity and the synchronization of mating behavior and estrous is disrupted. These findings demonstrate a greater role for growth factors in the adult brain. Rats and mice in the absence of E exhibit reproductive behavior 1 h after intracerebroventricular (icv) injection of Egf, Areg and Tgfa, demonstrating that growth factors can signal through the classical estrogen receptor (ERα) to alter *in vivo* function in rodent reproduction (Apostolakis et al. 2000).

In summary, while a central role for Egfr, ErbB4, Tgfa and Egf in the onset of female puberty and sexual maturity has been clearly demonstrated, no information is available about the role of Btc in this process.

2.2.2. Oocyte maturation and ovulation

Ovulation, initiated by the proestrus LH surge consists of several sequential follicular events involving enlargement of the antrum, expansion of the follicle and ovarian wall at the apex of the mature follicles and release of the fertilizable oocyte. This process involves a highly synchronized and exquisitely timed cascade of gene expression (Richards 1994, Richards et al. 1995, Richards et al. 2002). The LH analog hCG induces the transient and sequential expression of the EGF family members Areg, Ereg and Btc. An ovulatory dose of LH was shown to induce a rapid and transient expression of Btc mRNAs 1-3 h after the injection (Ashkenazi et al. 2005). The presence of EGF-like activity in follicular fluid (Hsu et al. 1987) supports this concept. Incubation of follicles with Areg, Ereg, and Btc recapitulates the morphological and biochemical events triggered by LH, including cumulus expansion and oocyte maturation (Park et al. 2004). Thus, these EGF-related growth factors are paracrine mediators that propagate the LH signal throughout the follicle.

A recently proposed model suggests that EGF family ligands, produced in a paracrine (Tgfa) or autocrine (Btc) fashion, bind with ErbB1 and/or ErbB4 receptors to activate Erk signaling that promotes enhanced expression of FSHR to initiate the process of granulosa cell differentiation subsequent to preovulatory follicle selection (Woods & Johnson 2006).

Btc and Ereg were shown to be expressed in an ovulation-dependent manner after an analysis of mouse preovulatory and postovulatory ovarian cDNA. (Hourvitz et al. 2006). In this interesting study, 43 of 98 cDNA clones showed a true ovulation-selective/specific expression pattern. Btc was also recently identified as a possible ovarian mediator of bone morphogenetic protein 15 (BMP-15) actions, an oocyte-specific growth factor that plays a major role in determining ovulation quota in mammals (Yoshino et al. 2006).

2.2.3 Implantation

In the mouse, the process of implantation can be classified into three stages: apposition, adhesion, and penetration. Apposition is the stage when embryonic trophectoderm cells become closely apposed to the uterine luminal epithelium. This is followed by the adhesion stage in which the association of the trophectoderm and the luminal epithelium is sufficiently intimate as to resist dislocation of the blastocyst by flushing the uterine lumen. The stage of penetration involves the invasion of the luminal epithelium by the trophectoderm. Stromal cell differentiation into decidual cells (decidualization) is more extensive, and the loss of the luminal epithelium is evident at this stage. These three stages of implantation form a continuum (Enders & Schlafke 1969; Schlafke & Enders 1975; Enders & Lopata 1999).

The attachment reaction coincides with a localized increase in stromal vascular permeability at the site of the blastocyst. This can be demonstrated by intravenous injection of a macromolecular blue dye resulting in discrete blue bands along the uterus (uterine blue reaction). Molecular signals coordinate the uterus receptivity and the interactions between the embryo and the uterus to initiate the process of implantation (Psychoyos 1973; Paria et al. 1993b; Lee & DeMayo 2004).

In mammals, the uterus differentiates into an altered state when blastocysts are capable of effective two-way communication to initiate the process of implantation. This state is termed uterine receptivity for implantation and lasts for a limited period, called window of implantation. At this stage, the uterine environment is able to support blastocyst growth, attachment, and the subsequent events of implantation (Paria et al. 1993b). The establishment of the receptive uterus to support embryo development and implantation is primarily coordinated by ovarian hormones, which modulate uterine events in a spatiotemporal manner. Estrogen and progesterone prime the uterus for implantation (Cross et al. 1994). In the mouse the attachment reaction occurs in the evening (22:00-24:00) of day 4 of pregnancy (Das et al. 1994b).

The expression of several growth factors, cytokines, and their receptors in the uterus in a temporal and cell-specific manner during the peri-implantation period suggests that these factors are important for implantation and that some of the effects of ovarian steroids in the implantation process are mediated by paracrine/juxtacrine effects of these growth factors (Paria et al. 1993b; Das et al.

1994b; Das et al. 1995; Carson et al. 2000; Paria et al. 2002; Lim et al. 2002; Norwitz et al. 2001).

The blastocyst and uterus generate various factors during implantation, but it is likely that the molecular "cross-talk" between them involves many more yet unknown factors. Indeed, it is more realistic to view the process of implantation as a condition of equilibrium in the up-regulation and down-regulation of a diverse set of genes. Identification of other essential regulatory steps is necessary to further understand the biologic basis for the establishment of pregnancy or the underlying causes of pregnancy failures. Many genes that are known to be associated with the implantation process fall into categories similar to the genes detected with increased expression at the implantation site, including growth factors/cytokines and their receptors, transcription factors, genes encoding structural proteins, or genes associated with cell proliferation. Genes with increased expression at the interimplantation site may act to guide the blastocyst to specific sites for implantation or be important for embryo spacing. 81 genes were reported with differential expression at the implantation site during both natural and induced implantation, suggesting their importance for implantation and some were members of the EGF family of growth factors that becomes intensely localized to the uterine luminal epithelium surrounding the blastocyst at the onset of implantation (Reese et al. 2001).

2.2.3.1 Role of EGFR family members in implantation

The expression of epidermal growth factor EGF-related ligands in the mouse uterus (Huet-Hudson et al. 1990; Tamada et al. 1991; Das et al. 1994b; Das et al. 1997a; Lim et al. 1998) and the Egfr and Erb-4 in the embryo (Paria & Dey 1990; Paria et al. 1993a; Wiley et al. 1992; Paria et al. 1999) suggests roles for these growth factors in embryo-uterine interactions during implantation. The expression of multiple receptors and ligands of the EGF family might be a protective mechanism to ensure the embryo development and implantation (Paria et al. 2000).

Hbegf gene is expressed in the mouse uterine luminal epithelium surrounding the blastocyst 6-7 hours before the attachment reaction that occurs at 22:00-23:00 hours on day 4 of pregnancy. *In vitro* studies showed that Hbegf induced blastocyst Egfr autophosphorylation, and promoted blastocyst growth, zona-hatching and trophoblast outgrowth. These results suggest possible interactions between the

uterine Hbegf and blastocyst Egfr very early in the process of implantation, earlier than any other embryo-uterine interactions defined to date at the molecular level (Das et al. 1994b).

Areg mRNA levels display a transient surge throughout the uterine epithelium on day 4 of pregnancy. With the onset of blastocyst attachment late on day 4, Areg mRNA accumulated in the luminal epithelium exclusively at the sites of blastocysts (Das et al. 1995).

The cell-specific "window" of expression of Btc and Ereg in the peri-implantation mouse uterus was also examined and it was demonstrated that both growth factors are expressed in the uterus exclusively at the sites of blastocyst apposition at the time of attachment reaction and during the initial phase of implantation. The distribution of Btc mRNAs in the peri-implantation uterus was examined by *in situ* hybridization on days 1-8 of pregnancy. Btc is present in the luminal epithelium and underlying the stroma at the site of blastocyst apposition at 23:00 on day 4 and on the morning of day 5 distinct autoradiographic signals was observed in an increased number of luminal epithelial and stromal cells. The expression of the *Ereg* gene was mostly similar to that of *Btc*, and both Btc and Ereg expression was not detected in implanting blastocysts (Das et al. 1997a).

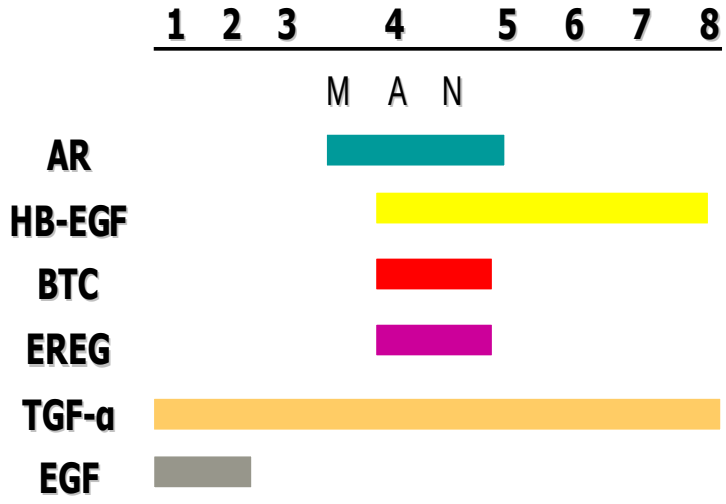

Figure 2.4 A schematic diagram showing the expression of EGFR ligands in the periimplantation mouse uterus (days 1-8 pregnancy; M = morning; A = afternoon; N= night). Adapted from Das *et al*. (*Dev. Biol.* **190**, 178-190, 1997).

Interestingly, the inappropriate expression of a transgenic human TGFA in the uterus delays the initiation of the implantation process, which was associated with

downregulation of the TGF-β receptor subtypes and deferral of uterine AREG expression (Das et al. 1997b).

The implantation depends not only on maternal events, but also on the blastocyst itself. Transgenic mice with null allele *Egfr* did not cause a blockage in the blastocyst formation, although the inner cell mass were degenerated in the peri-implantation period causing an embryonic death (Threadgill et al. 1995). This effect however, depends on the genetic background of the mouse strain.

The Egfr (erbB1) has been examined in several aspects during the peri-implantation period. ErbB1 was detected in the stroma, deciduum, and myometrium, but not in the luminal or glandular epithelium. These observations are supportive of the concept of paracrine or juxtacrine interactions between EGF-related growth factor ligands of luminal epithelial origin and blastocyst Egfr in the process of implantation (Das et al. 1994a).

A model has been established that shows cooperative interaction among preimplantation embryos and the role of growth factors on their development and growth. The embryos cultured with the addition of Egf or Tgfa have their development improved. Detection of Egf receptors on the embryonic cell surface at eight-cell/morula and blastocyst stages suggests beneficial effects of Egf on preimplantation embryo development and blastocyst functions. In the blastocyst, the binding was limited to trophectoderm. This study presents clear evidence that specific growth factors of embryonic and/or reproductive tract origin participate in preimplantation embryo development and blastocyst functions in an autocrine/paracrine manner. The mechanisms by which mitogenic and differentiating effects of EGF on preimplantation embryos are mediated are still unclear (Paria & Dey 1990).

The spatiotemporal uteroplacental expression pattern of the four ErbB receptors and their ligands was studied in the rabbit. The results provide evidence for the functional involvement of the Egf ligand-receptor system in embryo/feto-maternal cross-talk during the peri-implantation. Differential expression of Egfr-ErbB4 was observed in the trophoblast during implantation. Marked expression of ErbB1 mRNA and protein were observed in the cytotrophoblast of the embryonic trophectoderm, with the syncytiotrophoblast layer displaying weaker staining (Klonisch et al. 2001).

In vitro studies with human endometrial stromal cells had demonstrated that HBEGF has a function in endometrial maturation in mediating decidualization and

attenuating TNFA and TGF-β-induced apoptosis of endometrial stromal cells (Chobotova et al. 2005).

Placental growth is regulated by endocrine and local growth factors (Maruo et al. 1991) but little information is available regarding the expression of BTC in the placenta and uterus (Shing et al. 1993; Watanabe et al. 1994) and (Birdsall et al. 1996). A study was conducted to clarify the expression of Btc and its receptors, ErbB-1 and ErbB-4, in the trophoblasts in the human placenta over the course of pregnancy (4th to 5th, 6th to 12th, 18th to 21st, and 38th to 40th week placentas(Maruo et al. 1995). BTC from very early placentas until mid placentas was immunolocalized in syncytiotrophoblasts, and was most abundant in early placentas. The levels of BTC mRNA expression in early and mid placentas were significantly higher than those in term placentas. The levels of erbB-1 mRNA in the early and mid placentas were significantly higher than those in term placentas, whereas the levels of erbB-4 mRNA in early placentas were significantly lower than those in mid and term placentas, that shows evidence for changes in expression and cytological localization of BTC and its receptors in the trophoblasts in human placenta over the course of pregnancy. BTC may play a pivotal role as a local growth factor in promoting the differentiated villous trophoblastic function via ErbB-1 in early placentas and in contributing to placental growth through the maintenance of extra-villous trophoblast cell function via ErbB-4 in term placentas (Maruo et al. 1995).

2.3 Male reproduction

In sharp contrast to the abundant data supporting an important role for the EGFR ligands in the female reproductive tract, only little information is available about their function in the male reproductive organs.

EGF was measured in the seminal plasma of human patients attending an infertility clinic. No correlation was found between EGF concentration (~ 40 ng/ml) and age of donor, sperm count, sperm motility, sperm morphology or period of sexual abstinence before sample collection. There was no significant variation in mean EGF levels between fertile and infertile men, suggesting that this peptide plays no role in the density or motility of sperm associated with fertility (Elson et al. 1984; Hirata et al. 1987; Richards et al. 1988).

The circulating Egf appears to play a role in mouse spermatogenesis since the removal of the submandibular glands decreased the amount of circulating Egf to an undetectable level and caused a marked decrease in the number of mature sperm and spermatids (55 and 50%, respectively) and the number of spermatocytes increased 20%. Replacement of Egf to sialoadenectomized mice reversed completely both the sperm content of the epididymis and the number of spermatids in the testis to normal showing that Egf may play a role in the meiotic phase of spermatogenesis and may cause some cases of infertility like unexplained oligospermia (Tsutsumi et al. 1986). Interestingly, infertile men had mean blood plasma EGF concentrations lower than that of the fertile group. There were also statistically significant differences between the fertile and infertile men in sperm count, sperm viability, mean forward progression, testosterone, LH and FSH (Adekunle et al. 2000).

Transgenic male mice overexpressing Egf show only few post-meiosis II gametes, are sterile and have reduced serum testosterone (Wong et al. 2000). This is in contrast to transgenic mice overexpressing Tgfa in the testis. These transgenic animals had no abnormal testicular morphology or alterations in spermatogenesis (Mullaney & Skinner 1992). Observations demonstrate that gene expression of Tgfa and its receptor is high during early pubertal stages when somatic cell growth is predominant and low at late pubertal stages when somatic cell proliferation is reduced. Tgfa can act as an autocrine/paracrine mitogen for the mesenchymal-derived peritubular cell, while actions on the Sertoli cell population are not evident (Mullaney & Skinner 1992). Although Egf seems to be the major physiological ligand in germ cell development, mice with either single or triple null mutations in *Egf, Tgfa,* and *Areg* did not display reduced fertility (Luetteke et al. 1999). DNA synthesis in rat testis seminiferous tubules in vitro was stimulated with the addition of Egf, Tgfa and Btc. RT-PCR analysis revealed that EGFR, erbB2, erbB3 and erbB4 were all expressed at every stage of the spermatogenic wave, whereas differential expression was found in isolated Leydig, Sertoli and peritubular cells. These results show that EGFR-ligands are spermatogonial growth factors *in vitro*, suggesting a paracrine control of spermatogenesis *in vivo* (Yan et al. 1998; Wahab-Wahlgren et al. 2003).

Expression of the EGF-like ligands EGF, TGFA, AREG, BTC, HBEGF and all four ErbB receptors was detected in the human and mouse prostate showing an evidence that ErbB signaling contributes to mouse prostate function (Adam et al. 1999; Zhu & Jones 2004). Finally, aberrant ErbB family signaling contributes to the

development and metastatic progression of human prostate cancer (Klapper et al. 2000).

3 ANIMALS, MATERIALS AND METHODS

3.1 Animal Breeding

All animals were maintained under non-barrier conditions in the facilities of the Gene Center at 22°C, 65% humidity, and a 12 h light cycle and received standard food and water *ad libitum*. Mice used in expression studies and for phenotype analysis were weaned at an age of three weeks, marked by ear piercing and housed in cages separated by sex. At the time of weaning, tail tips were clipped and frozen on dry ice and stored at -80°C for genotype analysis. The generation of the transgenic mice used in this study was described previously (Schneider et al. 2005).

3.2 Mouse genotyping

Proteinase K digests of mouse tail tips

Reagents:

EDTA	Ethylene diamine tetraacetic acid, 0.5 M, ph 8.0
Proteinase K Solution	20 mg/ml dissolved in bidistilled H_2O
Isopropanol	100%
Ethanol	70%

Wizard ® Genomic DNA Purification Kit (Promega) containing:
Nuclei Lysis Solution
RNase Solution
Protein Precipitation Solution
DNA Rehydration Solution

Assay:

Each tail tip was incubated in a 1.5 ml tube with 600 µl of a solution containing 120 ml EDTA, 500 µl Nuclei Lysis Solution and 17.5 µl Proteinase K stock solution overnight at 56° C and with shaking. The next step was the addition of 3 µl of RNase Solution. The samples were mixed by inverting 25 times and incubated for 20 min at 37°C. After cooling down to room temperature, 200 µl of Protein Precipitation Solution was added to each sample. The samples were vortexed at high speed for 20 seconds, chilled on ice for 5 minutes and then centrifuged at 13,000 *g* x for 4 min. The supernatant was transferred into a clean tube containing 600 µl isopropanol. The DNA became visible as a small white pellet by gentle shaking and was centrifuged at 13,000 x *g* for 2 min. After removing the supernatant, 600 µl of 70% ethanol were added to wash the DNA and the tubes were inverted several times. A last centrifugation was made under the same conditions as above, the ethanol was removed and the pellet was air-dried for 10 min. 50 µl of Rehydration Solution were added and the DNA were rehydrated by incubating at 65°C for one hour.

Principle of the Polymerase Chain Reaction (PCR)

PCR is an *in vitro* method that allows up to a billion folds amplification of a selected DNA sequence. The reaction uses two oligonucleotide primers that hybridize two opposite strands and flank the target DNA sequence that is to be amplified. In the presence of deoxyribonucleoside triphosphates (dNTPs), a heat-stable DNA polymerase catalyzes the elongation of the primers. A repetitive series of cycles involving template denaturation, primer annealing, and extension of annealed primers by the polymerase results In exponential accumulation of the specific DNA fragment. Because the primer extension product synthesized in a given cycle can serve as a template in the next cycle, the number of target DNA copies increases exponentially corresponding to each cycle.

PCR protocol for detecting the BTC transgen sequence

Reagents:

Taq DNA polymerase Kit (Qiagen) containing:

PCR Buffer, 10x	trisCl, KCl, $(NH_4)_2SO_4$, 15mM $MgCl_2$, pH 8.7 (20°C)
Q-Solution	5x concentrated
$MgCl_2$	25Mm
Taq Polymerase	5U/µl, recombinant 94-kDa DNA polymerase, isolated from *Thermus aquaticus*, cloned in *E. coli*
dNTPs Set	100 Mm aqueous solutions of dATP, dCTP, dGTP and dTTP, each in a separated vial
Ethidium Bromide	0,1% solution in bidistilled H_2O
50x TAE running buffer	242 g Tris 57,1 ml Glacial Acetic Acid 100 ml EDTA, 0.5 M, ph 8.0 Ad 1 l bidistilled H_2O
6x Loading Dye	30% Glycerol Bromophenol Blue

To confirm the integrity of the DNA, a sequence of the β-actin gene was amplified using the following primers:

β-actin sense	5'-GGC ATC GTC ATG GAC TCC -3'
β-actin antisense	5'-GTC GGA AGG TGG ACA GGG -3'

For detection of the construct integration, following primers were use:

pTORUseq	5'-CTA CAG CTC CTG GGC AAC GTG-3'
globpA	5'- AGA TCT CAG TGG TAT TTG TGA GCC 3'

Assay procedure:

The 20 µl reaction was prepared in 100 µl PCR tubes on ice, containing:

DNA template (about 50 ng/µl)	2 µl
Sense primer (2 µM)	1 µl
Antisense primer (2 µM)	1 µl
dNTPs Mix (1 mM)	2 µl
PCR Buffer, 10x	2 µl
Q-Solution	4 µl
$MgCl_2$ (25Mm)	1.25 µl
Taq Polymerase, 5U/µl	0.1 µl
Bidistilled H_2O	6.65µl

The amplification took place in a Biometra Uno Thermocycler and was performed as follows:

1st step:	denaturation	94°C for 4 min
2nd step:	denaturation	94°C for 1 min
3rd step:	annealing	60°C for 1 min
4th step:	extension	72°C for 2 min
5th step:	extension	72°C for 10 min
6th step:	cooling	4°C

Steps 2 to 4 were repeated 35 times before progression to steps 5 and 6 (36 cycles). PCR products were mixed with 6x loading buffer, separated in 2% agarose TAE gels with ethidium bromide and visualized under UV light.

3.3 Analysis of Body and Organ Weights

Body, ovary and uterus weight of control and transgenic females at the age of 2 months were recorded with a scale (BP221S, Sartorius, Germany). Body weight was measured to the nearest 0.1 g and ovary and uterus to the nearest mg.

3.4 Fertility Analyses

Most of the routine mouse breeding for expanding the Btc transgenic lines involved mating of transgenic males to control females. A retrospective analysis shows that the number of successful matings (77/157, 49%) and pups per litter (8.2±0.2) was significantly reduced as compared to non-transgenic age-matched males (11/11, 100% successful matings and 9.9±0.4 pups per litter). A few matings involving transgenic females paired to control males were performed sporadically and these matings also revealed a clear impairment in the reproductive performance of transgenic females: the number of successful matings (8/13, 61.5%) and pups per litter (5.7±0.8) was significantly reduced. Thus, we decided to initiate extensive experiments to systematically analyze the reproductive fitness of transgenic females.

In order to analyze their breeding performance, Btc transgenic mice (lines 2 and 4) and control mice were paired with wild-type mice of the opposite sex. The mating pairs were:

- WT ♀ x WT♂, n=3
- Line 2 BTC ♀ x WT ♂, n=5
- Line 4 BTC ♀ x WT ♂, n=5
- WT ♀ x Line 2 BTC ♂, n=4
- WT ♀ x Line 4 BTC ♂, n=5

All animals were 2 months old at the beginning of the experiment. Each mating pair was housed together exactly three months and the litters were removed at the age of 4 weeks. The litter size of each mating pair was averaged. The interval to the first litter and litter size development were recorded.

3.5 Evaluation of female puberty onset and estrous cycle tracking

Wild-type (n=3) and Btc transgenic (n=4) mice were observed daily from the time of weaning at 21 days for the appearance of a vaginal opening (the loss of the vaginal closure membrane at puberty). Once this occurred, daily histological analysis of vaginal smears was made. Estrous cycles were monitored during three consecutives cycles. The cycle's length was defined as the interval between proestrus smears and the onset of estrous cyclicity was defined as the first day of the first cycle less than or equal to 6 days in length (Nelson et al. 1990).

3.6 Embryo Implantation Studies

Wild-type (n=6) and Btc transgenic (n=11) mice were mated with fertile males and used to examine the attachment reaction and progression of the implantation process. By the end of the day 4 or day 5 of pregnancy they were injected intravenously (200 µl/mouse) with a solution of Evans Blue dye (1% in PBS). Mice were killed 5 min after injection and blue bands along the uterus indicated the sites of blastocyst apposition (Das et al. 1994b). After the observation of the implantation sites the uterus were flushed to recover embryos that did not implant.

3.7 Ovulation and Fertilization Analysis

Female mice at 2 to 6 months of age were used for these experiments. Ovulation assays were performed with wild-type (n=15) and Btc transgenic (n=8) mice. Female were mated with wild-type vasectomized males. The females were examined every morning and evening for the presence of a copulatory plug (positive=day 0). The oviducts and ovaries were excised from mice and were placed into dishes containing medium (M2 supplemented with BSA). The cumulus-oocyte complexes (CCOs) were recovered by dissection of both ampullas on day 0.5. The number of ovulated oocytes was determined after enzymatic disassociation with 0.3 % hyluronidase from the surrounding cumulus. Fertilization assays were performed with wild-type (n=12) and Btc transgenic (n=7) female mice which were 2 to 6 months old. The females were mated with wild-type males with proved fertility. The methodology to detect copulatory plugs and the COCs collection was the same as described for determination of the ovulation rate. The fertilized oocytes (zygotes) were counted.

3.8 Cumulus-Oocyte Complex Isolation and Culture

Female mice, 2 to 6 months old, were induced to superovulate with pregnant mare serum gonadotropin (PMSG, Intergonan) and sacrificed 48 h later to obtain the immature oocytes. The ovaries were removed and stored in Opti-MEM medium

(Gibco, Grand Island, NY) with 10% fetal calf serum (FCS; Biochrom AG). Large antral follicles were punctured with 28 in. × 0.5 in. 18G syringe needles to release COCs. Only COCs that consisted of an oocyte surrounded by at least three complete layers of cumulus cells were selected for further culture. COCs were taken through three washes of Opti-MEM medium plus 10 IU/ml PMSG and 10% FCS before being cultured in groups of 20 in 80µl drops of preequilibrated medium and overlaid with silicone oil (Sigma) in an atmosphere of 5% CO_2 in air at 37°C. These oocytes were cultured for 16 h. At the end of the culture period, COCs were removed from the drop and the oocyte denuded of cumulus cells by repeated gentle pipetting in medium containing 0.1% hyaluronidase (Sigma). Morphological changes of the cultured oocytes were evaluated and classified. Immature oocytes were classified as GV when the germinal vesicle (GV) was present, as metaphase I (MI) when GV was broken down, as metaphase II (MII) when the first polar body was extruded, and as degenerated when oocytes were dark, granulated, or fragmented. The degree of cumulus expansion was assessed according to a subjective scoring system, a scale of 0 (no expansion) to +4 (maximal expansion) (Yoshino et al. 2006).

3.9 Sperm isolation and *in vitro* fertilization

Sperm were collected from the *cauda epididymis* and *vasa deferentia* of fertile mice in IVF medium (Vitrolife). The percentages of motile and progressive spermatozoa and the sperm concentration were recorded. The oocytes were washed three times in the fertilization medium (IVF, Vitrolife) and placed in fertilization drops, where the spermatozoa were added with a concentration of 1 x 10^6/ml. Six hours after fertilization, oocytes were denuded from the remaining cumulus cells by repeated gentle pipetting. We evaluated and classified the morphological changes of the zygotes, as appearance of two pronuclei and after 24 hours the cleavage.

3.10 Sperm Analysis

Sperm analyses were performed with wild-type (n=7) and Btc transgenic (n=11) males mice ranging from 3 to 5 months of age. After retro-orbital puncture for blood collection under ether anesthesia, the animals were killed by cervical dislocation. The two caudae epididymides and vasa deferentia were dissected and

placed into 0.9% NaCl in a 4-well dish (on ice). Under a microscope all remaining fat and visible blood vessels were removed with scissor. The epididymis was cut several times and the spermatozoa were left to swim out for 3-5 minutes at 37°C. The suspension was shacked carefully until the solution was homogenous. The sperm quality is checked using a computerized semen analysis system (IVOS, v 12.2, Hamilton Thorn Research, Beverly, MA). The percentage of motile and progressive sperm and concentration of sperm were recorded. We thank Dr. Auke Boersma (GSF) for the sperm analysis.

3.11 Histological and Immunohistochemical studies

Excised ovarian and uterus tissues were fixed in 4% paraformaldehyde, routinely processed for paraffin embedding, and serially sectioned at a thickness of 5 µm. The sections were stained with hematoxylin and eosin and examined by light microscopy. Sections were deparaffinized and hydrated in PBS for 20 min. Blocking of endogenous peroxidase activity was achieved by incubation in 3% H_2O_2 in PBS for 10 min at 37°C. The sections were incubated with the primary antibody, a goat anti-mouse Btc antibody (R & D Systems, Wiesbaden, Germany), for 1 h at 37°C. Then, the sections were rewashed in TBS (Tris-buffered saline) two times for 5 min and incubated with the secondary antibody, a biotinylated rabbit anti-goat Ig (Dako, Denmark). After immunostaining, sections were stained lightly with diaminobenzidine (DAB), mounted, and examined under a brightfield microscope. Red deposits indicated the sites of immunoreactive protein. IHC was kindly performed by Maik Dahlhoff and evaluated by Prof. Dr. Sinowatz (Institute of Veterinary Anatomy).

3.12 Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed using paired or unpaired Student's *t* tests to detect significant differences between transgenic and control mice. Values of $p<0.05$ were considered significant.

4 RESULTS

4.1 Analysis of Body and Organs Weight

To detect a possible effect of the Btc overexpression on the weight of uterus and ovaries, the weight of these organs in females at 2 months of age was recorded. Table 4.1 shows that in comparison with wild-type females, Btc transgenic females exhibited a lower body weight. The absolute weight of ovary and uterus did not differ significantly between the two genotypes. While the same was true for the relative weight of the uterus, an increase in the relative weight of the ovary in transgenic females was detected.

Table 4.1 Body weight and absolute and relative weights of the ovary and uterus of non-transgenic (wt) and Btc-transgenic (tg) females at the age of 2 months. The table shows the calculated mean values (standard deviation values are shown in brackets). A *t*-test was used to show the absence (-) or presence (*: $P<0.05$) of a statistically significant difference (Δ) between groups.

	Weight			**% Body weight**		
	wt	**tg**	**Δ**	**wt**	**tg**	**Δ**
Body (g)	22.86 (2.9)	19.67 (2.6)	*			
Uterus (mg)	78.1 (34)	81.6 (21)	-	0.35 (17)	0.42 (0.11)	-
Ovary (mg)	10.1 (3.9)	11.5 (2.8)	-	0.04 (0.01)	0,06 (0.01)	*
n	10	11				

4.2 Fertility measurements

4.2.1 Males

To study systematically the consequences of transgene expression on male and female fertility, Btc transgenic mice from Lines 2 and 4 were mated with wild-type animals.

In average, control litters consisted of 9.9 ± 0.3 pups, while L2 Btc and L4 Btc transgenic males produced an average litter size of 7.9 ± 0.8; and 8.0 ± 0.8 pups, respectively ($p<0.05$). The interval to the first litter was not significantly different between groups (Table 4.2).

Table 4.2 Mean values (± SEM) and statistical analysis of the results of the fertility measurements of BTC transgenic male's mice.

Parameter	**Control** (n=3)	**L2** (n=4)	**L4** (n=5)
Number of successful matings	3 (100%)	3 (80%)	5 (100%)
Number of litters/animal	4.3 ± 0.3	3.6 ± 0.3	2.6 ± 0.5
Interval to the first litter (days)	21.3 ± 0.6	21.0 ± 0.3	31.0 ± 3.6
Litter size	9.9 ± 0.3	7.9 ± 0.8[a]	8.0 ± 0.8[a]

[a] $P < 0.05$

4.2.2 Females

Successive matings of Btc transgenic female mice with wild-type male mice revealed a decrease in litter size compared with litters produced by control mating. Control litters had an average size of 9.9 ± 0.3 pups, L2 Btc transgenic females gave birth to an average of 5.3 ± 0.7 pups/litter, and L4 Btc transgenic females had an average of 4.8 ± 0.5 pups/litter ($p<0.001$). The interval to the first litter was not significantly different between groups (Table 4.3).

Table 4.3 Mean values (± SEM) and statistical analysis of the results of the female's fertility study.

Parameter	**Control** (n=3)	**L2** (n=5)	**L4** (n=5)
Number of successful matings	3 (100%)	5 (100%)	4 (80%)
Number of litters/animal	4.3 ± 0.3	2.8 ± 0.6	2.6 ± 0.5[a]
Interval to the first litter (days)	21.3 ± 0.6	23.0 ± 1.0	23.4 ± 2.3
Litter size	9.9 ± 0.3	5.3 ± 0.7 ***	4.8 ± 0.5***

[a] $P = 0.0533$
*** $P < 0.001$

Litter size development showed a significant difference between Btc transgenic females and controls females (Figure 4.1).

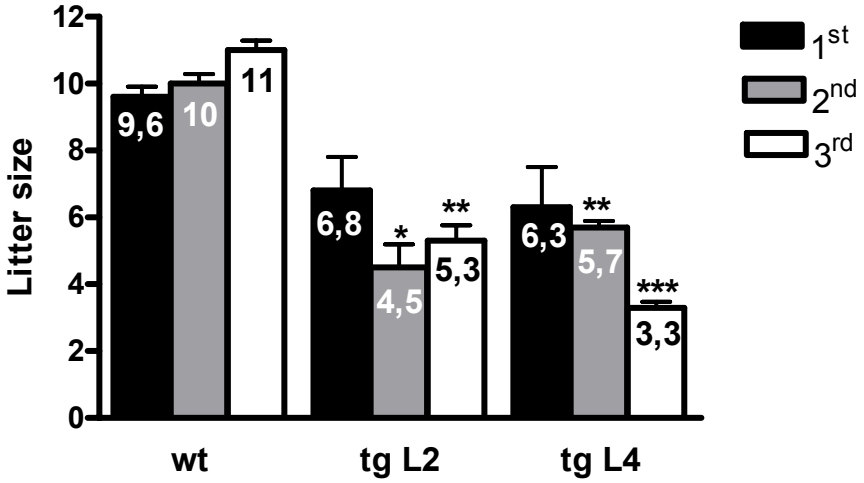

Figure 4.1 Litter size development. A reduction in the mean number of pups per litter in the second and third litters as compared to the first litter was observed in Btc transgenic females but not in control females (***$P < 0.05$).

4.3 Evaluation of female puberty onset and estrous cycle tracking

Vaginal opening, the first observable consequence of the rise in circulating E that accompanies the onset of puberty in rodents, occurred essentially at the same age in transgenic and non-transgenic females (days 24 to 26, data not shown). Similarly, the interval between vaginal opening and the first estrus was

undistinguishable between both genotypes (data not shown). The interval between vaginal opening and the onset of estrous cyclicity, which takes several days in normal mice, was slightly increased in the transgenic females, but the difference did not reach statistical significance (Figure 4.2A). Figure 4.2B shows the duration of the first 3 cycles, demonstrating that, although the first cycle was significantly longer in the transgenic group, this difference disappeared after the second cycle.

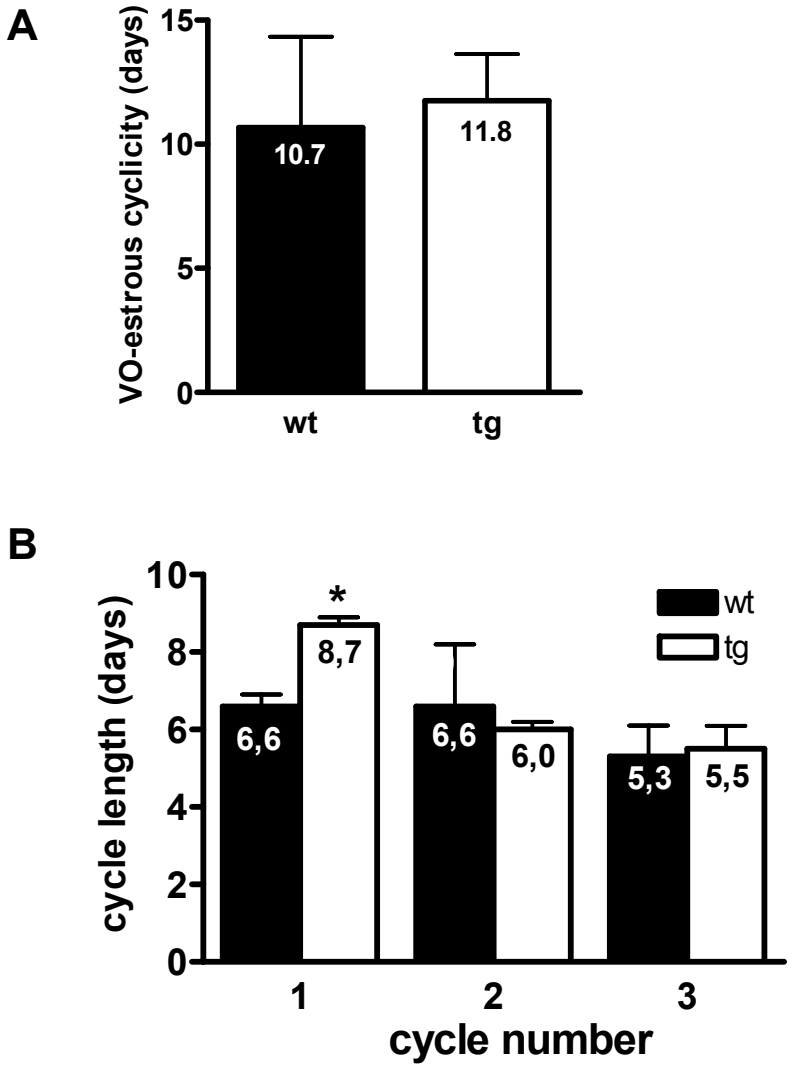

Figure 4.2 Evaluation of the onset of estrous cyclicity and cycle length in non-transgenic (wt, n= 3) and transgenic animals (tg, n = 4). A) The interval between vaginal opening (VO) and estrous cyclicity (defined as the first day of the cycle with duration of 6 or less days) is not different between the groups. B) The first estrous cycle has a longer duration in transgenic females. Cyclicity is attained at the third cycle in both groups.

4.4 Embryo Implantation

In order to evaluate the kinetics of blastocyst attachment, plugged females were injected intravenously with a blue dye solution to make the implantation site visible (Figure 4.3).

Figure 4.3 The attachment reaction. The left uterus is from a wild-type animal at 24:00 h on day 4 with the blue bands indicating the implantation sites. The right uterus with no visible blue sites is from a BTC transgenic female at the same time after copulation.

The attachment reaction was observed in all of the wild-type mice at 24:00h on day 4 of pregnancy and only 13 blastocysts were recovered. In contrast, none of the pregnant transgenic mice exhibited blue reaction at this time and many zona-enclosed and hatched blastocysts were recovered. At 9:00h on day 5 of pregnancy, 100% of the transgenic females showed attachment reaction and only a few embryos were recovered (Table 4.4).

Table 4.4 Initiation of blastocyst attachment reaction in transgenic and age-matched wild-type females. Mice without blue sites or blastocysts were not included.

day	genotype	mice with implantation sites (%)	implantation sites (n)	embryos recovered
4	wt (n=6)	6 (100)	51	13
4	tg (n=11)	0 (0)	0	53
5	tg (n=5)	5 (100)	22	8

Interestingly, the mean number of total embryos either attached to or recovered from the uterus of transgenic females at day 4 (53:11= 4.8) or day 5 (30:5= 6) was markedly reduced when compared to the number of embryos present in the uterus of control females at day 4 (64:6= 10.7). This finding is particularly informative since these values correspond quite well to the observed litter sizes in both groups. This observation strongly suggests that, although implantation delay is certainly a feature of Btc transgenic mice, it does not explain the reduction in the number of pups produced. The reason for this reduction must be found in processes taking place before implantation (ovulation or fertilization).

4.5 Ovulation and Fertilization analysis

To evaluate whether ovulation or fertilization rates were reduced in transgenic animals and could be responsible for the reduction in litter size, we mated transgenic and non-transgenic females with vasectomized non-transgenic males. As shown in Figure 4.4A, the number of ovulated oocytes obtained did not differ between the two genotypes. Next, to evaluate whether fertilization was impaired, we mated females from both genotypes to fertile, non-transgenic males and recorded the number of fertilized oocytes. While the mean number of ovulated oocytes was again similar in both groups, confirming our previous observation (data not shown), we observed a statistically significant (P=0.01) reduction in the percentage of fertilized oocytes in transgenic (54.7 $\pm$ 8.9 %) as compared to control (81.7 $\pm$ 5.3 %) females (Figure 4.4B). The mean number of fertilized oocytes observed in transgenic (41:7 = 5.8 $\pm$ 0.9) and control (119:12 = 9.9 $\pm$ 0.5) females is again extremely informative because it reflects quite closely the mean litter size observed in these groups. Thus, since no significant further embryonic losses will occur after this stage, we have identified impaired fertilization as the mechanism responsible for the reduction in the litter size.

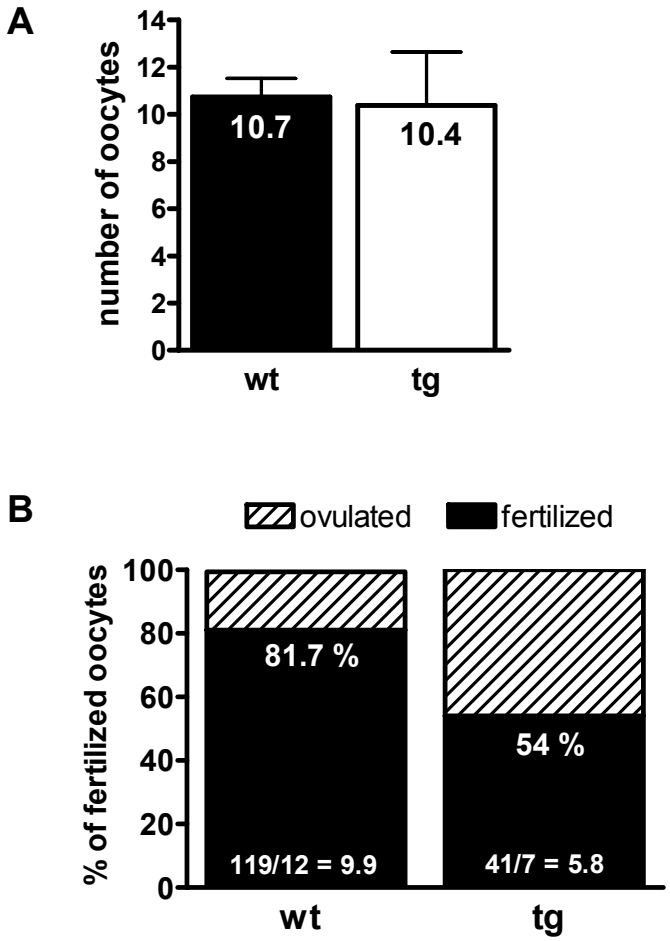

Figure 4.4 Ovulation and fertilization rates. A) The mean number of ovulated oocytes is not different between control (n = 15) and transgenic (n = 8) animals. B) The proportion of fertilized oocytes is significantly reduced in transgenic females (n = 7) as compared to non-transgenic females (n = 12).

4.6 *In vitro* maturation

In this study, oocytes were isolated from control (n=11) and Btc transgenic females (n=15), and matured *in vitro*. After *in vitro* maturation, oocytes were classified, under a light microscope, as germinal vesicle (GV, Figure 4.5A), metaphase I (MI, Figure 4.5B), or metaphase II (MII, Figure 4.5C).

The percentage of oocytes matured to MII was significantly lower in the transgenic group as compared to control animals. Conversely, oocytes from transgenic females showed a higher rate of oocytes at MI, indicating a blockage or delay in the maturation process (Table 4.5).

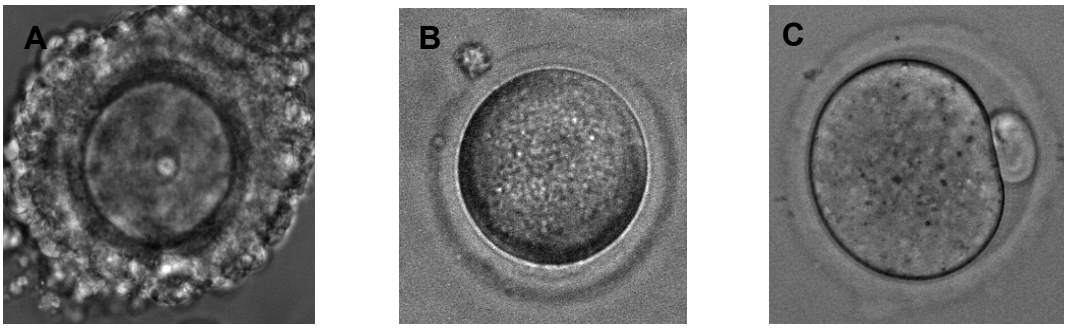

Figure 4.5 Light micrographs of mouse oocytes at the germinal vesicle (**A**), metaphase I (**B**), and metaphase II (**C**) stages of nuclear maturation.

Table 4.5 Analysis of IVM oocytes from Btc transgenic (n=15) and control (n=11) mice. Values are the mean ± SEM. Nuclear maturation and the degree of *cumulus* expansion were assessed after 16 hours of culture.

Genotype	Oocytes employed	IVM		
		Metaphase I	Metaphase II	CE
	n	n (%)	n (%)	(1 to 4)
control	146	16 (9.8 ± 3)	129 (89.7 ± 3.1)	4
transgenic	187	36 (19.4 ± 2.8)	150 (80.2 ± 2.6)	4
P		0.0327	0.0308	

The transgenic and wild-type animals had 1 degenerated oocyte each after IVM. CE = cumulus expansion; Metaphase I = neither GV nor polar body; Metaphase II = 1st polar body present.

4.7 *In vitro* fertilization

Our analysis of the *in vitro* fertilization rate, shown as the appearance of two pronuclei after 6 h of fertilization, revealed a reduction in the percentage of fertilized oocytes of the transgenic animals as compared with control animals. The cleavage

rate, evaluated 24 h after the fertilization, was also reduced in oocytes from transgenic mice (Table 4.6). Interestingly, in the transgenic group, the number of cleaved oocytes was higher than that of oocytes with 2 PN after IVF (82 vs. 75, see Table 4.6). This finding supports the idea of delayed oocyte maturation.

Table 4.6 Analysis of IVF of oocytes from Btc transgenic (n=11) and control (n=7) mice. Values are the mean ± SEM. The observation of two pronuclei and the cleavage to a 2-cell stage were considered an indicative of successful fertilization and evaluated 6 and 24 hours after the IVF, respectively.

Genotype	Oocytes employed	IVF	
		2 PN	Cleavage
	n	n (%)	n (%)
control	87	73 (87.4 ± 4.6)	71 (84.6 ± 5.6)
transgenic	150	75 (50.6 ± 3.8)	82 (57.5 ± 5.1)
P		0.0001	0.0034

2PN = 2 pronuclei present.

4.8 Sperm analysis

Although the fertility of Btc transgenic males appears to be impaired, these animals do not display evident alterations in sperm production. Analysis of sperm parameters in Btc transgenic males showed no difference compared with non-transgenic males (Table 4.7).

Table 4.7 Analysis of sperm parameters of Btc transgenic and control mice.

Genotype	Motility (%)	Progressivity (%)	Concentration (Mio./ml)
Wt mice	78.3±2.1 (7)	37.1±2.6 (7)	120.3±18.2 (7)
BTC tg mice	82.0±1.6 (11)	41.0±3.0 (11)	148.3±19.2 (11)

Values are the mean ± SD, with the number of mice in parentheses.

4.9 Immunohistochemical analysis

Using Western blot analyses, we have previously detected increased levels of Btc expression in the ovary and uterus of transgenic females as compared to non-transgenic littermates (data not shown). Since our studies uncovered impaired fertilization as the reason behind the reduced litter size, we decided to study the expression pattern at the cellular level by employing immunohistochemistry. The results confirmed the Western blot analyses, showing increased levels of Btc expression in the uterus and ovary (Figure 4.6). Particularly interesting is the high Btc expression in granulosa and cumulus cells of the follicles of transgenic females (Figure 4.6f).

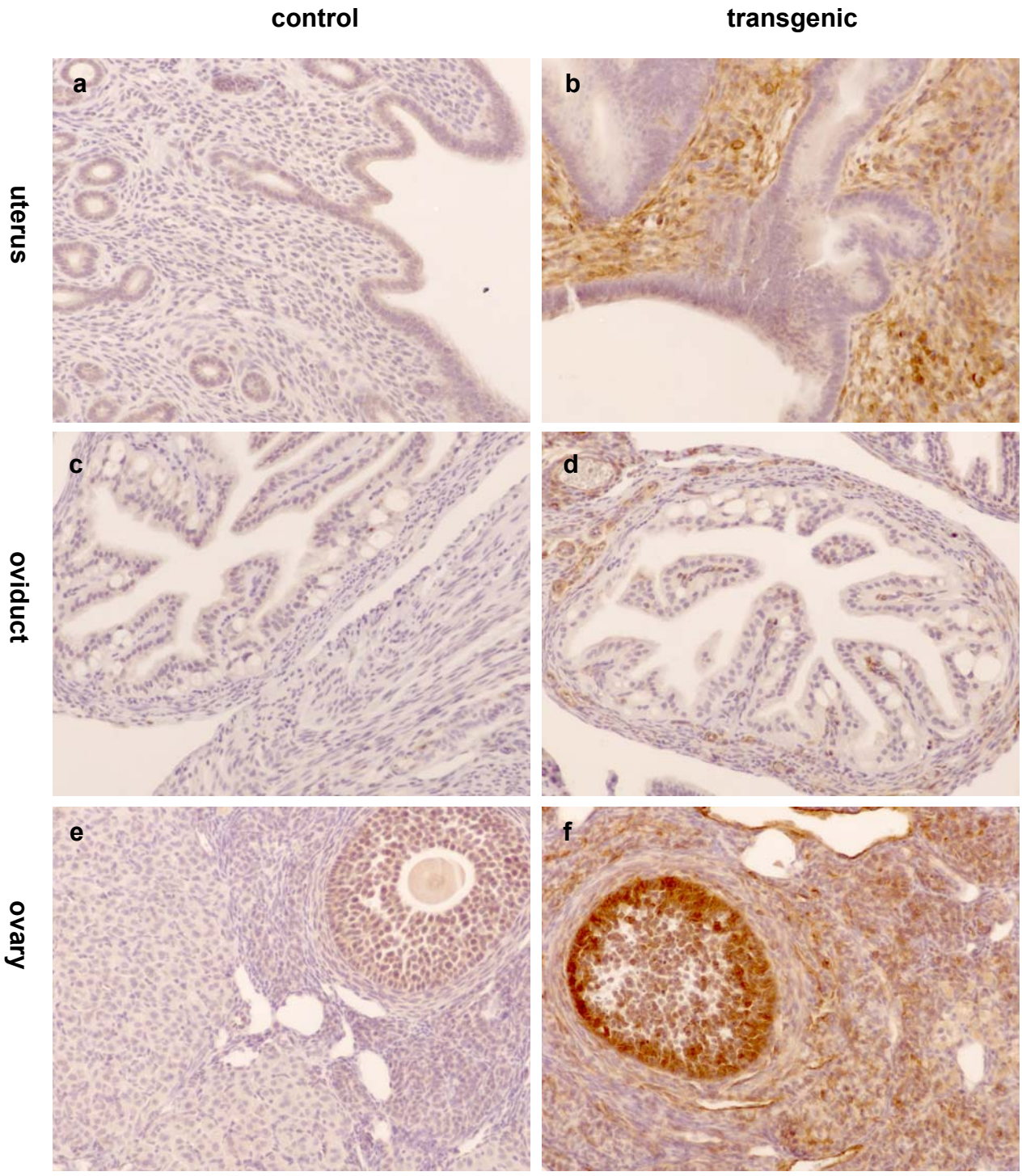

Figure 4.6 Immunohistochemistry of Btc mouse reproductive tract on day 0.5. Note the distribution of Btc in uterus and ovary from transgenic mouse (b, f). No immunostaining was noted when similar sections from wild-typ mice were used (a, c, e)

5 DISCUSSION

Gene targeting by homologous recombination in embryonic stem cells represents a powerful tool for evaluating gene function in whole organisms. Not infrequently, the targeted gene encodes a protein belonging to a family of molecules sharing structural and functional properties. In this case, gene deletion may be rather uninformative because the related proteins can compensate for the loss of the targeted proteins (a process called functional compensation). In this case, the opposite strategy, that means, increasing the expression levels of the gene of interest by means of overexpression may be a much more appropriate approach. This has been shown to be true for many families of peptides including the IGF binding proteins (Schneider et al. 2000; Wolf et al. 2005). Betacellulin, a member of a family of seven Egfr ligands is a further example. Mice lacking *Btc* develop normally, are fertile and display no overt phenotype (Jackson et al. 2003). Recently, transgenic mice overexpressing Btc were generated (Schneider et al. 2005). In addition to growth retardation, a variety of pathological alterations including cataract and abnormally shaped retinal layers, bone alterations and severe lung alterations, were observed. Thus, this model uncovers multiple consequences of Btc overexpression *in vivo* and provides a useful model for examining the effects of Btc excess on different organs. In the present study, Btc transgenic mice were employed to study the effects of increased growth factor levels in female and male reproduction. The observation of relatively ineffective matings involving transgenic females (non-productive matings and reduced litter size) during routine breeding led us to functionally evaluate the different stages of the reproductive process.

5.1 MALES

Retrospective analysis revealed that matings involving transgenic males were often unsuccessful. Systematic fertility measurements revealed that, although the mean number of litters produced and the interval to the first litter was unaltered, a significant reduction in the number of pups per litter was observed for transgenic males as compared to non-transgenic animals. Analyses of sperm parameters (concentration, motility, and progressive motility) failed to reveal any difference

between transgenic and control age-matched males. Although an impairment of the fertilization rate is a probable explanation, an impairment of the early embryonic development or blastocyst attachment by increased levels of Btc in the seminal plasma of transgenic males cannot be excluded. Thus, the exact cause of the reproductive deficit of transgenic males remains to be determined.

Growth factors of different families have been shown to play a role in male reproduction (Bartke et al. 1999; Burns & Matzuk 2002; Cho et al. 2001; Camp et al. 2005). Particularly interesting are studies showing the effects of depleting circulating Egf by sialoadenectomy. After the sialoadenectomy a reduced spermatid count was observed and returned to normal with Egf treatment, suggesting a relationship between EGF and spermatogenesis (Tsutsumi et al. 1986; Liu et al. 1994). Increased testicular Egf concentrations have been shown in synchronized rat testes that were closed to the meiotic stages (Bartlett et al. 1990).

Egf seems to have a physiological role in germ cell development, as demonstrated by *in vitro* stimulation by Egf of spermatogonial proliferation in seminiferous tubules of adult rats (Yan et al. 1998; Wahab-Wahlgren et al. 2003). The first *in vivo* evidence that Egf overexpression can adversely affect spermatogenesis (hypospermatogenesis) was demonstrated by Wong and colleagues (Wong et al. 2000). This report demonstrated that the transgenic male mice overexpressing Egf had few post-meiosis II gametes, the animals were sterile and had reduced serum testosterone levels. Mice with either single or triple null mutations in Egf, Tgfa, and Areg did not show reduced fertility, raising the possibility of functional redundancy with Hbegf, Btc, and Ereg (Luetteke et al. 1999).

This study provides evidence that Btc overexpression does not negatively affect spermatogenesis, sperm motility, progression and concentration values. This observation is in agreement with a previous report that mice overexpressing Tgfa did not show abnormal testis morphology or spermatogenesis (Mullaney & Skinner 1992), suggesting that the growth factors may have different roles in spermatogenesis.

5.2 FEMALES

5.2.1 Fertility Analyses

Despite the extensive research and a lot of information obtained from *in vitro* and *in vivo* experiments, the precise roles of the Egfr ligands for female and male fertility remain largely undefined. As a first step, systematic fertility experiments were carried out and confirmed under controlled conditions that the litter size is significantly reduced when a transgenic female or male were paired with a control animal. Similarly to the effect observed in males, the major reproductive deficit of females was the litter size. The interval to the first litter was not altered. Interestingly, the litter size development was altered: the size of the second and the third litters was reduced as compared with the first litter.

An effect on litter size has not been reported so far for Btc or other Egfr ligands, while this is not an uncommon observation in other growth factor families. For instance, female transgenic mice overexpressing IGFBP-6 showed severe reproductive defects (Bienvenu et al. 2004). 5–20% of the IGFBP-6 transgenic females were sterile and the litter size of fertile transgenic females was small. Litters sired by transgenic males were in the normal range. The cause of the reproductive deficiency appeared to be an alteration of ovulation and a dramatic decrease in plasma LH concentrations, suggesting some hypothalamo-pituitary disorder resulting from hIGFBP-6 overexpression in the brain (Bienvenu et al. 2004).

5.2.2 Female Puberty Onset and Estrous Cycle Tracking

In mammals, puberty begins with a pulsatile release of GnRH, activation of hypothalamic neurons secreting LHRH and activation of hypothalamic membrane-bound growth factor receptors (Ma et al. 1994). An overexpression of Tgfa or a central blockade can delay puberty or advance it (Ojeda & Ma 1998; Ojeda et al. 2003; Junier 2000). Apostolakis and colleagues (Apostolakis et al. 2000) demonstrated that Egf, Areg and Tgfa can signal through the ERα and alter rodent reproduction. The absence of erbB4 in transgenic mice also delayed sexual maturation and a diminished reproductive capacity, related to impaired release of

LHRH (Prevot et al. 2003; Prevot et al. 2005).

Our studies revealed that onset of puberty, evaluated by vaginal opening, was not different between Btc transgenic and non-transgenic animals. Furthermore, with the exception of a longer first estrous cycle, the transgenic females behaved quite similar to control females during the first weeks of puberty. Importantly, starting with the third cycle, the mean cycle length in both groups was shorter than 6 days in duration, the maximal cycle length considered normal for in different mouse strains (Nelson et al. 1990). Since body weight plays (at least partially) a role in triggering puberty, the longer first estrous cycles may be related to the fact that transgenic females have a lower body weight at the time of puberty as compared to control animals (Schneider et al. 2005). Collectively, these results indicate that, with the exception of a longer first estrous cycle, onset of puberty is, in essence, not affected in transgenic females.

5.2.3 Embryo Implantation

Attachment of the blastocyst to the uterine epithelium initiates the implantation process and is followed by locally increased vascular permeability which can be visualized in the mouse uterus as distinct blue bands after an intravenous injection of a blue dye solution (Reese et al. 2001). Several evidences strongly suggest an important role for Egfr in blastocyst attachment. Thus, Tgfa (Tamada et al. 1991); (Paria et al. 1994), Hbegf (Das et al. 1994b), Areg (Das et al. 1995) and Ereg and Btc (Das et al. 1997a) genes are expressed in the mouse uterus only at sites and time-points relevant to the implantation process. Interestingly, the Egfr and ErbB4 are expressed in blastocysts (Paria et al. 1993a; Paria et al. 1999; Lim et al. 1998). Even more convincingly is the report of delayed blastocyst-attachment reaction in transgenic mice with timely inappropriate expression of Tgfa in the uterus (Das et al. 1997b). In this study, delayed implantation was associated with deferred expression of Areg, which is probably the most relevant Egfr ligand associated with blastocyst implantation. However Tgfa null mice are viable and fertile, showing that a possible compensatory mechanism by others Egfr ligands exists. *In vitro* experiments showing that Hbegf can stimulate embryo development and zona hatching provide further evidence for a role of this growth factor in the implantation process (Raab et al. 1996; Martin et al. 1998). Btc is present in the luminal epithelium and stroma of the mouse

uterus (Das et al. 1997a). However, its significance in implantation has not been studied so far.

The reduced fertility of Btc transgenic females could be attributed to one or more reproductive dysfunctions, such as a decreased ovulation, fertilization or implantation. In light of the abundant experimental data indicating an important role for Egfr ligands in this process, we evaluated whether the process of implantation was perturbed in these animals. Our studies revealed that delayed blastocyst implantation is certainly present in Btc transgenic mice. However, since the total number of embryos either attached (visualized as a blue band) or recovered from the uterus of transgenic females at day 4 or 5 is almost identical to the observed litter size, the reduction in the number of viable embryos did already take place at this stage. Consequently, the explanation for the reduced litter size must be disturbed ovulation or fertilization rates. Thus, we next evaluated whether these processes were negatively affected in transgenic females.

5.2.4 Ovulation and Fertilization Rates *in vivo*

The implantation process of the Btc transgenic mice was delayed, but this was not the explanation for the litter size reduction. For this reason, we decided to evaluate the processes taking place before the implantation that is ovulation and fertilization.

Recent studies have implicated selected Egfr ligands (including Btc) as paracrine mediators of LH actions in the ovulatory follicle (Hsieh et al. 2005; Conti et al. 2006). Specifically, LH stimulated the expression of Areg, Ereg and Btc in mouse preovulatory follicles, and these growth factors triggered meiosis and cumulus expansion (and the expression of genes associated with this process) in cultured follicles in a Egfr-dependent manner (Park et al. 2004; Ashkenazi et al. 2005). Recently, prostaglandin synthase 2 and progesterone receptor were identified as key molecules involved in this process (Shimada et al. 2006). These findings may help to explain the puzzling observation that, in spite of complex actions on the oocyte, granulosa and cumulus cells, LH receptor expression is restricted to mural granulosa cells (Peng et al. 1991). Importantly, at least for Ereg, the release of the soluble form into the follicular fluid by protease-dependent shedding appears to be essential for this effect (Ashkenazi et al. 2005). Tgfa and Btc can activate the Erk signaling

pathway, which promotes the initialization of the differentiation of granulosa cells subsequent to preovulatory follicle selection (Woods & Johnson 2006). Recently, Yoshino and colleagues showed that Btc expression is increased by treatment of cumulus cells with bone morphogenetic protein 15 (BMP-15), an oocyte-specific growth factor that plays a major role in determining ovulation quota in mammals (Yoshino et al. 2006). This is an interesting finding because female mice lacking BMP-15, although exhibiting no obvious defects in folliculogenesis, produce oocytes with a reduced ability to develop into viable embryos (Yan et al. 2001).

We have evaluated the number of ovulated oocytes and observed that this rate did not differ between the transgenic and wild-type animals. However when fertilized oocytes were collected, a statistically significant reduction in the percentage of fertilized oocytes in transgenic as compared to control females was observed. Thus we have identified an impaired fertilization as the reason for the reduction in the litter size.

5.2.5 *In vitro* Maturation and Fertilization

To verify if the fertilization problem was due to an oocyte development competence we decided to determine the *in vitro* maturation rate of the Btc transgenic oocytes. Previous investigations showed that Areg, Epi, Hbegf and Btc, when added in the culture medium can also recapitulate the oocyte maturation *in vitro* (Richards 1994; Richards et al. 1995; Richards et al. 2002; Park et al. 2004; Pan et al. 2004).

Our studies revealed that the rate of oocytes showing germinal vesicle breakdown and reaching metaphase II after *in vitro* maturation was significantly reduced in Btc transgenic females as compared to control littermates. This observation can be attributed to a direct or indirect effect of a high expression of Btc in the transgenic *cumulus* cells (as demonstrated by immunohistochemistry). Thus, the reduced fertilization observed in Btc transgenic mice could be attributed to a maturation defect. Therefore, *in vitro* fertilization was employed to confirm the fertilization ability on the mature oocytes *in vitro*.

Our analysis of *in vitro* fertilization revealed a reduction in the percentage of fertilized oocytes of the transgenic animals as compared with control animals. This

result makes a negative influence of the oviduct during the fertilization process unlikely, uncovering a problem in the cumulus-oocyte complex itself.

The fact that the maturation of oocytes from transgenic animals is affected to a relatively low degree (about 10% less oocytes at MII) makes an impaired maturation an improbable explanation for the reduction in litter size (a decrease of about 50%). Thus, an impaired fertilization of the mature oocyte is the most likely explanation for the fertility defect of Btc transgenic females.

6 SUMMARY

Impaired fertility in transgenic mice overexpressing Betacellulin

Peptide growth factors regulate many cellular functions by autocrine, paracrine, juxtacrine or endocrine mechanisms. The epidermal growth factor (EGF)-like peptides are emerging as major players in regulating different aspects of animal and human physiology and pathology. The EGF family elicits essential actions in reproduction. For instance, different Egfr ligands have been shown to be involved in oocyte maturation and ovulation, preimplantational embryonic development, and implantation.

Btc, a member of the Egf family, was initially isolated from a mouse insulinoma cell line, and it is expressed in a wide range of tissues in the mouse, with particularly high levels in the heart, lung, liver, kidney, pancreas, small intestine, colon, testis, ovary and uterus. Btc was identified as one of the Egfr ligands expressed in the mouse uterus exclusively at the time of implantation and can also participate as a mediator of luteinizing hormone (LH), prostaglandins (PGs) and progesterone receptor (PGR).

Mice lacking *Btc* are viable, fertile and show no overt phenotype, but transgenic mice overexpressing Btc exhibit a whole array of phenotypical alterations.

In the present study, Btc transgenic mice were employed to study the effects of increased growth factor levels in female and male reproduction. The observation of relatively ineffective matings involving transgenic females (non-productive matings and reduced litter size) during routine breeding led us to functionally evaluate the different stages of the reproductive process.

The reduced fertility of Btc transgenic females could be attributed to one or more reproductive dysfunctions, such as a decreased ovulation, fertilization or implantation. Therefore, we have studied different aspects of Btc transgenic female's and male's reproduction, including puberty initiation, ovulation, *in vivo* and *in vitro* oocyte maturation, sperm parameters, *in vivo* and *in vitro* fertilization, and implantation in order to uncover the reason for their reduced fertility.

Successive matings of Btc transgenic males and females mice with wild-type mice revealed a decrease in litter size as compared with litters produced by control matings. However, the interval to the first litter was not significantly different between

groups. Litter size development showed a significant difference between Btc transgenic females and controls females. The onset of puberty occurred essentially at the same age in transgenic and non-transgenic females. The implantation of the Btc transgenic mice was delayed, but this was not the reason for the litter size reduction, because the mean number of total embryos either attached or recovered from the uterus of transgenic females was already markedly reduced when compared to the number of embryos present in the uterus of control females. For this reason, the explanation must be found in processes taking place before implantation (ovulation or fertilization). We evaluated the number of ovulated oocytes and observed that this parameter did not differ between the two genotypes, however, we observed a statistically significant reduction in the percentage of fertilized oocytes in transgenic as compared to control females, identifying the reason for the reduction in the litter size. Next, we carried out *in vitro* maturation of oocytes. The timing of nuclear maturation differed between transgenic and control oocytes. Therefore, we decided to evaluate the *in vitro* fertilization rate, which turned out to be impaired in the transgenic group. The expression pattern at the cellular level, studied by immunohistochemistry, revealed a high expression of Btc in the transgenic *cumulus* cells, which could be an explanation for the altered *in vivo* and *in vitro* fertilization.

Although the fertility of Btc transgenic males appears to be impaired, these animals do not display evident alterations in sperm production. This study provides evidence that Btc overexpression does not negatively affect spermatogenesis, sperm motility, progression and concentration values.

Future studies are needed to clarify whether the altered fertilization is in fact caused by the high expression of Btc in the transgenic *cumulus* cells. Furthermore, experiments involving the overexpression of a non-sheddable form of Btc are underway and will help to clarify the actions of precursor (membrane-bound) versus mature Egfr ligands during oocyte maturation and fertilization.

7 ZUSAMMENFASSUNG

Fruchtbarkeitsstörungen bei transgenen Mäusen, die Betacellulin überexprimieren

Wachstumsfaktoren regulieren viele zelluläre Funktionen durch autokrine, parakrine, juxtakrine oder endokrine Mechanismen. Bei Mensch und Tier spielen die EGF- (epidermale Wachstumsfaktoren) ähnliche Peptide eine übergeordnete Rolle, weil sie in den unterschiedlichsten Bereichen von Physiologie und Pathologie Einfluss nehmen können. In der Fortpflanzung werden durch die EGF-Familie essentielle Mechanismen ausgelöst. Es konnte zum Beispiel gezeigt werden, dass unterschiedliche Egfr Liganden für die Oozytenentwicklung, deren Ovulation, in die präimplantative embryonale Entwicklung, und in die Implantation von Bedeutung sind.

Btc, ein Mitglied der Egf Familie, wurde erstmals bei einer Mäuseinsulinomzelllinie beschrieben. Eine Btc-Expression kann in vielen Geweben der Maus nachgewiesen werden und weist ein besonders hohes Niveau im Herzen, in der Lunge, in der Leber, in der Niere, im Pankreas, im Dünndarm, im Dickdarm, im Hoden, im Eierstock und in der Gebärmutter auf. Es wurde festgestellt, dass Btc einer der Egfr Liganden ist, die in der Gebärmutter der Maus ausschließlich zur Zeit der Implantation exprimiert werden. Außerdem kann es als Mediatoren des luteinisierenden Hormons (LH), der Prostaglandine (PGs) und des Progesteron Rezeptors (PGR) fungieren.

Btc-knock-out Mäuse sind lebensfähig, fruchtbar und zeigen keinen offensichtlichen Phänotyp. Transgene Mäuse, die Btc überexprimieren, haben jedoch eine Reihe von phänotypischen Veränderungen.

In der vorliegenden Untersuchung wurden Btc-transgene Mäuse eingesetzt, um die Effekte eines erhöhten Spiegels dieses Wachstumfaktors in der weiblichen und männlichen Fortpflanzung zu studieren. Die Beobachtung, dass die Anpaarung von transgenen Weibchen im Rahmen der Routinezucht oft erfolglos (keine Trächtigkeiten oder verringerte Wurfgröße) war, veranlasste uns dazu die unterschiedlichen Stadien der Fortpflanzung funktionell auszuwerten.

Die verringerte Fruchtbarkeit der Btc-transgenen Weibchen kann mit einer oder mehreren reproduktiven Funktionsstörungen wie einer verringerten Ovulation,

Befruchtung und/oder Implantation zusammenhängen. Deswegen haben wir die unterschiedlichen Phasen in der weiblichen und männlichen Fortpflanzung der Btc-Mäuse untersucht. Um den Grund für ihre verringerte Fruchtbarkeit aufzudecken, wurde der Anfang der Pubertät, die Ovulation, die *in vivo* und *in vitro* Oozytenreifung, Spermienzellenparameter, die *in vivo* und in *vitro* Befruchtung und die Implantation untersucht.

Im Rahmen von aufeinanderfolgenden Anpaarungen (Daueranpaarungen) von Btc-transgenen Männchen und Weibchen mit Wildtyp-Mäusen konnte eine Reduzierung der Wurfgröße im Vergleich zu Würfen von Wildtyp-Anpaarungen errechnet werden. Die Intervalle zwischen Anpaarung und erstem Wurf unterschiede sich in den Gruppen nur unerheblich. Die Wurfgrößenentwicklungen im Laufe der Daueranpaarung von Btc-transgenen Weibchen zeigten jedoch erhebliche Unterschiede im Vergleich zu der von Wildtyp-Weibchen auf. Der Anfang der Pubertät trat im Wesentlichen im gleichen Alter bei transgenen und Wildtyp-Weibchen auf. Die Implantation in Btc-transgenen Mäusen war verzögert, das war allerdings nicht der Grund für die kleineren Wurfgrößen, da die Anzahl der implantierten oder aus der Gebärmutter gespülten Embryonen von transgenen Weibchen im Vergleich mit der Zahl der Embryonen aus wild-typ Weibchen bereits deutlich reduziert war. Somit musste die Erklärung für die verringerte Fruchtbarkeit in der Zeit vor der Implantation (Ovulation oder Befruchtung) gesucht werden. Wir werteten die Anzahl der ovulierten Oozyten (Ovulationsrate) aus und konnten hierbei keinen Unterschied zwischen den Genotypen feststellen. Da wir jedoch eine statistisch signifikante Reduktion des Anteils an befruchtetet Oozyten in transgenen, verglichen mit wild-typen Mäusen, beobachteten konnten, war damit eine mögliche Erklärung für die geringere Fruchtbarkeit und die verminderte Wurfgröße gefunden. Mittels *in vitro* Maturation konnte gezeigt werden, dass der Anteil an Oozyten im Meiose II Stadium in der transgenen Gruppe signifikant reduziert war. Die Auswertung der Befruchtungsrate nach in vitro Fertilization zeigte, dass auch dieser Parameter bei den transgenen Weibchen signifikant reduziert war. Das Expressionsmuster auf zellulärer Ebene, untersucht mittels Immunhistochemie, ergab eine hohe Expression von Btc in den transgenen Cumuluszellen. Dies könnte eine Erklärung für die gestörte *in vivo* und *in vitro* Befruchtung sein.

Diese Studie zeigt, dass eine Btc-Überexpression die Spermatogenese, Spermienzellenmotilität, progressive Motilität und die Konzentration der Spermien

nicht negativ beeinflusst.

Zukünftige Studien müssen mögliche Zusammenhänge zwischen der hohen Expression von Btc in transgenen Cumuluszellen und der veränderten Befruchtungsfähigkeit abklären. Außerdem sind Experimente, die die Überexpression einer „non-sheddable“ (nicht abspaltbar) Form von Btc berücksichtigen in Planung. Diese werden helfen, die Wirkung des membrangebundenen Precursorproteins im Vergleich zu reifen Egfr Liganden während der Oozytenreifung und der Befruchtung zu erklären.

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