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ABSTRACT

Members of the HER family (including EGFR) are overexpressed in a wide range of human tumors, including those of the brain, breast, colon, prostate, and ovary. In human breast cancers, c-Src is overexpressed in ~ 70% of cancers, suggesting that it interacts functionally with this family of receptors. In many human cancers, including breast cancer, EGFR is activated in an autocrine or paracrine manner by TGFα. To test whether interactions between TGFα, EGFR and c-Src result in synergistic increases in breast tumor development, transgenic mice expressing each of these genes under the control of the MMTV promoter are being developed. The MMTV promoter responds transcriptionally to glucocorticoids and steroids and causes expression of the transgene in steroid hormone responsive organs. We have generated MMTV EGFR mice and demonstrated the presence of the transgene by PCR and Southern analyses. At the present time, although we have evidence for elevated expression of the EGFR in hormonally responsive tissues (especially in multiparous animals), only one of the EGFR transgenic mice that we have developed had a visible tumor; however, 4 of 12 females showed focal hyperplasia of the mammary gland, 9 of 12 females showed varying degrees of cystic endometrial hyperplasia and dysplasia in the uterus or uterine horn and 6 of 12 females exhibited follicular or luteal cysts in ovary or oviducts and also exhibited a mild to moderate hypertrophy or dysplasia. Male reproductive tissues examined did not show any signs of preneoplastic conversion. The ability of TGFα to enhance tumor formation in MMTV EGFR transgenic mice was tested by crossing MMTV TGFα transgenic mice. Bigenic mice carrying both of these transgenes have been generated and are being examined for rates of tumor formation. It is expected that breast tumors will form in these bigenic mice, providing evidence for the role of both EGFR and TGFα in breast tumorigenesis. MMTV c-Src transgenic mice are under preparation, and the strategy and progress in generating such a strain will be discussed. Eventually, a trigenic mouse that overexpresses TGFα, EGFR, and c-Src will be generated to test the interactions between these three molecules in an animal model.

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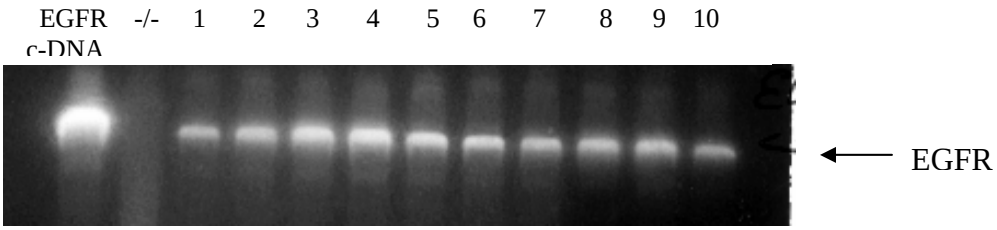
Introduction

Members of the HER family (including EGFR) are overexpressed in a wide range of human tumors, including those of the brain, breast, colon, prostate, and ovary. In human breast cancers, c-Src is overexpressed in ~ 70% of cancers, suggesting that it interacts functionally with this family of receptors. In many human cancers, including breast cancer, EGFR is activated in an autocrine or paracrine manner by TGFα. To test whether interactions between TGFα, EGFR and c-Src result in synergistic increases in breast tumor development, transgenic mice expressing each of these genes under the control of the MMTV promoter are being developed. The MMTV promoter responds transcriptionally to glucocorticoids and steroids and causes expression of the transgene in steroid hormone responsive organs. We have generated MMTV EGFR mice and demonstrated the presence of the transgene by PCR and Southern analyses. At the present time, although we have evidence for elevated expression of the EGFR in hormonally responsive tissues (especially in multiparous animals), only one of the EGFR transgenic mice that we have developed had a visible tumor; however, 4 of 12 females showed focal hyperplasia of the mammary gland, 9 of 12 females showed varying degrees of cystic endometrial hyperplasia and dysplasia in the uterus or uterine horn and 6 of 12 females exhibited follicular or luteal cysts in ovary or oviducts and also exhibited a mild to moderate hypertrophy or dysplasia. Male reproductive tissues examined did not show any signs of preneoplastic conversion. The ability of TGFα to enhance tumor formation in MMTV EGFR transgenic mice was tested by crossing MMTV TGFα transgenic mice. Bigenic mice carrying both of these transgenes have been generated and are being examined for rates of tumor formation. It is expected that breast tumors will form in these bigenic mice, providing evidence for the role of both EGFR and TGFα in breast tumorigenesis. MMTV c-Src transgenic mice are under preparation, and the strategy and progress in generating such a strain will be discussed. Eventually, a trigenic mouse that overexpresses TGFα, EGFR, and c-Src will be generated to test the interactions between these three molecules in an animal model.

Ī. Research accomplishments associated with the tasks outlined in the approved Statement of work.

Task 3. To monitor tumor formation in MMTV-EGFR transgenic mice (month 18-36)

As I have reported previously, I have generated MMTV-EGFR transgenic mice. Now I have a stable colony for 2 years with 100% transmission of the transgene.



1-10 samples of transgenic mice genomic DNA
EGFR- plasmid DNA, positive control
-/- non- transgenic mice genomic DNA, negative control

Figure 1. MMTV-EGFR transgenic mice . PCR.

Expression analysis

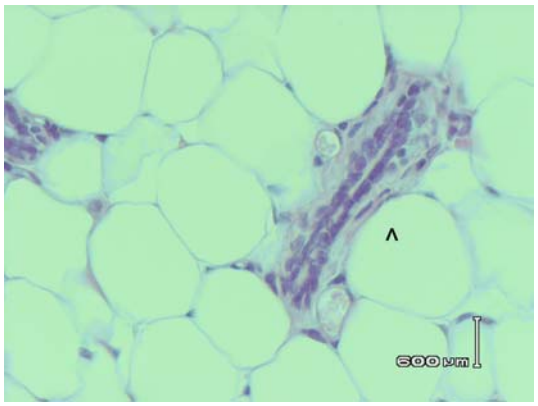
10 from 12 analyzed MMTV-EGFR transgenic female mice had hEGFR protein expression in mammary gland and uterus, 3 from 6 analyzed animals had weak EGFR overexpression in ovaries, 1 from 3 mice had weak hEGFR expression in salivary gland. None of the transgenic mice had hEGFR protein overexpression in muscle. Male reproductive tissues examined did not have EGFR protein overexpression. (Table 1 – see supporting data).

Pathological analysis of tissue samples.

Tissue samples from the same animals underwent histopathological evaluation. Animals were euthanized at the age of 1.7 –2.0 years. Age-matched nontransgenic C57BL mice were used as a control.

At the present time, although we have evidence for elevated expression of the EGFR in hormonally responsive tissues (especially in multiparous animals), only one of the EGFR transgenic mice that we developed had a tumor - a well-differentiated squamoused cell carcinoma at the age of 1 year and nine month; however, 4 of 12 females showed focal hyperplasia of the mammary gland, (figure 2), 9 of 12 females showed varying degrees of cystic endometrial hyperplasia and dysplasia in the uterus or uterine horn and 6 of 12 females exhibited follicular or luteal cysts in ovary or oviducts and also exhibited a mild to moderate hypertrophy or dysplasia. Male reproductive tissues examined did not show any signs of preneoplastic conversion. These findings are consistent with the weak oncogenic potential of overexpressed EGFR in tissue culture and xenograft studies and suggest that it contributes to the early neoplastic process in a significant but incremental way.

Figure 2.



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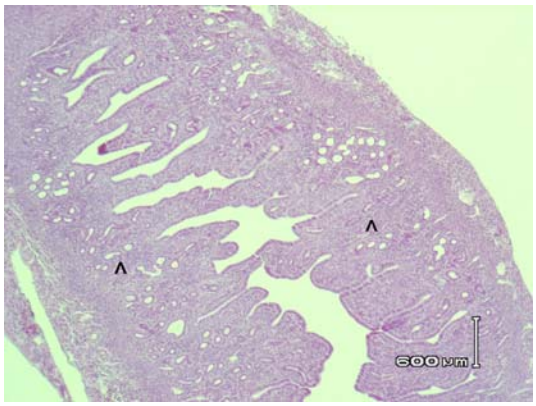

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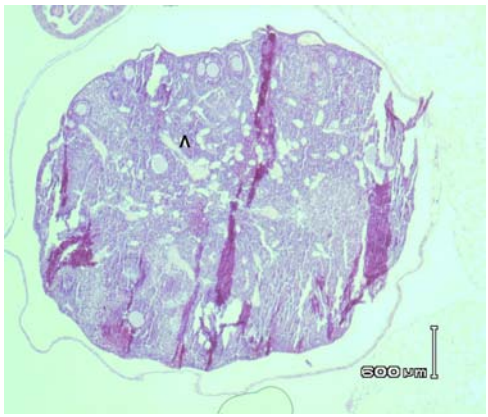

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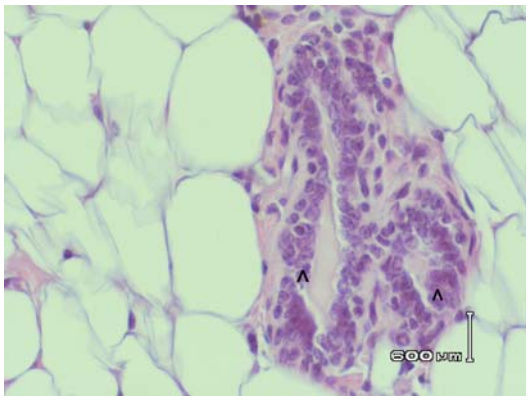

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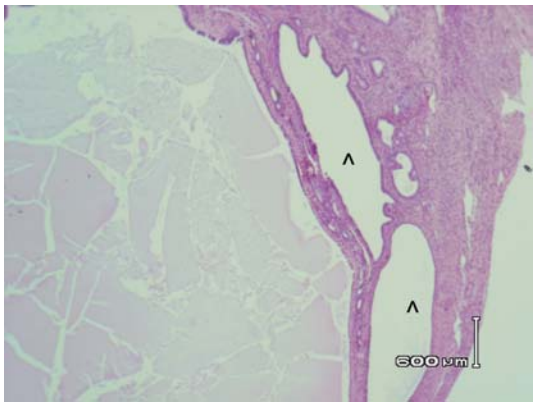

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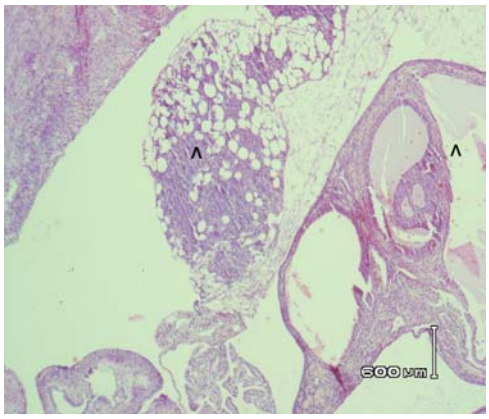

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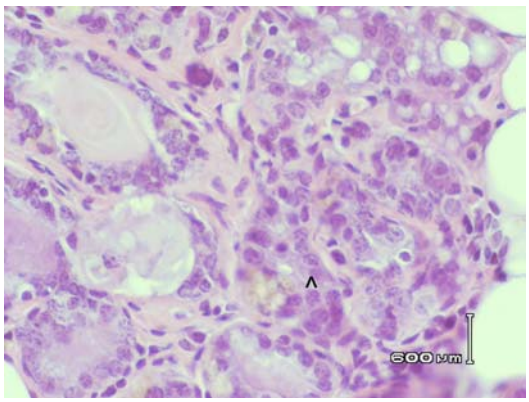

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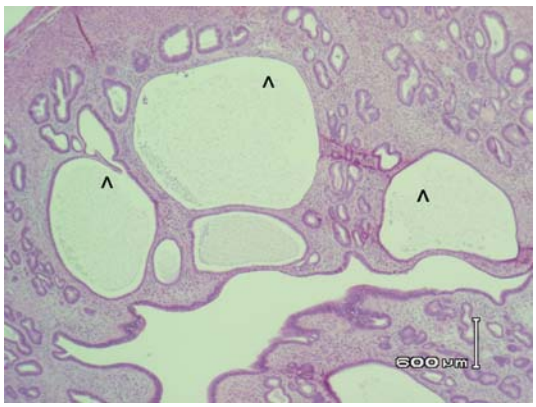

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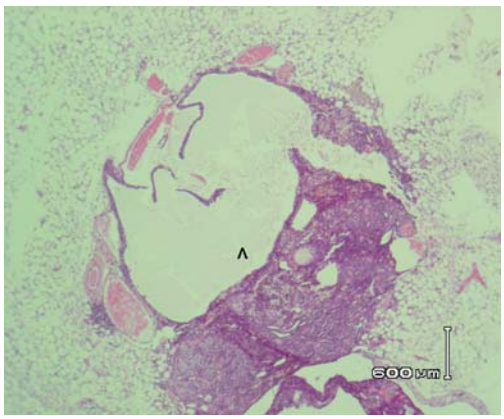


2i

Figure 2. Histological evaluation of MMTV-EGFR transgenic mouse tissue samples.

2a -Intralobular Ducts – Within normal limits. (x400).
2b -Uterus – Within normal limits (x40).
2c -Ovary – Within normal limits. (x40).
2d - Intralobular Duct – Arrows show a minimal to mild hyperplasia occurring within the epithelial cell layer lining the intralobular duct (x400).
2e- Uterine Horn - mild to moderate cystic hyperplasia is evident within the endometrium of the uterine horn (x40).
2f -Ovary – Arrows depict a follicular cyst of the ovarian cortex and multifocal areas of subacute inflammation occurring within the periovarian adipose tissue. (x40).
2g -Mammary Gland- Hyperplasia and/or dysplasia of the epithelial lining cells of the glandular acini of a mammary gland is depicted by the arrows. (x400).
2h - Uterus – moderate to marked cystic endometrial hyperplasia depicted by the arrows within cystic structures. (x40).
2i - Arrow depicts a cystic follicular structure within the cortex of the ovary (x40).

Task 4. To generate EGFR/ TGFα bigenic mice and monitor tumor formation.

a. TGF a transgenic mice.
It has been reported previously, that we obtained MMTV-TGFα transgenic mice from Jackson Laboratories and have established our lab's own colony of MMTV-TGFα transgenic mice. (Figure 3 – see supporting data).

b. *Bigenic MMTV-EGFR-TGFa mice.*

I bred MMTV-TGFα transgenic mice with MMTV-EGFR transgenic mice to derive bigenic MMTV-EGFR/ TGFα transgenic mice.

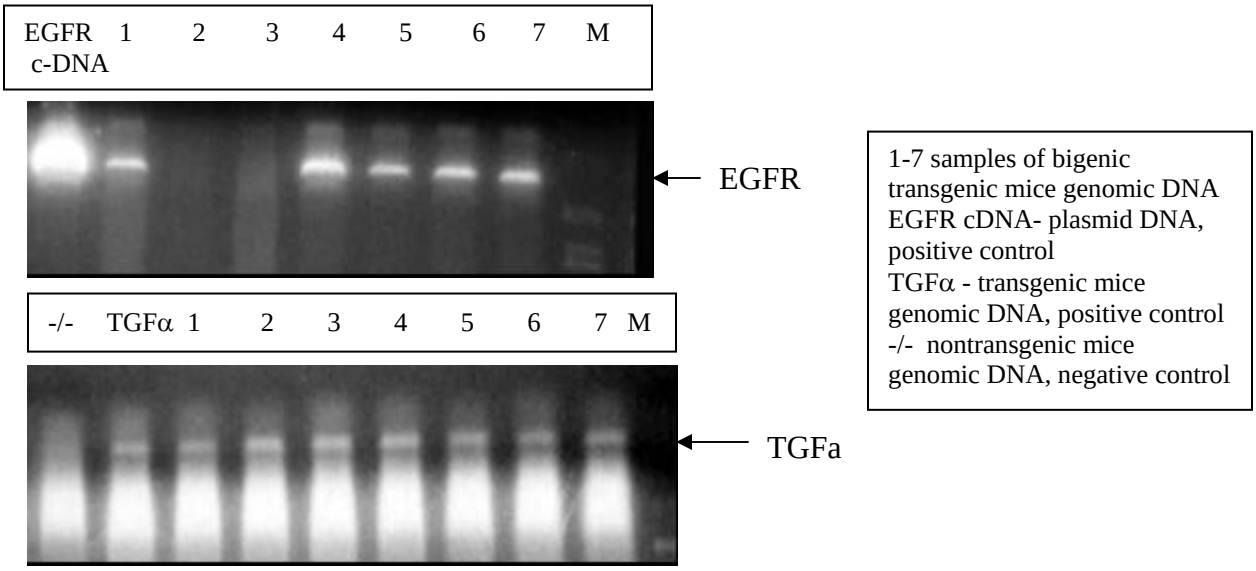


Figure 4. Bigenic MMTV-EGFR/ TGFα transgenic mice PCR.

5/7 positive. Positive mice are being interbred to generate a line with 100% transmittance of the transgenes.

Animals that scored positively for the transgenes were assessed for EGFR protein overexpression by Western blotting of excised and solubilized mammary gland tissue, ovary, uterus and prostate (Figure 5). Serum from these mice are being analyzed by ELISA for TGFa expression.

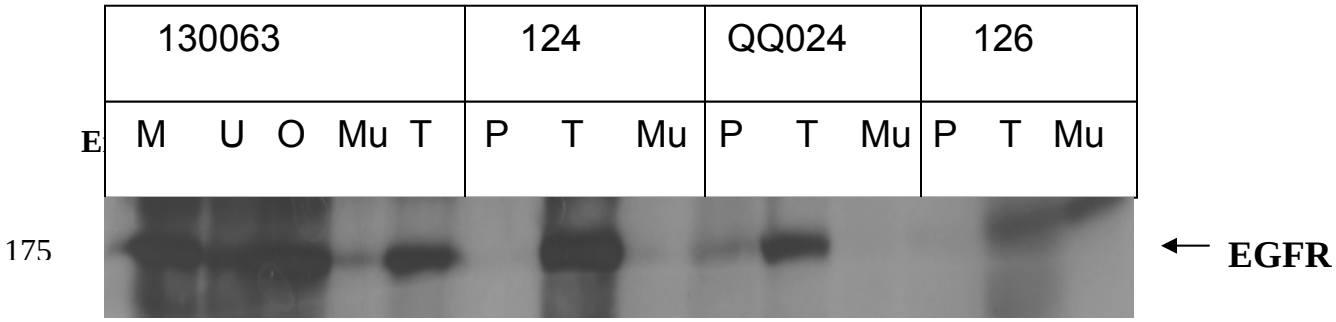


Figure 5. Bigenic MMTV-EGFR/ TGFα transgenic mice. EGFR Western Blot.

M- mammary gland O- ovary T- tumor P- prostate
U- uterus Mu – muscle C- control, nontransgenic mice

130063 , 124 , QQ024, 126 – transgenic mice

Task 7. To test the significance of EGFR/TGFa interaction on breast cancer development (month 28-36)

1. Compare the tumor formation between non-transgenic, the transgenic monogenic MMTV-EGFR, MMTV-TGFa mice and transgenic bigenic MMTV TGFa/EGFR, mice.

a) **Tumor formation in bigenic MMTV TGFa/EGFR** transgenic mice. 7 of 22 (33%) bigenic MMTV TGFa/EGFR transgenic mice developed tumors between the ages of 7 months –1 year. bigenic mice having tumors shown on Figure 6 (supporting data) The histological types of tumors varied from sebaceous gland adenoma to acytic mammary tumor (Figure 7, supporting data)

b) **Tumor formation in MMTV EGFR transgenic mice.** Only 1 of 50 MMTV-EGFR mouse developed tumor – a squamous cell carcinoma at the age of 2 years.

c) **Tumor formation in TGFa transgenic mice**. 9 of 34 (26%) MMTV TGFa transgenic mice developed tumors between the ages of 1 year 7 months – 2 years. The types of tumors varied from sebaceous gland adenoma to uterosarcoma. (Figure 7, supporting data)

III. Technical and unexpected difficulties.

A new MMTV BSL1- STOP VENUS c-Src plasmid construction that I made and successfully expressed in COS-7 cells and SYF-/- fibroblasts (has been reported previously), was microinjected into ICR mice, but none of the pups that were born were positive for the c-Src transgene. It is possible that c-Src did not integrate into the mouse genome. It is also possible that the c-Src transgene could be toxic to mice.

Several approaches to overcome this problem:

1. I can obtain constitutively active c-Src^{527} mice and breed them with the MMTV-EGFR transgenic mice.
2. I can use blastocyst injection of ES cells (embryonic stem cells) after appropriate selection of c-Src clones

Key research accomplishments

1. The colonies of MMTV-EGFR, MMT-TGF a, MMTV-EGFR/TGFa transgenic mice have been established.

2. The EGFR protein expression in bigenic MMTV-EGFR/TGFa transgenic mice was confirmed by Western Blot.

3. MMTV-EGFR and MMT-TGFa transgenic mice were monitored and analyzed for tumor formation. Bigenic MMTV-EGFR/TGFa transgenic mice have been generated and are being examined for rates of tumor formation. Bigenic MMTV-EGFR/TGFa transgenic mice developed tumors more frequently than single MMTV-EGFR or MMT-TGFa transenic mice and at an earlier age. This provides evidence for the synergistic effect of EGFR and TGFα in tumorigenesis

Reportable outcomes

1. N.V.Marozkina, and S.J. Parsons: Testing the Synergy Between Epidermal Growth Factor Receptor, Transforming Growth Factor α and c-Src in Breast Tumorigenesis in Transgenic Mice // AACR. 97 Annual Meeting. Washington. Virginia. 2006.

2. Marozkina N. V. Parsons S.J. MMTV-EGF Receptor transgene promotes preneoplastic conversion of multiple steroid hormone-responsive tissues. Submitted to Oncogene.

3. Marozkina N. V. Parsons S.J. Synergistic effect of EGF receptor and TGFα transgenes in tumorigenesis. Manuscript in preparation.

Abstract is enclosed. See appendices.

Conclusions

1. At the present time, although we have evidence for elevated expression of the EGFR in hormonally responsive tissues (especially in multiparous animals), only one of the EGFR transgenic mice that we developed had a tumor - a well-differentiated squamous cell carcinoma at the age of 1 year and nine months; however, 4 of 12 females showed focal hyperplasia of the mammary gland, 9 of 12 females showed varying degrees of cystic endometrial hyperplasia and dysplasia in the uterus or uterine horn and 6 of 12 females exhibited follicular or luteal cysts in ovary or oviducts and also exhibited a mild to moderate hypertrophy or dysplasia. Male reproductive tissues examined did not show any signs of preneoplastic conversion. These findings are consistent with the weak oncogenic potential of overexpressed EGFR in tissue culture and xenograft studies and suggest that it contributes to the early neoplastic process in a significant but incremental way.

2. 9 of 34 MMTV TGFa (26%) transgenic mice developed tumors between the ages of 1 year 7 months and 2 years. The histological types of tumors varied from sebaceous gland adenoma to uterosarcoma.

3. 7 of 22 bigenic MMTV TGFa/EGFR (32%) transgenic mice developed tumors between the ages of 7 months and 1 year. The histological types of tumors varied from sebaceous gland adenoma to acytic mammary tumor.

4. Bigenic MMTV TGFa/EGFR transgenic mice developed more tumors (32% vs 26% of MMTV TGFa) at an earlier age (on the average of 9 months vs 22 month of MMTV TGFa mice), thus providing evidence for the synergistic effect of EGFR and TGFα in tumorigenesis.

Appendices

Abstract from AACR meeting

Testing the Synergy Between Epidermal Growth Factor Receptor, Transforming Growth Factor α and c-Src in breast tumorigenesis in transgenic mice.

N.V.Marozkina, S.J. Parsons.

University of Virginia Health System, Department of Microbiology and Cancer Center, P.O.Box 800734, Charlottesville, VA, 22908, USA

Key words: epidermal growth factor receptor, transforming growth factor α, c-Src, transgenic mice, breast tumorigenesis

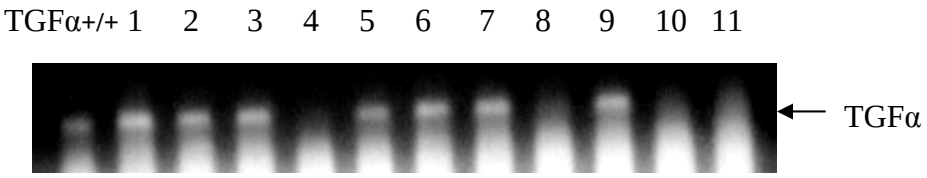
Members of the HER family (including EGFR) are overexpressed in a wide range of human tumors, including those of the brain, breast, colon, prostate, and ovary. In human breast cancers, c-Src is overexpressed in ~ 70% of cancers, suggesting that it interacts functionally with this family of receptors. Previous studies from our laboratory have demonstrated that when c-Src is overexpressed with the EGFR, synergistic increases in sub-cutaneous tumor formation in nude mice are observed as compared to cells that overexpress only one of the pair. In many human cancers, including breast cancer, EGFR is activated in an autocrine or paracrine manner by TGFα. To test whether interactions between TGFα, EGFR and c-Src result in synergistic increases in breast tumor development, transgenic mice expressing each of these genes under the control of the MMTV promoter are being developed. The MMTV promoter responds transcriptionally to glucocorticoids and steroids and causes expression of the transgene in steroid hormone responsive organs. We have generated MMTV EGFR mice and demonstrated the presence of the transgene by PCR and Southern analyses. At the present time, although we have evidence for elevated expression of the EGFR in hormonally responsive tissues (especially in multiparous animals), none of the EGFR transgenic mice that we have developed have visible tumors; however, they will be examined for evidence of displasia, particularly in steroid-responsive tissues, such as mammary gland, uterus, ovary and prostate. The ability of TGFα to enhance tumor formation in MMTV EGFR transgenic mice will be tested by crossing MMTV TGFα transgenic mice. Bigenic mice carrying both of these transgenes have been generated and are being examined for rates of tumor formation. It is expected that breast tumors will form in these bigenic mice, providing evidence for the role of both EGFR and TGFα in breast tumorigenesis. MMTV c-Src transgenic mice are under preparation, and the strategy and progress in generating such a strain will be discussed. Eventually, a trigenic mouse that overexpresses TGFα, EGFR, and c-Src will be generated to test the interactions between these three molecules in an animal model.

Supporting data

	Mammary	Uterus	Salivary gland	Muscle	Ovaries
Number of positive by Western blot analysis of immunoprecipitated human EGFR	10	10	1	0	3
Number of negative by Western blot analysis of immunoprecipitated human EGFR	2	2	2	12	3
Total number	12	12	3	12	6

Table 1. MMTV-EGFR transgenic mice tissue samples.

Figure 3.



1-10 samples of transgenic mice genomic DNA
11 –negative control, non-transgenic mice genomic DNA
TGFα+/+ – positive control for mice genomic DNA

Human-specific primers for TGFα were used for PCR reactions to detect the presence of the transgene, against a mouse background. The MMTV- TGFα primers gave a PCR product of about 0.192 kb.

MMTV-human TGFα primers structure:

TGF1 – AGTTCTGCTTCCATGCAACC
TGF2 – TGATGATAAGGACAGCCAGG

Figure 3. MMTV-TGFα transgenic mice. PCR.

Figure 6.

A)

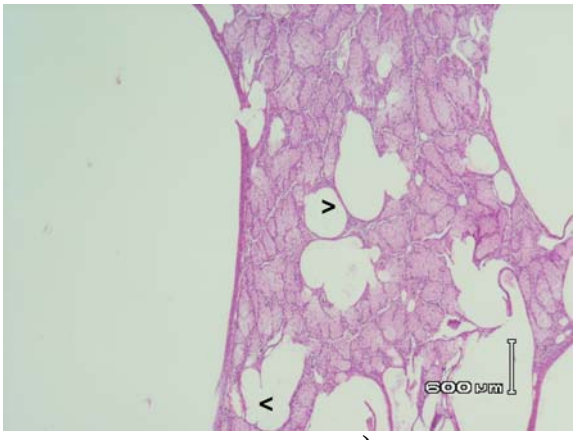
B)

C)

Tumor formation in bigenic MMTV-EGFR/ TGFa transgenic mice.
photo was taken at the age of 7 month. A) sebaceus gland adenomas, B) basal cell adenoma
C) mammary gland acytic tumor

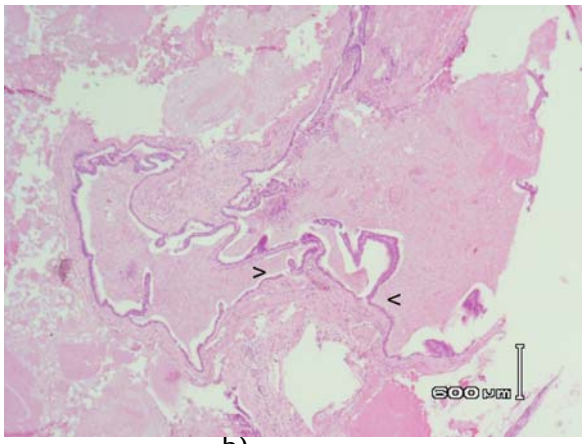
Figure 7.

EGFR/TGFα



a)

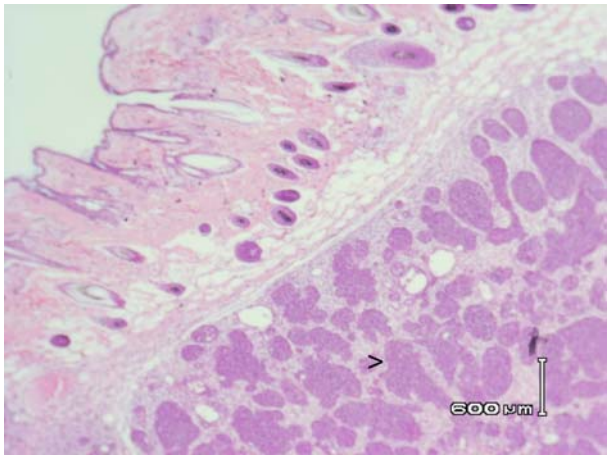
TGFα



b)

a) Arrows depict moderate cystic ductal hyperplasia, as well as an increase in the number of sebaceous glands within the dermis. (x40 Magnification).
MMTV-EGFR/TGFa transgenic mice, male. **sebaceus gland adenoma**

b) Arrows depict a condition of cystic, glandular hyperplasia of sebaceous glands. The identification of the sample region was hard to discern. (x40 Magnification).
MMTV-TGFa transgenic mice, male. **sebaceus gland adenoma**



c)

c) Arrows depict region in dermis/subcutaneous region of haired skin that may represent an epithelial cell or Basal Cell tumor. (x40 Magnification).
MMTV-EGFR/TGFa transgenic mice, male. **Basal cell adenoma**