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MMTV-EGF Receptor Transgene Promotes Preneoplastic Conversion of Multiple Steroid Hormone-Responsive Tissues

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Abstract Correlative analyses of tumors and patient-derived cell lines of the human reproductive system suggest that overexpression of EGF contributes to the oncogenic phenotype. However, it is unclear at what stage in disease overexpression of the EGFR is most critical. To assess its role as an initiator of reproductive tissue tumor development, transgenic mice were derived with mouse mammary tumor virus (MMTV)-regulated overexpression of the human EGFR. Although elevated expression of the EGFR in hormonally responsive tissues was observed, only one EGFR transgenic mouse developed a visible tumor over a 2-year period. However, of 12 females monitored over the same time, hyperplasia, hypertrophy, or slight dysplasia was found in mammary glands of 55% of the animals examined, in the uterus or uterine horn of 89%, and in ovaries or oviducts of 100%. None of the reproductive tissues of the male transgenic animals or age-matched, normal mice displayed these changes. These results revealed a role for the EGFR in the initiation of ovarian and uterine cancer and supported previous studies in breast cancer that the receptor can contribute to the neoplastic process in a significant albeit incremental way. J. Cell. Biochem. 103: 2010–2018, 2008. © 2007 Wiley-Liss, Inc.

Key words: epidermal growth factor receptor; transgenic mice; MMTV-promoter; mammary gland; uterus; ovary

The human epidermal growth factor receptor (hEGFR) is one of a family of receptor tyrosine kinases (RTK) that consists of four known members (EGFR, HER2, ErbB3, ErbB4). Upon binding of ligand, receptors dimerize and become catalytically activated, mediating transphosphorylation of their partners on carboxyl-terminal tyrosine residues, which in turn act as docking sites for multiple signaling proteins, such as Shc and Grb2, phosphatidylinositol-3 kinase (PI-3 kinase), phospholipase Cγ (PLCγ), p120catenin, c-Cbl, p130Cas, p120RasGAP, phosphotyrosine phosphatases (PTPases), and many others [Hunter, 2000; Schlessinger, 2000; Lo et al., 2006]. Activation of these proteins, in turn, initiates a cascade of signaling events that culminates in a variety of biological responses, including cell proliferation, differentiation, survival, and migration [Moller et al., 1989; Adamson and Wiley, 1997; Wiesen et al., 1999].

EGFR is found in ductal epithelial cells of normal breast, in uterine tissue, and in ovarian follicles where it plays key roles in normal tissue development and function [Moller et al., 1989; Wiesen et al., 1999; Wong et al., 2004; Ejskjaer et al., 2005; Jamnongjit et al., 2005]. Because many prominent signal transduction pathways feature EGFR, it has also been postulated to enhance malignant conversion and progression of these tissues [Chrysogelos and Dickson, 1994; Zeineldin and Hudson, 2006]. In support of this idea is the finding that specific abrogation of EGFR by antisense oligonucleotides, neutralizing antibodies, or pharmacological inhibitors

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results in cell cycle arrest, apoptosis, or dedifferentiaton of breast, uterine, ovarian, and prostate cancer cells [Baselga and Mendelsohn, 1994; Jamnongjit et al., 2005; Solbach et al., 2005; Wilding et al., 2006].

Members of the EGFR family are overexpressed in a wide range of human tumors, including the brain, lung, breast, stomach, liver, prostate, colon, ovary, and bladder [Biscardi et al., 1999a; Hunter, 2000; Olayioye et al., 2000; Schlessinger, 2000; Di Lorenzo et al., 2002]. This overexpression can be induced by multiple extracellular regulators, such as the EGFR ligands, phorbol esters, and steroid hormones [Chrysogelos and Dickson, 1994; Ejskjaer et al., 2005; Jasonni et al., 2005]. Studies examining TGF-α synthesis and secretion, an EGFR ligand, suggest that autocrine or paracrine signals involving the EGF/EGFR loop contribute significantly to oncogenesis [Chrysogelos and Dickson, 1994; Salomon et al., 1995]. Overall, evidence points to the involvement of elevated levels of EGFR in later stages of cancer, particularly metastasis [Sainsbury et al., 1987; Ruibal et al., 2006].

The vast majority of the evidence for involvement of the EGFR in the initiation of human cancers is correlative in nature, although causal studies in tissue culture and xenografts do support a role for the receptor in early stages of tumor formation [Velu et al., 1989; Biscardi et al., 1999a; Ishizawar and Parsons, 2004]. However, whether abberant overexpression of the receptor alone can contribute to tumor initiation in vivo has not been carefully examined. Transgenic mice represent a useful model to assess tissue-specific action of oncogenes or growth factors in vivo and to study their role in cancer formation [Hinrichs et al., 1991]. To address the question about the role of EGFR in the initiation of reproductive tissue tumors, we generated transgenic mice whose expression of the hEGFR is under the control of the mouse mammary tumor virus (MMTV) promoter and monitored the animals for rates and incidence of tumor formation and/or pathological evidence of a neoplastic phenotype. While only one animal developed a visible tumor after a long latency, hEGFR expression and concomitant phenotypic alterations occurred in a high percentage of transgenic mice. Involved tissues included the uterus, ovary, and mammary gland but not the prostate or testis. These findings indicate that overexpressed EGFR can promote preneoplastic conversion in a subset of targeted tissues, setting the stage for subsequent genetic and physiological alterations and tumor progression. Thus, these data suggest that the EGFR can play a role in early as well as late stages of disease.

MATERIALS AND METHODS

pMSG-MMTV-EGFR Plasmid Construction and Validation

The pMSG vector (Pharmacia Biotech, Piscataway, NJ), which drives the expression of the cDNA of choice from the MMTV-LTR promoter, was utilized as a steroid hormone-responsive vector system. A 3.8 kbp KpnI/XhoI fragment of hEGFR cDNA from the plasmid pcDNA-EGFR [Biscardi et al., 1999b] was inserted by blunt end ligation into the pMSG vector, digested with NheI/XhoI.

The resulting plasmid, pMSG-MMTV-EGFR, was then tested for steroid hormone responsiveness. MCF7 breast cancer cells (which express the steroid-responsive estrogen receptor) were transfected with pMSG-MMTV-EGFR using Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. Twenty-four h later, cells were either left untreated or stimulated for an additional 24h with 1% dexamethasone in complete culture media, harvested, and lysed, as previously described [Boerner et al., 2004]. The protein content of the lysate was measured and samples were subjected to Western blotting with human EGFR-specific primary antibodies (EGFR 1,2 cocktail from NeoMarker, Fremont, CA) and ERK1/2 mouse monoclonal antibody (from M. Weber of the University of Virginia) and horseradish-conjugated anti-mouse secondary antibody (GE Healthcare, Piscataway, NJ), as previously described [Boerner et al., 2004]. Antigen–antibody complexes were localized by chemiluminescence (ECL) (Pierce, Rockford, IL) and viewed by autoradiography.

Generation and Identification of Transgenic Mice

A 6.9 kbp fragment encoding MMTV-EGFR (Fig. 1) was released by BstZ17I/Aat11 digestion from the pMSG-MMTV-EGFR plasmid and purified by the NUCLEOSPIN method (BD Biosciences/Clontech, Franklin Lakes, NJ). The purified fragment was then microinjected into the male pronuclei of C57B6/CBAF2 fertilized eggs, and blastocysts were

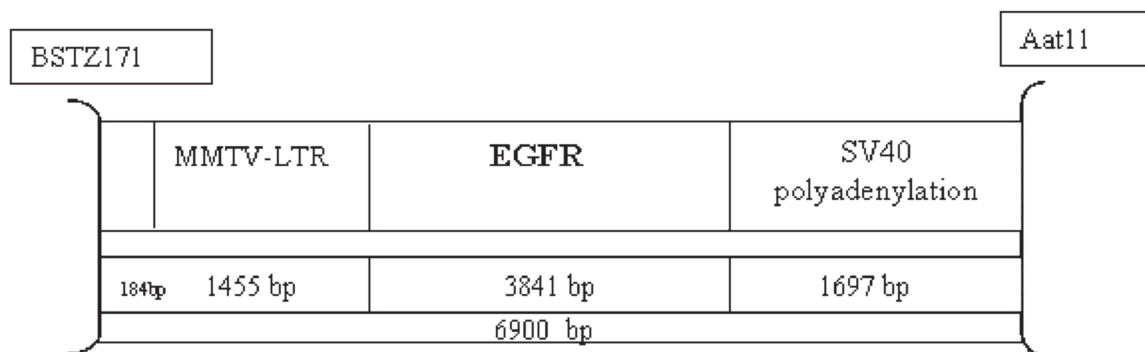

Fig. 1. MMTV-EGFR construction for microinjection. A 6.9 kbp region of the pMSG-MMTV-EGFR plasmid, containing the MMTV-LTR, the coding sequence for the human EGFR, and SV40 polyadenylation signal, was excised by BSTZ171 and Aat11 digestion, purified, and microinjected into B6CBAF2 fertilized eggs as described in Materials and Methods.

implanted into ICR pseudopregnant females. These and subsequent phases of the process were carried out according to established procedures in conjunction with the Gene Targeting and Transgenic Core Facility of the University of Virginia.

Genomic DNA from tail crops of F1 pups was tested for the presence of the hEGFR transgene by PCR, utilizing hEGFR-specific primers (Qiagen, Valencia, CA), 5′-GATCGGCCTCTTCATCGG-3′ (forward) and 5′-TCTTTCATCCCCCTGAATG-3′ (reverse), capable of distinguishing hEGFR from mouse genomic DNA and murine EGFR of the mouse fibroblast C3H10T1/2 cell line. As a control for the quality of tail DNA, a fragment of the OMO gene was amplified by PCR, as previously described [Pearson-White, 1993; Chang et al., 1999]. The Titanium Taq Polymerase kit from BD Biosciences/Clontech was used for the PCR screens, with C3H10T1/2 genomic DNA serving as a negative control and pMSG-MMTV-hEGFR cDNA as a positive control. Briefly, 1 μl taq polymerase was mixed with 2 μl DNA (at a concentration of 0.1 μg/μl), 10 μl hEGFR-specific primers (0.2 μM), 1 μl dNTP mix (Invitrogen) and 5 μl reaction buffer. Conditions for the thermal cycler were as follows: 95°C for 2 min, 95°C for 30 sec, 68°C for 1.5 min for 30 cycles. PCR product was then analyzed on an agarose gel.

Five founder mice that were identified by PCR (see Results) were back-crossed to C57B6 mice to generate stable, congenic lines, and progeny of these lines, that were positive for the hEGFR transgene by PCR and Southern blot analysis, were cross-bred for four generations to establish a colony with 100% transmission of the transgene.

Southern Blot Analysis

Integration of the hEGFR transgene into mouse genomic DNA was confirmed by Southern blot analysis, carried out according to established procedures [Pearson-White, 1993]. Tail clip DNA was digested with BamHI and Hind III.

Immunoprecipitaion and Western Blot Analysis

EGFR protein overexpression was assessed by immunoprecipitaion and Western blotting of excised and detergent-extracted mammary glands, uteri, ovaries, salivary glands, muscle, and prostates. Excised tissues were frozen in liquid nitrogen, placed into aluminum foil packets, and pulverized with a hammer. Powdered tissue was solubilized in cold RIPA buffer [Boerner et al., 2004], supplemented with protease inhibitors (Complete Mini from Roche Diagnostics, Indianapolis, IN). The cellular homogenate was centrifuged, and 1 mg extract protein was immunoprecipitated with EGFR antibody (BD Transduction Laboratories, Rockville, MD) and immunoblotted with hEGFR 1,2-specific antibodies (Neomarker), as described previously [Boerner et al., 2004].

Histological Evaluation

For histological analysis, excised tissue was fixed in zinc formalin, embedded in paraffin, sliced into 4 μm thick sections, and stained with hematoxylin and eosin.

RESULTS

Generation of Transgenic Mice

To assess the role of EGFR overexpression in the initiation and development of reproductive

tissue neoplasias, we created transgenic mice expressing the hEGFR under the specific control of the MMTV promoter. The MMTV promoter responds transcriptionally to ligand-activated glucocorticoid and steroid hormone receptors [Beato, 1996], found predominantly in reproductive organs. Figure 1 is a schematic of the pMSG-MMTV-hEGFR plasmid that was constructed as described in Materials and Methods and utilized in this study.

The inducible expression of this construction was tested by transient transfection in MCF7 breast cancer cells, which endogenously express estrogen receptors that bind estrogen (or dexamethasone) and become transcriptionally active. Figure 2 shows that the hEGFR was expressed from the pMSG-MMTV-hEGFR construct only upon dexamethasone treatment, as expected, while expression from the original pcDNA-hEGFR vector was constitutive and independent of dexamethasone treatment.

MMTV-hEGFR transgenic mice were generated through the Gene Targeting and Transgenic Core Facility at the University of Virginia, using established techniques as summarized in Materials and Methods. Five of 37 pups derived from blastocysts were positive for the transgene by PCR analysis (Fig. 3A). All five were chosen as founders for colony formation and back-crossed with C57B6 mice. Forty-two of 57 pups analyzed from these crosses were also positive for the transgene. A representative PCR is shown in Figure 3B. Results of the PCR reaction were confirmed by Southern blot analysis (Fig. 3C). Eight of 42 mice (4 breeding pairs) were selected for further breeding, and 3 pairs, representing 3 independent founder lines, gave litters. Animals were further in-bred for four generations, yielding lines with 100% transmission of the transgene (Fig. 3D).

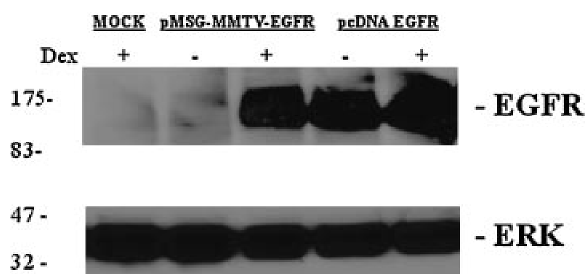

Fig. 2. Inducible expression of MMTV-EGFR in MCF7 breast cancer cells. pcDNA-EGFR and pMSG-MMTV-EGFR plasmids were transfected into MCF7 breast cancer cells and either left untreated or stimulated for 24 h with 1% dexamethasone, as described in Materials and Methods. On the second day after transfection, cells were harvested and lysed, and 100 ug protein extract was immunoblotted with hEGFR-specific antibodies. MOCK represents cells transfected with reagents alone, lacking DNA. ERK was used as a protein loading control.

EGFR Expression

Animals of the fully established, colony were monitored over 1.7–2.0 years for evidence of tumor formation. Only one animal developed a visible or palpable tumor, a well-differentiated squamous cell carcinoma in the vulvar region, which was visible at the age of 1 year and 9 months. Twenty additional animals (12 females and 8 males) were assessed for hEGFR protein overexpression by immunoprecipitaion and Western blotting of excised and solubilized mammary gland, uterus, salivary gland, ovary, muscle, prostate, and testis in the appropriate sex. Tissues from age and sex-matched C57B6 mice were used as negative, normal controls, and the C3H10T1/2 cell line overexpressing the hEGFR was used as a positive control. Figure 4A–C shows representative immunoprecipitations/Western blots of the samples, while Table I lists the outcomes of the analyses of the 12 female animals. hEGFR protein expression was seen in mammary glands and uteri of 10 of 12 animals tested, in 2 of 4 ovaries, and in 2 of 5 salivary glands. All 12 females were positive for hEGFR in at least one tissue. No male reproductive tissues of the 8 animals examined expressed hEGFR protein (data not shown), and none of the transgenic mice were positive for hEGFR protein in muscle.

Pathological Analysis of Tissue Samples

Tissue samples from the same animals underwent histopathological evaluation. Sections of skin containing mammary gland exhibited numerous intralobular ductal structures located randomly within the subcutaneous adipose tissue. These ductal structures were lined by a single layer of low cuboidal epithelial cells rimming a central lumen. Occasionally, this ductal epithelium was mildly hyperplastic, but regions of overt dysplastic change or neoplastic transformation occurring within the epithelial cells lining these mammary ducts were not observed. Among the subcutaneous intralobular ductal cross-sections were occasional small mammary gland acini, most of which appeared to be active and secretory in nature. Figure 5, panels A–C, shows examples of

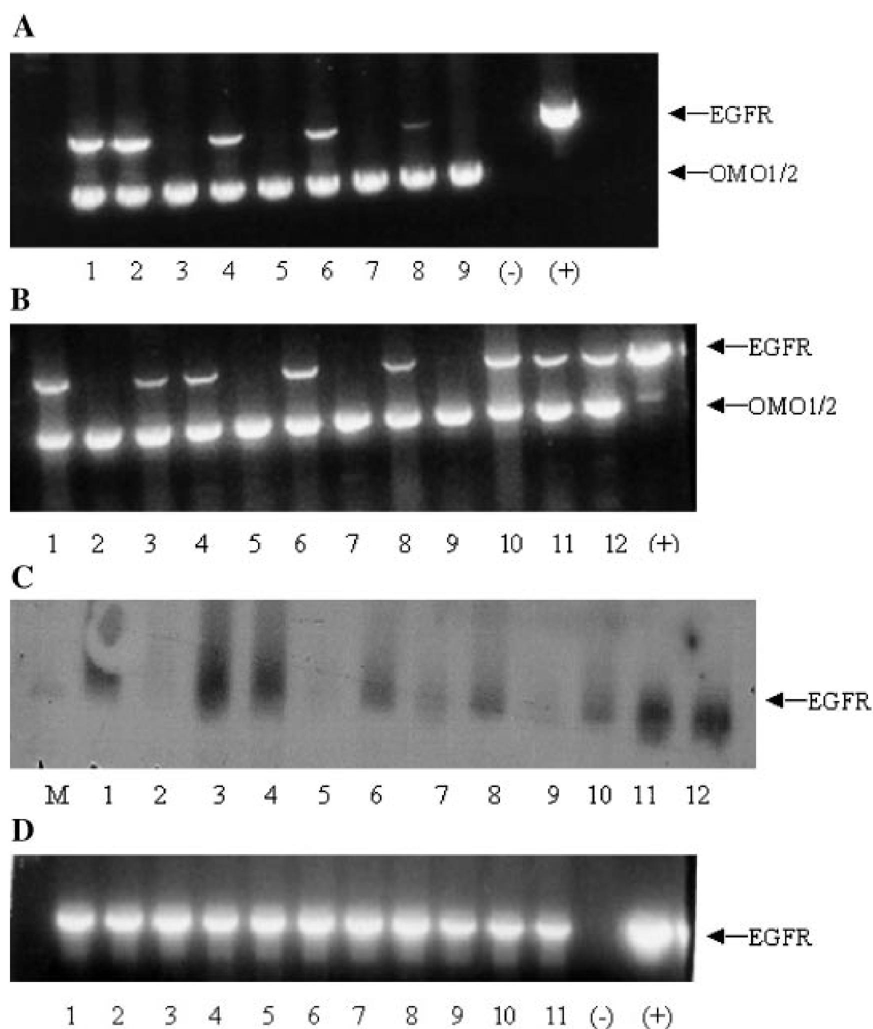

Fig. 3. Transgene integration into mouse genomic DNA. Transgene integration into mouse genomic DNA was analyzed by RT-PCR using hEGFR-specific primers and DNA extracted from tail crops, as described in Materials and Methods. **Panel A**: PCR analysis of the original litters yielded five founders (samples 1, 2, 4, 6, 8). (+) is the positive control (pMSG-MMTV-EGFR plasmid DNA), and OMO1/2 is an internal control for the quality of genomic DNA. **Panel B**: Results of a representative PCR analysis of a typical back-cross are shown. Genomic DNA from 12 pups was analyzed. **Panel C**: The same genomic DNA samples analyzed in panel B were subjected to Southern blot analysis. The results corroborate one another. ''M'' represents the 4 kbp marker. **Panel D**: Establishment of a transgenic colony in which transmission of the hEGFR gene is 100% (see Materials and Methods). (+) pMSG-MMTV-EGFR plasmid DNA, positive control. (–) genomic DNA from nontransgenic mice, negative control.

5/9 mice examined (56%) (see Table I for animal by animal summary) that contained foci of minimal to mild hypertrophy or early dysplasia of the secretory cells lining the acinar lumens.

A more distinctive phenotype was observed in uterine or uterine horn samples (8/9 animals examined (89%)). Figure 5, panels D–F, shows varying degrees of cystic endometrial hyperplasia that occurred in the uterine glands within the endometrial mucosal layer, as compared to normal. The degree of cystic hyperplasia varied from mild to moderate.

Figure 5, panels G–I and Table I show that all ovaries evaluated (8/8 (100%)) contained varying numbers of active follicles and corpus luteum. Fifty percent of the samples, however, exhibited variably sized cystic structures within the cortex, compatible with a diagnosis of follicular or leuteal cysts. Occasional oviducts also exhibited a mild to moderate hypertrophy or dysplasia occurring in the epithelial cell lining of the oviduct's central lumen. Often these hypertrophied or enlarged cells displayed a mild to moderately vacuolated cytoplasm,

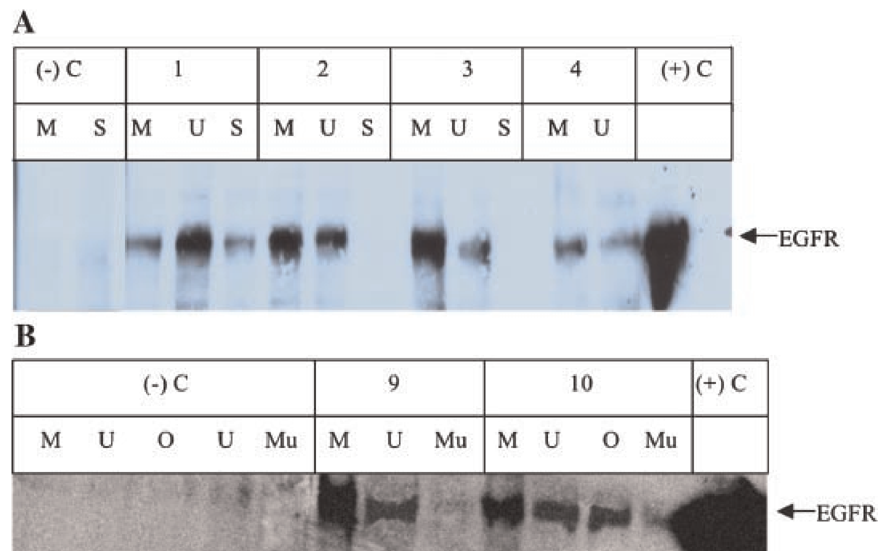

Fig. 4. hEGFR expression in tissues of MMTV-hEGFR transgenic mice. Expression of hEGFR in various tissues of MMTV-hEGFR transgenic mice was assessed by immunoprecipitaion and Western immunoblotting. Mammary glands, uteri, ovaries, salivary glands, and muscle were excised and solubilized as described in Materials and Methods. One milligram protein extract was immunoprecipitated with hEGFR antibodies from BD Transduction Laboratories and blotted with hEGFR-specific antibodies from NeoMarker. M, mammary gland; U, uterus; S, salivary gland; Mu, muscle; O, ovary. (−)C, nontransgenic mouse negative control; samples 1–4, 9, and 10 correspond to tissues from animals listed in Table I; (+)C is the positive control, a C3H10T1/2 mouse fibroblast cell line (5HR) that stably overexpresses hEGFR. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

representing possible secretory activity. This cellular hypertrophy often bordered upon dysplastic change.

All sections of prostate, testes, epididymis, vesicular glands (seminal vesicles), and coagulating glands of male transgenic mice were considered to be within normal limits.

DISCUSSION

The oncogenic nature of the EGFR as a single causative agent has not been rigorously tested in an animal model, in which the specific tissues with which it has been linked have been targeted. This study sought to examine the role of the EGFR in the initiation of steroid hormone-responsive tissues, such as breast and prostate. To this end, transgenic mice expressing the hEGFR under the control of the MMTV promoter were generated. hEGFR expression was verified in mammary gland, uterus, and ovary by immunoprecipitation and Western immunoblotting, but no expression

TABLE I. Expression Patterns and Pathology of Various Tissues From 12 Representative Female EGFR Transgenic Mice

	Mammary gland	Uterus	Ovary	Salivary gland	Muscle
Mouse	EGFR[a]/path[b]	EGFR/path	EGFR/path	EGFR/path	EGFR/path
1	+/ND[c]	+/ND	−/ND	+/ND	−/−
2	+/ND	+/ND	ND/ND	−/ND	−/−
3	+/−	+/+	+/+	−/ND	−/−
4	+/−	+/+	ND/+	ND/ND	−/−
5	−/+	+/+	−/+	ND/ND	−/−
6	+/+	+/+	ND/+	−/ND	−/−
7	+/+	+/+	ND/ND	ND/ND	−/−
8	+/+	−/+	ND/+	ND/ND	−/−
9	+/−	+/+	ND/+	ND/ND	−/−
10	+/+	+/+	+/+	ND/ND	−/−
11	−/−	−/−	ND/+	ND/ND	−/−
12	+/ND	+/ND	ND/ND	+/ND	−/−

[a]EGFR denotes expression of the transgene as determined by immunoprecipitation and Western blot.
[b]Path denotes pathology—hyperplasia, dysplasia, cystitis, etc., as described in the text.
[c]ND denotes not done.

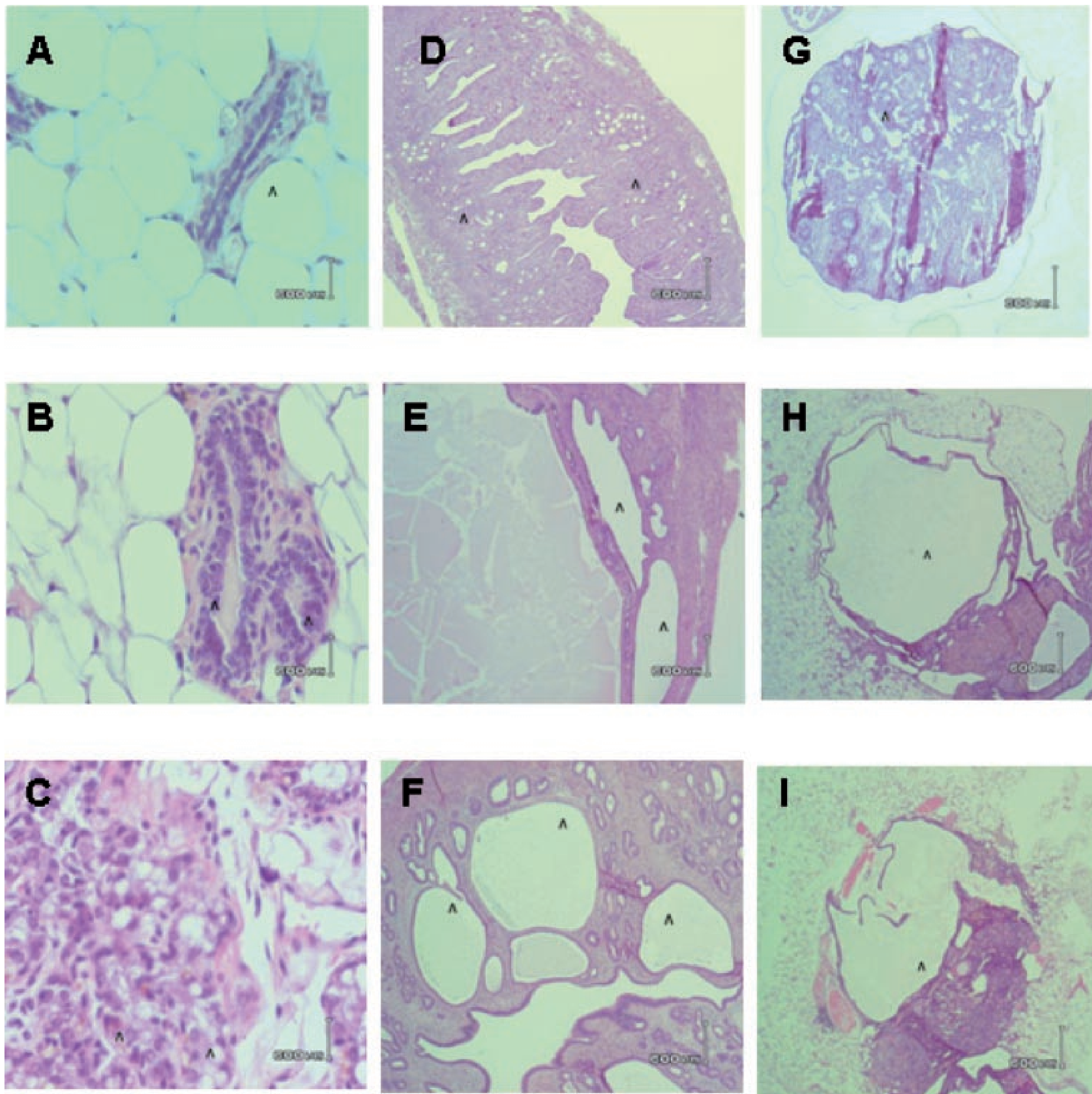

Fig. 5. Histological evaluation of tissues from transgenic mice. Formalin-fixed, paraffin-embedded sections were stained and examined microscopically. Five random microscopic fields were analyzed for each section. **Panel A**: Intralobular duct of a normal, age- and strain-matched mouse mammary gland (40×). **Panel B**: Intralobular duct of a transgenic mammary gland—arrows show minimal to mild hyperplasia within the epithelial lining of the intralobular duct (40×). **Panel C**: Transgenic mammary gland—arrows depict hyperplasia and/or dysplasia of epithelial cells lining glandular acini (400×). **Panel D**: Normal uterus (40×). **Panel E**: Transgenic uterine horn—mild to moderate cystic hyperplasia is evident within the endometrium (40×). **Panel F**: Transgenic uterus—arrows depict moderate to marked cystic endometrial hyperplasia (40×). **Panel G**: Normal ovary (40×). **Panel H**: Transgenic ovary—arrows depict a follicular cyst of the ovarian cortex (40×). **Panel J**: Transgenic ovary—arrow depicts a cystic follicular structure within the cortex of the ovary (40×).

was detected in prostate, testis, or other male reproductive tissues.

At the histological or cellular level, no neoplastic transformation was evident in any of the male tissues examined. This is likely due to the failure of the transgene to express in these tissues. In female mice, however, abnormal architectural appearance, hyperplasia, and/or mild dysplasia were observed in reproductive tissues of 100% of the animals, with the highest percentage of abnormalities appearing in ovarian tissue (100%). Eighty-nine percent of the

female mice displayed neoplastic changes occurring within uterine tissue, while 56% exhibited changes consisting only of mild hypertrophy or early dysplasia of the mammary gland. These results indicate that simple overexpression of the EGFR alone can initiate preneoplastic changes in both cell function and architectural structure of female reproductive organs with particularly significant effects occurring in ovarian and uterine tissues. However, secondary changes are necessary for tumor formation and progression.

Transgenic mice expressing various oncogenes specifically in the mammary gland have been generated by other investigators who employed either the MMTV-LTR (MMTV-TGFα, MMTV-c-myc, MMTV-tTA, MMTV-neu) or milk protein-specific promoters such as those of the whey acidic protein, β-lactalbumin or β-lactoglobulin [Muller, 1991; Krane and Leder, 1996; Chepko et al., 2005; Kaminski et al., 2006]. In the majority of these cases, mammary tumors develop in a stochastic fashion with a latency period of 5–10 months. In the case of transgenic mice expressing wild type HER2/ErbB2/*Neu*, induction of mammary tumors occurs after a long latency and correlates with the frequent occurrence of activating mutations in the *Neu* transgene [Jhappan et al., 1990; Siegel et al., 1994; Muller et al., 1996]. Recent studies using multiparous MMTV-Neu mice suggest that parity also induces a population of cells that are susceptible to ErbB2/Neu-induced transformation [Landis et al., 2005]. These findings also suggest that overexpression of individual oncogenes is necessary but not sufficient for tumor formation.

Brandt and colleagues [Brandt et al., 2000] reported previously the development of hEGFR transgenic mice, wherein expression of the hEGFR was under the control of the MMTV-LTR (MHERc) or the β-lactoglobulin promoters (BLGHERc). The BLGHERc transgene was expressed exclusively in the female mammary gland, whereas the MHERc transgene was expressed more promiscuously in other organs as well, such as ovary, salivary gland, and testis. While both strains of transgenic mice had impaired mammary gland development, which progressed to hyperplasias in virgin mice and dysplasias and some tubular adenocarcinomas in lactating animals, little to no phenotype was described in ovarian and none at all in uterine tissue. These results are quite different from those obtained from the MMTV-hEGFR transgenic mice we have developed, where hEGFR protein is undetectable in male tissues and the uterine/ovarian phenotype is more frequent and pronounced than that in the mammary gland. The cause of these differences is unclear but could be due to differences in promoter-transgene construction or usage and in mouse strains employed. No follow-up studies employing these mice have been forthcoming from Brandt and/or colleagues that might lend insight into these questions. In any event, our transgenic strain provides a suitable model in which to explore secondary events required for tumor formation in uterine and ovarian tissues in particular, as well as in mammary tissue.

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