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Signal Crosstalk Promoted Proliferative Lesions in Mouse Mammary Glands As a Consequence of ET-1 Overexpression

ET-1 Aşırı Anlatımının Bir Sonucu Olan, Sinyal Çapraz Etkileşimi 'Crosstalk', Fare Meme Bezlerinde Proliferatif Lezyonların Oluşumunu Teşvik Eder

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ABSTRACT

Objective: As a distinct cellular signaling model, a receptor crosstalk between *G protein-coupled receptors (GPCR)* and *epidermal growth factor receptor (EGFR)* has been demonstrated in various *in vitro* studies. In addition, recent *in vitro* studies had focused on the signaling pathways of *endothelin-1 (ET-1)* in the pathophysiology of cancer. Accordingly, a growing interest in the analysis of the receptor crosstalk between *ET-1* receptors and EGFR and functional consequences of EGFR activation of proliferative diseases evoked us to the analysis of this phenomenon *in vivo*.

Materials and Methods: We performed a comparative study between *ET-1* transgenic mice and control mice during the late pregnancy (n=7), early lactation (n=6) the mid of lactation (n=10) and involution day 14 (n=7) periods. Hematoxylin and eosin (HE)-stained parallel sections from mammary glands were microscopically examined. The key signal proteins (*ETAR, ETBR, ERK1/2, pEGFR*) in transactivation of *EGFR* were analyzed employing Western blot techniques. Genes (*amphiregulin, TGFa, EGF, HB-EGF, ADAM 17*) known to play an important role in these activities were analyzed using real-time PCR (RT-PCR) techniques.

Results: *ET-1* transgenic mice exhibited hyperproliferative lesions (*lactational hyperplasia*) during the middle of the lactation period. Our RT-PCR analyses showed a prominent up regulation of *amphiregulin* and *ADAM 17* in *ET-1* transgenic mice. Moreover, we found higher *EGFR* and *ERKs* activations in the transgenic mammary glands.

Conclusion: This study highlights a causative effect of upregulated *ET-1* gene expression on the induction of proliferative lesions via *EGFR* transactivation in mammary glands. Further, *ET-1* overexpression induced an upregulation of *amphiregulin* and *ADAM17* expressions in the transgenic mammary glands. These results suggests that the enhanced *ET-1* gene expression and its receptors might have a crucial role in proliferative diseases maintaining *EGFR* activation.

Keywords: *Endothelin-1*, *EGFR* transactivation, lactational hyperplasia, *amphiregulin*, transgenic mice

ÖZ

Amaç: Farklı bir hücresel sinyalleşme modeli olarak, *G proteini ile eşleşmiş reseptörler (GPCR)* ve *epidermal growth faktör reseptör (EGFR)* arasındaki reseptör çapraz etkileşimi (crosstalk), çeşitli *in vitro* çalışmalarda gösterilmiştir. Ayrıca, son dönem *in vitro* çalışmalar, *endotelin-1 (ET-1)* sinyal yolağının kanser patofizyolojisi üzerindeki ilişkisine odaklanmıştır. Bu bağlamda, *ET-1* reseptörleri ve *EGFR* arasındaki reseptör çapraz etkileşim analizine ve *EGFR* aktivasyonunun fonksiyonel sonuçlarından proliferatif hastalıklara olan artan ilgi, bize bu fenomeni *in vivo* analiz etme fikrini oluşturmuştur.

Gereç ve Yöntem: *ET-1* transgenik fareler ve kontrol fareleri arasında geç gebelik dönemi (n=7), erken laktasyon dönemi (n=6) orta dönem laktasyon (n=10) ve involusyon 14. gün (n=7) sırasında karşılaştırmalı bir çalışma gerçekleştirildi. Meme bezlerinden hematoksilen-eozin (HE) ile boyanmış paralel kesitler mikroskobik olarak incelendi. EGFR'nin transaktivasyonunda görev alan anahtar sinyal proteinleri (*ETAR, ETBR, ERK1/2, pEGFR*), Western blot teknikleri kullanılarak analiz edildi. Bu aktivitelerde önemli bir rol oynadığı bilinen genler (*amfiregulin, TGFa, EGF, HB-EGF, ADAM 17*), gercek zamanlı polimeraz zincir reaksiyonu (RT-PCR) teknikleri kullanılarak analiz edildi.

Bulgular: *ET-1* transgenik fareler, laktasyon döneminin ortasında hiper-proliferatif lezyonlar (*laktasyonel hiperplazi*) geliştirdi. *RT-PCR* analizlerimiz, transgenik farelerde *amfiregulin* ve *ADAM 17* gen anlatımlarında belirgin bir artış olduğunu göstermektedir. Ayrıca transgenik meme bezlerinde daha yüksek *EGFR* ve *ERKs* aktivasyonları tespit edilmiştir.

Sonuç: Bu çalışma, yüksek seviyede *ET-1* gen anlatımının *EGFR* yi transaktive etme yoluyla meme bezlerinde proliferatif lezyonları tetiklediğini göstermektedir. Ayrıca, *ET-1* aşırı anlatımı, transgen meme bezlerinde *amfiregulin* ve *ADAM17* anlatımlarının belirgin bir şekilde yukarı regülasyonuna neden olmaktadır. Bu sonuçlar bize *ET-1* gen ekspresyonunu ve reseptörlerinin *EGFR* aktivasyonunu sağlayarak proliferatif hastalıklarda önemli bir role sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Endotelin-1*, *EGFR* transaktivasyonu, laktasyonel hiperplazi, *amfiregulin*, transgenik fareler

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INTRODUCTION

Endothelin-1 (ET-1), an important substance in the maintenance of vascular tone, was firstly isolated and characterised by Yanagisawa from cultured supernatant of porcine aortic endothelial cells (6). *ET-1* is expressed as an inactive precursor peptide comprising of 212 amino acid residues, named preproendothelin. Two endopeptidases control the activation process of preproendohelin (1). Firstly, the prepro form of endothelin is cleaved by a dibasic endopeptidase to form big *ET-1* or *proET-1* which is biologically inactive. Secondly, the conversion of *proET-1* to the biologically active *ET-1* is catalysed by a furin like protease named *endothelin converting enzyme (ECE)*. The cleavage occurs in the intracellular compartments and on the cell surfaces and leads to the active *ET-1*. The active *ET-1* comprises 21 amino acids and is folded by two disulphide bridges designating the half-life of the biological activity (2). The half-life of *ET-1 in vivo* is less than one minute whereas *proET-1* lasts approx. 20-25 mins (3). Therefore, under normal physiological conditions *ET-1* is not a circulating hormone but rather *ET-1* functions in an autocrine or paracrine manner at multiple locations in the body (4,5).

ET-1 exerts its effect by binding to two distinct *G protein-coupled receptors (GPCR)*, the *endothelin A (ETAR) and endothelin B receptor (ETBR)*. However, on the functional level both receptors differ, *ETAR* predominantly mediates vasoconstriction in vascular smooth muscle cells (6) whereas, *ETBR* activation results in vasodilatation in vascular endothelial cells (7).

In addition to typical *GPCR* signal characteristics, these receptors are also able to communicate with structurally unrelated receptors (transactivation) such as the epidermal growth factor receptor (*EGFR*), the most prominent receptor tyrosine kinase, (8-10) resulting in *EGFR* characteristic intracellular signals (Figure 1). The *EGFR* mediated signaling pathway is positioned to affect duct formation as well as the outgrowth and branching of the mammary gland during pregnancy (11-13).

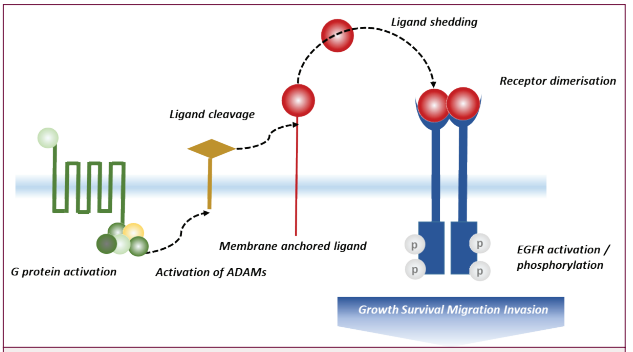

Figure 1. *EGFR* Transactivation mechanism: G Protein Coupled Receptor (*ETAR / ETBR)* activates metalloproteases *(ADAM 17*) which cleave and release the inactive *EGFR* ligands (*amphiregulin*) from cell surface. Free active ligand amphiregulin binds to its receptor (*EGFR*) and lead the downstream signaling cascade of *EGFR* (*illustrated by Nadir Gül*).

Further, recent studies reported that morphogenesis of the mammary gland requires paracrine activation of the *EGFR* via metalloprotease dependent shedding of *amphiregulin* (14,15). Transactivation of the *EGFR* is regulated by various cellular responses such as overexpression, amplification or mutation of critical pathway elements with variable functional outcomes which are frequently linked to hyperproliferative diseases.

Indeed, *GPCR* induced *EGFR* transactivation was found to mediate cell proliferation in breast cancer cells (16,17) and an increase of tumorigenicity in ovarian cancer cells (18). Activation of *EGFR* affects a wide range of cellular responses, depending on the coordinate expression of the cognate ligand (19). Up to now, eight EGF like ligands that directly activate the *EGFR* have been identified: *EGF, TGFα, heparin binding-EGF (HB-EGF), amphiregulin, betacellulin, epiregulin, epigen and cripto* (20-25). Each of these molecules activates receptors of the HER family of tyrosine kinase receptor by autocrine or paracrine stimulation (26). The upregulation of these ligands is believed to be critical for the tumor growth.

A number of studies point out receptor crosstalk under controlled experimental models such as cancer cell lines. The purpose of this study was to compare the results of the above-mentioned *in vitro* studies under *in vivo* and non neoplastic physiology. Therefore, we also systematically analyzed possible tumorigenic effect of *ET-1* via receptor transactivation on *ET-1* overexpressing mice model.

MATERIALS AND METHODS

Mouse Models

In this study the outbred strains NMRI (Harlan-Winkelmann, Paderborn) as wild type and NMRI originated homozygotic human ET-1 transgenic mice were used. These transgenic mice have been extensively characterized for their cardiovascular and renal phenotypes (27).

The animal experiments were maintained by dividing the groups of animals under three physiologic conditions; Pregnancy day 18 (n=7), lactation day 3 (n=6), lactation day 14 (n=10) and involution day 14 (n=7).

Mammary gland samples were obtained free of the muscles of the anterior abdominal wall and skin by blunt dissection on late pregnancy and lactation physiological stages. Animals had free access to standard mouse chow (mouse chow 5015) and tap water. The mice were routinely screened for common mouse pathogens.

The animals were sacrificed by cervical dislocation. In general, male and female mice were held separately in groups of 2-6 animals. All animal experiments were conducted in accordance with the German Law for animal protection (Tierschutzgesetz).

Histology, Immunohistochemistry; The mammary glands were fixed in paraformaldehyde for a minimum of 12 hours at room temperature. After the fixation process, the tissue sam-

ples were embedded in paraffin blocks and cut in at 5mm sections. The sections were submitted to hematoxylin–Eosin (HE) staining or incubated with SMA antibody. These slides were evaluated with blinded analytical protocol.

Western Blots Analyses

Frozen mammary gland samples were pulverized in LN2 with a mortar and a pestle. 100 mg tissue samples were homogenized in urea lysis buffer. After the centrifugation, the supernatants were placed into new tubes. Protein concentration was measured with the Bradford colorimetric assay.

Samples (25µg) were run on SDS (12%) polyacrylamide gels, blotted onto nitrocellulose membrane (Amersham) and incubated with blocking solution in TBS with 0.1% tween 20 for 1 hour at room temperature. Membranes were incubated with primary antibody diluted in blocking solution overnight at 4°C and detected using horseradish peroxidase conjugated secondary antibodies in conjunction with ECL (Amersham).

Immunoprecipitation; Grinded mammary gland samples were lysed in ice cold RIPA buffer. Lysates containing 400 µg of proteins in RIPA buffer were incubated with 2µg/ml *EGFR* antibody at 4°C overnight. The immune complex was precipitated with protein A sepharose CL-4B beads at 4°C for 1hr. The beads were washed (3 times) with PBS containing tween-20 (1%) and spin down by centrifugation (2000 rpm for 1 min), re-suspended in RIPA buffer and boiled for 5 min at 95°C in a thermomixer (Eppendorf). The samples were spin-down at 2000 rpm for 1 min. The supernatants were carefully placed in new tubes and immunoblotted with *EGFR (pY845)* and *EGFR (Y1005),* respectively (Table 1).

Gene Expression Analyses

Total RNA was extracted from quick-frozen mammary glands (50-100 mg per sample) using standard trizol extraction method (Invitrogen). RNA quantification was performed with a bio-analyzer (Agilent 2100) using a microfabricated chip (Agilent RNA 6000 Nano). The system was able to report not only the amount of RNA but also the 260/280 Nm ratio and the RNA integrity number (RIN). RIN is a scale (from 1 to 10) to measure degradation and quality of RNA. Higher RIN value represents a better quality of RNA. Less than 9 RIN scaled samples were not used.

Prior to cDNA synthesis, all crude RNA samples were routinely treated with DNase1 (TurboDNase, Ambion). cDNA was synthetized from 1000 ng of total RNA using Promega MMLV reverse transcriptase enzyme with oligo(dT)18 Primers according to the manufacturer's protocol.

For the quantitative detection, the primers were designed using Ensembl gene data base and employing a commercial software (Primer 3) and web based algorithms (NCBI Blast, multi align) otherwise, the primer sets used from the literature were specified. All these primers were purchased from Invitrogen, Oligo (dT)18 were obtained from Promega (Table 2). The quantitative PCRs were performed using sybergreen mastermix (Applied Biosystems) and were carried out (25 ng template per test) in triplicate in MX 3700p Real-time PCR (RT-PCR) system (Stratogene).

Statistical Analyses

Western blots analyses; The digitized membranes were evaluated by special software (AlphaEaseFC) and the densitometric data were analyzed using a student's t-test by a statistical analysis program (Graphpad prism).

Gene Expression Analyses; Primer efficiency, standard curve and expression analyses were performed with the software Relative Expression Software Tool (REST 2008) which is using Pair Wise Fixed Reallocation Randomisation Test for statistical analyses (28).

RESULTS

ET-1 acts through its two G-protein coupled receptors, the *ETAR* and the *ETBR*. To determine whether overexpression of *ET-1* in transgenic animals was accompanied by changes in the expression levels of these two receptors, expression of both re-

Table 1. The following antibodies were used in immunoprecipitation and Western blot analyses

Antibody	Origins and Dilutions	Molecular weight (kDa)	Reference
pEGFR	Rabbit polyclonal antibody (tyr 845)	170	Cell signaling Tech.
EGFR	Rabbit polyclonal antibody (tyr 1005)	170	Santa Cruz Biotech.
ETAR	Rabbit polyclonal antibody	69	Cell signaling Tech.
ETBR	Rabbit polyclonal antibody	49/34	Cell signaling Tech.
pERK	Rabbit polyclonal antibody	42/44	Cell signaling Tech.
ERK	Rabbit polyclonal antibody	42/44	Cell signaling Tech.
SMA	Mouse monoclonal antibody	--	Sigma
Antimouse Ig	Rabbit, HRP linked polyclonal antibody	--	Dakocytomation
Antirabbit Ig	Goat, HRP linked polyclonal antibody		Dakocytomation

Table 2. The following primer sets were used in RT-PCR analysis.

Gene	Forward Primers (5'-3')	Reverse Primers (5'-3')
ADAM 17 (mouse) (56)	ACTCTGAGGACAGTTAACCAAACC	AGTAAAAGGAGCCAATACCACAAG
Amphiregulin (mouse)	TCTTGGGCTTAATCACCTGTTC	GGGGACTACGACTACTCAGAG
EGF (mouse)	CTAAGGATCCTGACCCCGAACT	GTACAGCCGTGATTCTGAGTGG
EGFR (mouse)	GCCAATAATGTCTGCCACCT	TCCCAGTGGCAATAGATGGT
ETAR (mouse)	GCTGGTTCCCTCTTCACTTAAGC	TCATGGTTGCCAGGTTAATGC
ETBR (mouse)	TGTGCTCTAAGTATTGACAGATATCGAG	GGCTGTCTTGTAAAACTGCATGA
HB-EGF (mouse)	TGAACCTTTTCAAAGTTGCTTTCT	CGTGGATGCAGTAGTCCTTGTA
GAPDH (mouse)	CTTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGAG
TGFa (mouse)	CAGAGGGCAGTACAGTTGATTCAG	GAAGACATCCTGGGCAAGC

ceptors in the mouse mammary glands of day 3 lactating wild type and transgenic *ET-1* mice was determined at the mRNA and the protein level by RT-PCR and Western blotting, respectively. The mRNA expression of the *ETAR* and *ETBR* in *ET-1* transgenic mice did not differ from wild type animals. Additionally, this result was confirmed at the protein level for the two receptors (Figure 2).

Throughout the second half of lactation, some parts of the mammary gland displayed intense lobular proliferation with cells characterized by increased cytoplasmic volume and enlarged nuclei (Figure 3 C, D) in the transgenic group.

Although no definite signs of pathological dysplasia were detected, alveolar organization was lost in parts of these areas. Therefore, immunohistostaining of smooth muscle actin was employed in order to rule out an underlying malignant transformation. HE and *smooth muscle actin (SMA)* immunohistostaining was performed on parallel sections obtained from wild type and *ET-1* transgenic mammary glands. The slides were immunostained with an anti-*SMA* antibody and show brown colored myoepithelial cells enclosing the alveolar epithelial cells blue colored (Figure 3, F, H). The slides not treated with *SMA* antibody were utilized as negative control, ensuring specifity of the *SMA* immunohistostaining (Figure 3 E, G). *SMA* immunostaining clearly manifests the non-neoplastic structure by the presence of myoepithelial cells surrounding alveoli in the *ET-1* transgenic mice.

EGFR activation has an important role in mammary ductal outgrowth and branching (29,30). Deregulation of the activity of this receptor has a strong correlation with tumor progression

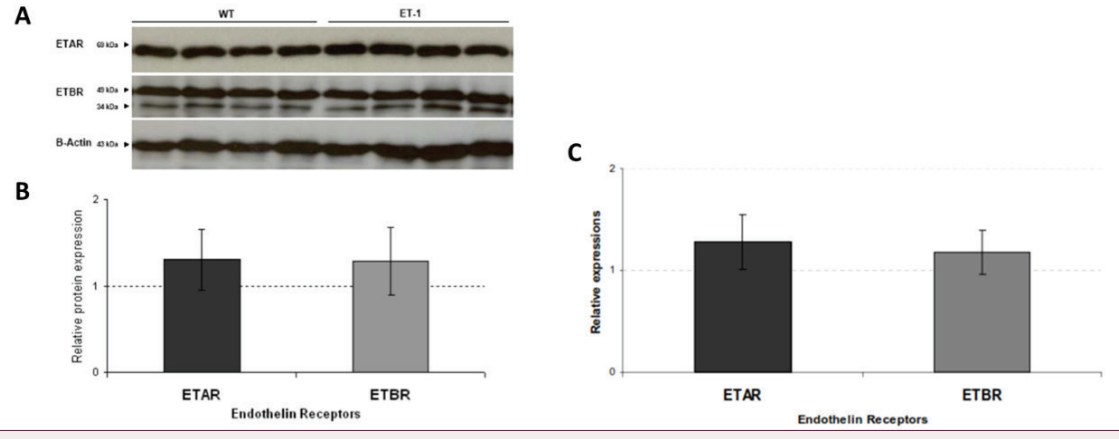

Figure 2. A. Western blot analysis of the two *ET-1* receptors *ETAR* (69kDa) and *ETBR* (49/34kDa) in mammary glands of wild type and transgenic mice. *Beta-actin* (43kDa) served as loading control. B. Densitometric analysis of endothelin receptors at lactation day 3, densities of the receptors were normalized with Beta-actin density n=4 females for each group. C. Analysis of the relative mRNA expression levels of the two *ET-1* receptors, *ETAR* and *ETBR*, in mammary glands at lactation day 3 employing RT-PCR. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). n=4 females for each group no significant difference was observed.

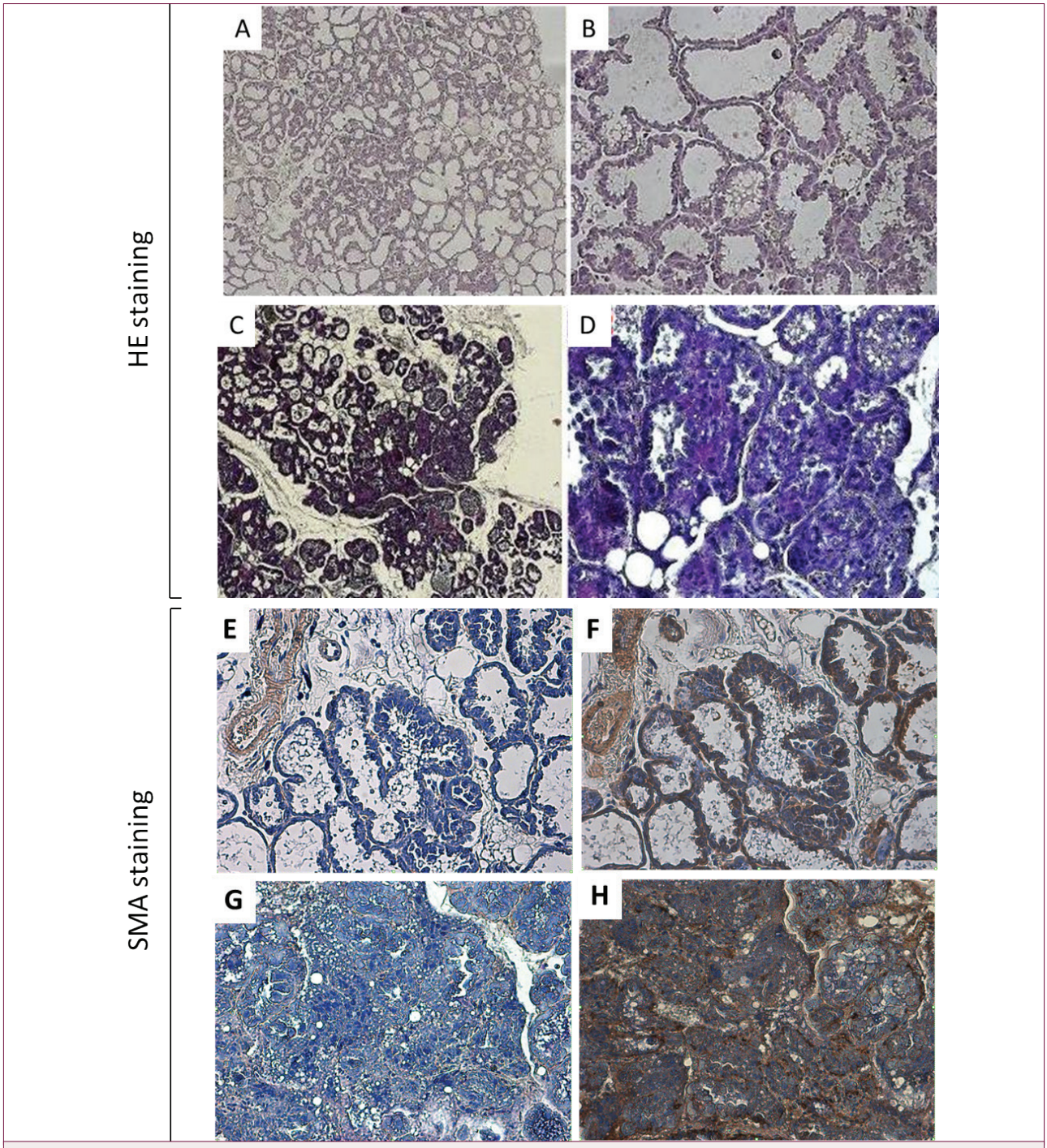

Figure 3. Histological features of the mammary gland at the lactation day 14. HE stained tissue sections; wild type: A; wild type: B; *ET-1*: C (x 50 magnification); *ET-1*: D (x 200 magnification). Wild type mammary glands exhibited fully expanded alveoli reflecting the highly active secretory capacity (A, B). *ET-1* transgenic mammary glands exhibited focal hyper proliferative lesions (C, D). SMA stained tissue sections; in order to visualize any possible cross reactivity for *SMA* staining parallel slides were treated without *SMA* antibody as negative control. Wild type: E; *ET-1*: G (x 200 magnification), myoepithelium was stained by a *SMA* specific immunohistostaining method; brown colored areas in both groups indicated the *SMA*, wild type: F; *ET-1*: H (x 200 magnification), the wild type group demonstrated well organized alveoli and the individual alveoli are surrounded by blood vessels. On the other hand, the *ET-1* group demonstrated hyper proliferative epithelium with *SMA* staining alveolar lumen and borders of alveoli become visual.

(31). Lactational hyperplasia (Figure 3 G, H) could be related to *EGFR* activation due to its central role on tumorigenesis. Therefore, *EGFR* phosphorylation was analyzed at lactation day 14 using immunoprecipitation techniques. As demonstrated in figures 5, an increased phosphorylation at residue *Y845* of the *EGFR* was observed in *ET-1* transgenic mice compared to wild type. The phosphorylation at residue *Y998* and *Y1068* of the *EGFR* did not changed (data is not shown).

*ERK*s represent critical downstream molecules for *ET-1* induced *EGFR* transactivation and might therefore be related to initiation of lactational hyperplasia. Hence, *ERK* activation was analyzed at pregnancy day 18 and lactation day 3 (Figure 4 C, D). The Western blot analysis represented that during pregnancy day 18, as well as during lactation day 3, a pronounced activation of *ERK* 1 and *ERK*2 signaling could be detected in the mammary glands of *ET-1* transgenic mice when compared to wild type mice (Figure 4E).

In order to identify ligands which are potentially involved in the observed activation of the *EGFR*, RT-PCR analyses were employed and the expression of *EGF, TGFa, HB-EGF* and *amphiregulin* was quantified at the transcript level.

As shown in figure 5A, expression analyses on the 14th day of lactation demonstrated only a significant increase for the *amphiregulin* level in *ET-1* transgenic compared to wild type mice, whereas the expression level of other ligands remained unchanged.

It is known that, *amphiregulin* expression was found to be increased during pregnancy but after parturition its transcription dramatically decreased due to its functional role on ductal development of the mammary gland (32,33). Whether this significant upregulation of *amphiregulin* synthesis caused by high *ET-1* expression, RNA levels of *amphiregulin* were analyzed at pregnancy day 18. As depicted in Figure 5B *amphiregulin* is also significantly upregulated at pregnancy day 18.

ADAM 17, the transmembrane metalloproteinase, is responsible for *amphiregulin* activation (54). Therefore, *ADAM 17*, as a key component of this signaling mechanism, was also analyzed employing RT-PCR techniques. As shown in figure 5C, *ADAM 17* expression was significantly upregulated in *ET-1* transgenic mice.

Following the lactation period, *ET-1* transgenic mice were analysed with HE staining in order to find out the presence of

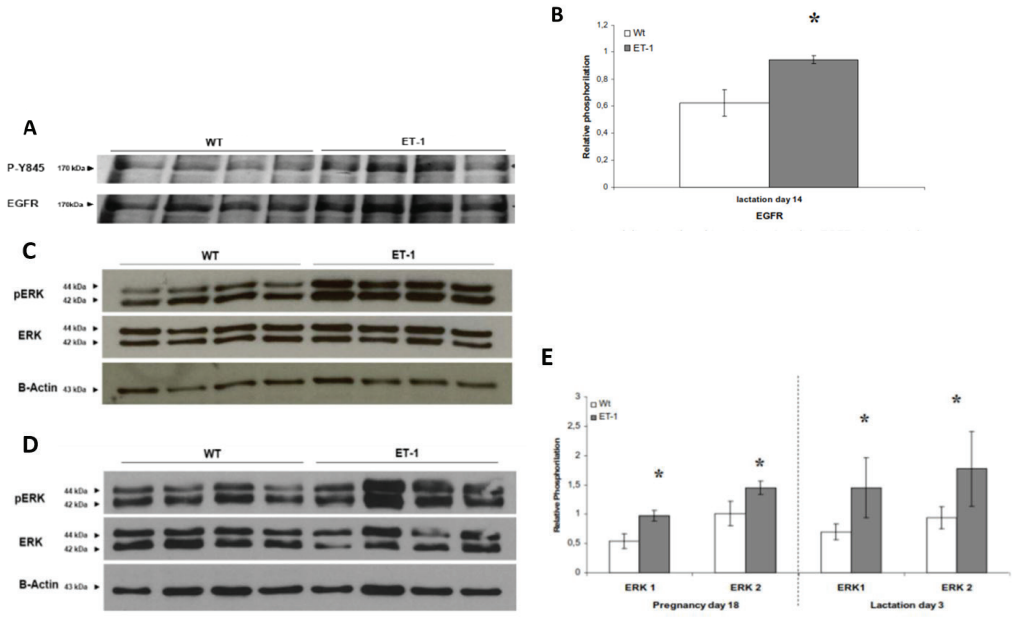

Figure 4. A. Analysis of the *EGFR* phosphorylation at lactation day 14 was determined by immunoprecipitation. ~400 µg protein lysate for each sample has been used for immunoprecipitation with an *EGFR* specific antibody (with *Y1005*), activation of *EGFR* has been monitored by phosphor specific antibody *Y845* and *EGFR* served as loading control. B. The representative densitometric analysis of relative *EGFR* phosphorylation in wild type and *ET-1* transgenic mice on the 14th day of lactation. *ET-1* transgenic mammary glands exhibited significantly more phosphorylated *EGFR* than the wild type counterparts C. At 18th day of pregnancy, protein expression and phosphorylation of *ERK* 1 (44kDa) and *ERK* 2 (42 kDa), proteins were determined by Western blotting. Beta-actin served as control for loading. 25µg protein extract was loaded for each sample. D. 3 rd. day of lactation protein expression and phosphorylation of *ERK* 1 (44kDa) and *ERK* 2 (42 kDa). E. The representative densitometric analysis of relative *ERK* 1 and *ERK* 2 phosphorylation *ET-1* transgenic mammary glands exhibited significantly more phosphorylated *ERK* 1 and *ERK* 2 than the wild type counterparts (n=4 mice for each group. (*) p<0.05 Student´s t-test).

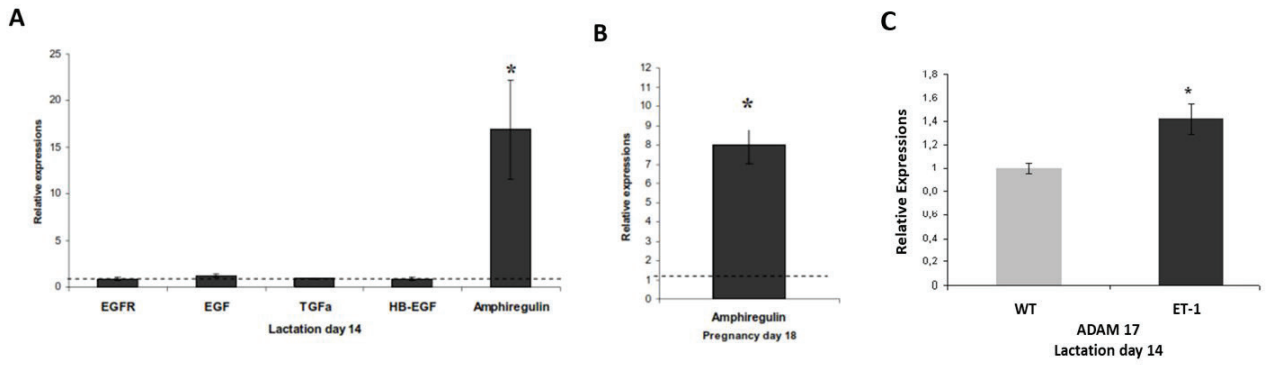

Figure 5. A. Relative expression of *EGFR* and its prominent ligands EGF, TGFa, HB-EGF, Amphiregulin at lactation day 14. B. Relative expression of *amphiregulin* at pregnancy day 18 C. Relative expression of ADAM17 at lactation day 14. The relative expression levels were compared to wild type mice expression levels, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (28) $p<0.05$ n=5 mice for each group.

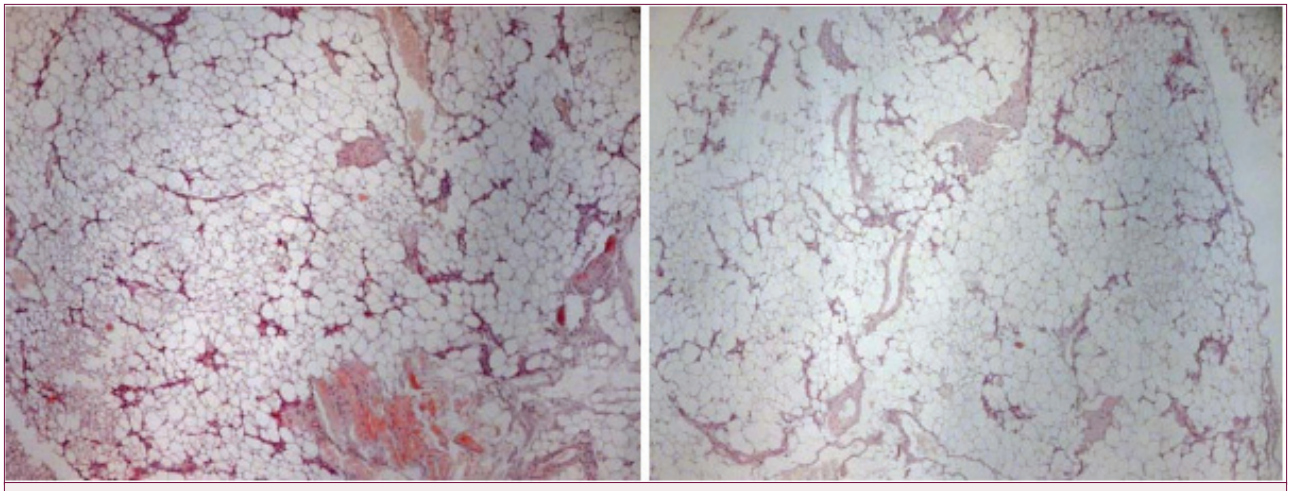

Figure 6. Histological features of the mammary gland at the 14th day of involution. The tissue sections were stained with HE of wild type (left) and *ET-1* transgenic mammary glands (right) (x 50 magnification). Both mammary glands showed same histological patterns of involution with the adipocytes and rudimentary epithelium. No hyperproliferative lesion or any type of irregularity was detected in both animal groups.

benign tumors at the involution day 14 stage. Throughout the involution no proliferative lesions were detected (Figure 6).

DISCUSSION

Along with its well-known effect on cardiovascular biology, *ET-1* has also been associated with a wide range of biological activities including mitogenic and proliferative responses in vascular smooth muscle cells and other cell types (34). However, this proliferative aspect of *ET-1* is not only involved in developmental progress. For instance, increased *ET-1* expression has been demonstrated in a variety of solid tumors thereby promoting growth and inhibiting apoptosis in breast cancer while decreasing tumor cell differentiation (35-38). However, none of these reports described the causative effect of *ET-1* on hyperproliferative lesions.

In this study, *ET-1* transgenic mice displayed intense lobular proliferation in various grades in mammary glands during lactation. Further, alveolar organization was lost in parts of these areas (Figure 3). Suggesting the overexpression of *ET-1* might contribute to mammary tumorigenicity.

One indication of a neoplastic progression is the loss of normal tissue architecture, including polarity. Generally, primary breast carcinomas show a dramatic increase in the ratio of luminal to myoepithelial cells, and many invasive breast carcinomas essentially lack myoepithelial cells completely (39). After the *SMA* immunohistostaining, these histological observations pointed to lactational hyperplasia in *ET-1* transgenic mammary glands during the middle of the lactation period (Figure 3 G, H).

The lactational hyperplasia is known as a benign mammary lesion unique to pregnancy and lactation. This lesion may occur in response to the alteration of physiological conditions which represent pregnancy and lactation and it diminish spontaneously after the lactation (40,41).

A possible causative effect of *ET-1* overexpression on the development of lactational hyperplasia might have been mediated by the interaction of its *GPCR*s with the *EGFR*s, since *ET-1* is known to possess growth regulatory properties (3,8,42).

ET-1 exerts its effect by binding to two distinct *GPCR*, *ETAR* and *ETBR*. In addition to typical *GPCR* signal characteristics, these receptors are also able to communicate with structurally unrelated receptors such as the *EGFR*, the most prominent receptor tyrosine kinase (8,9), resulting in *EGFR* characteristic intracellular signals. The expression level of both endothelin receptors was determined using RT-PCR and Western blotting (Figure 2) and no difference could be detected between wild type and transgenic animals, suggesting that the increase in expression of *ET-1* is not influencing the expression of its receptors in this biological system.

Initial Western blot studies performed with mammary glands derived from *ET-1* transgenic mice were not able to detect an *EGFR* signal during the lactation period. Therefore, immunoprecipitation was employed to detect total *EGFR* and phosphor- *EGFR*. Structurally, *EGFR* contains multi phosphorylation sites in intracellular domains, and several tyrosine (Y) phosphorylation domains *(Y845, Y998 and Y1068)* were analyzed during initial studies on *EGFR* activation (data not shown). Figure 4 *ET-1* transgenic mice exhibits significantly more active *EGFR* phosphorylation on the specific domain tyrosine 845 residue. It has been reported that various *GPCR* agonists including *ET-1* could stimulate the phosphorylation of *Y845* in the *EGFR in vitro* (43).

Due to the technical difficulty to demonstrate the *EGFR* transactivation *in vivo*, we have analyzed also *ERK* protein activation which is the most well defined signalling pathway from the cell membrane to *ERK* 1 and *ERK* 2 is that mediated by the *EGFR* (44). *ET-1* stimulates *ERK* 1 and *ERK* 2 through cognate *GPCR* receptors by transactivation of the *EGFR* in various cell types and tissues (45,46). Additionally, increased *ERK* activation is documented in tubular hyperplasia as a result of *ET-1* induction (47,48). In Figure 4 C, D, E our Western blot studies represent that the transgenic group has significantly more active *ERK*1 and *ERK*2 during pregnancy and lactation stages which implies us *ET-1* induced *EGFR* transactivation *in vivo*.

To further characterize the assumed signaling network in *ET-1* transgenic mammary glands, the most widely expressed ligands *TGFa, EGF, HBEGF* and *amphiregulin*, involved in the activation of the *EGFR*, were analyzed using RT-PCR. As presented in the Figure 5 the *amphiregulin* expression was significantly upregulated in *ET-1* transgenic mice at lactation day 14 and pregnancy day 18 which is suggesting that the regulation of *amphiregulin* is influenced by the *ET-1* overexpression. None of the three other ligands did exhibit significant differences in their expression level.

It was demonstrated that *amphiregulin* is a unique *EGFR* ligand for ductal branching and ductal outgrowth (49). Normally, *amphiregulin* expression is increased during pregnancy and decreased dramatically after parturition (32,33). However, *amphiregulin* gene expression is upregulated in *ET-1* transgenic mice both during pregnancy and lactation periods.

Moreover, it has been noted that high *amphiregulin* expression is strongly associated with breast carcinomas and neoplastic progression (50,51). Several studies reported an increased *amphiregulin* expression in parallel to breast cancer. Therefore, *amphiregulin* was chosen as a pharmacological target for breast cancer treatment (52,53).

ADAM 17, the transmembrane metalloproteinase, is responsible for *amphiregulin* activation. This is the upstream of *EGFR* dependent of signal pathway (54). *ADAM* 17 was analyzed employing RT-PCR techniques. As shown in Figure 5C, *ADAM* 17 expression was significantly upregulated in *ET-1* transgenic mice during the lactation day 14.

The mechanism of *ET-1* induced *EGFR* transactivation might provide a molecular explanation how overexpression of *ET-1* could regulate the proliferative behaviour of tumor cells. Indeed, *ET-1* transgenic mice were found with an increased *EGFR* activation in their mammary glands during lactation. Moreover, *EGFR* activation is maintained by an increased *amphiregulin* expression (55) which again might be regulated by the observed *ET-1* overexpression.

Figure 6 represents mammary histology of both *ET-1* transgenic and wild type mice mammary glands at involution day 14. There was no residue of benign tumors found in *ET-1* transgenic mice. Probably apoptotic process was stronger than cell survival. Therefore, those benign lesions were diminished due to the absence of *ET-1* expression during the involution process. Since ET-1 expression does not take place within the involution we can not further elaborate tumorigenicity of ET-1in mammary gland physiology. However, the key components of signal transactivation (Figure 1) were found to be upregulated or more active while *ET-1* expressions were higher than normal state. This result suggests the causative effect of *ET-1* on pathophysiological alteration of the mammary glands taken place via *EGFR* transactivation.

Taken together, various studies underlining *amphiregulin* expressions and activation of *EFGR,* are positioned at the center of breast cancer or neoplastic transformation, as in this study, we focused on factors that might affect the regulation of this system. In this regard, the *ET-1* axis reaches an important point in the understanding of cancer biology by increasing and therefore activating the characteristic genes observed in neoplastic transformation. Indeed, recent studies confirm our thoughts on the development of anticancer drugs to block endothelial receptors (57,58).

In future studies, by further studying the properties of the signal downstream of the *ET-1* axis, we can find out more about the neoplastic transformation of the mammary glands on a larger scale.

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