American Journal of Pathology, Vol. 147, No. 2, August 1995

Short Communication

Coexpression of Transforming Growth Factor-α and Epidermal Growth Factor Receptor in Capillary Hemangioblastomas of the Central Nervous System

Guido Reifenberger,*† Julia Reifenberger,*† Thomas Bilzer,*Wolfgang Wechsler,* and V. Peter Collins†

From the Department of Neuropathology, Heinrich-Heine-University, Düsseldorf, Germany; and Ludwig Institute for Cancer Research,† Stockholm Branch and Institute of Oncology and Pathology, Division of Tumor Pathology, Karolinska Hospital, Stockholm, Sweden*

The expression of epidermal growth factor receptor (EGFR) and the pre-pro form of one of its ligands, transforming growth factor-α (TGF-α), was studied by Northern blotting in a series of 14 capillary hemangioblastomas of the central nervous system. A constant coexpression of EGFR and pre-pro-TGF-α mRNAs was found. Immunocytochemical investigation of an extended series of 51 capillary hemangioblastomas revealed that the stromal cells in these tumors showed immunoreactivity with monoclonal antibodies to EGFR and TGF-α. Analysis of gene dosage by Southern blotting in 20 tumors indicated a normal gene copy number of EGFR and TGFα in all cases. Our findings suggest that autocrine and/or juxtacrine growth stimulation via the EGFR may contribute to tumor growth in capillary hemangioblastomas. (Am J Pathol 1995, 147:245–250)

Capillary hemangioblastomas are benign, vessel-rich, and usually cystic tumors of uncertain histogenesis that most frequently occur in the cerebellum of adults.[1] These tumors arise either in the setting of von Hippel-Lindau syndrome or, more often, as solitary sporadic lesions without extracerebellar stigmata or family history.[2] Histologically, capillary hemangioblastomas consist of stromal or interstitial cells that lie in a dense network of capillary vessels.[1] Despite extensive immunocytochemical and electronmicroscopical studies, the nature and origin of the stromal cells is still uncertain. Different studies have characterized these cells as showing phenotypes as diverse as undifferentiated mesenchymal,[3] neuroendocrine,[4] and even fibrohistiocytic.[5]

The molecular genetic alterations underlying the growth of capillary hemangioblastomas are poorly understood at present. The recent identification and cloning of the von Hippel-Lindau (*VHL*) tumor suppressor gene[6] in conjunction with the finding of mutations and/or deletions of the *VHL* gene in sporadic capillary hemangioblastomas[7] and other neoplasms associated with von Hippel-Lindau disease[8–10] suggest a loss of function of the *VHL* gene as a critical step in the development of these tumors. In addition, there are studies showing that the characteristic proliferation of capillary vessels may be due to the production and secretion of vascular endothelial derived growth factor, a potent angiogenic factor, by the stromal cells.[11–12]

In the present study we have investigated a series of capillary hemangioblastomas by Northern blotting for the expression of epidermal growth factor receptor (EGFR) mRNA and the precursor transcript of one of

Supported by grants from the Swedish Cancer Fund, the Child Cancer Foundation of Sweden, the Konung Gustav V Forsknings Fund, Stockholms Cancer Society, and the Deutsche Forschungsgemeinschaft (Re938/2–1).

Accepted for publication April 26, 1995.

Address reprint requests to V. Peter Collins, Institute of Oncology and Pathology, Division of Tumor Pathology, Box 100, Karolinska Hospital, S-17176 Stockholm, Sweden.

its ligands, transforming growth factor-α (TGF-α). Here we show that EGFR and pre-pro-TGF-α mRNAs are consistently coexpressed in capillary hemangioblastomas. Immunocytochemistry for the respective protein products revealed that the expression of both is localized to the stromal cells. Our findings thus suggest a potential role of EGFR and TGF-α in the growth of capillary hemangioblastomas by providing autocrine and/or juxtacrine growth-stimulatory signals for the stromal cells.

Materials and Methods

Materials

Formalin-fixed and paraffin-embedded tumor tissue from 46 cerebellar (39 primary and 7 recurrent tumors) and 5 spinal capillary hemangioblastomas was immunocytochemically analyzed for the expression of EGFR and TGF-α. The tumors were derived from 43 patients (23 males, 20 females; mean age at operation, 45 years; range, 14 to 68 years). Three of the patients had a known family history of von Hippel-Lindau disease. Unfixed frozen tumor tissue for the extraction of DNA and RNA was available from 20 of these cases and peripheral blood for the extraction of leukocyte DNA was collected from 12 patients. Sufficient RNA for Northern blotting could be extracted from 14/20 tumors. Tumor tissue and blood had been frozen immediately after operation and stored at −135°C for up to 4 years. Controls for the mRNA expression studies included nonneoplastic adult human brain tissue (cortex and white matter from the temporal lobe of a patient operated on for epilepsy), two pilocytic astrocytomas and two medulloblastomas of the cerebellum, and the A431 epidermoid carcinoma cell line (see Figure 2). A431 was obtained from American Type Culture Collection (ATCC CRL1555).

DNA and RNA Extraction and Analysis

Extraction of high molecular weight DNA and total RNA as well as Southern and Northern blotting were carried out as previously described.[13,14] Plasmid probes for *TGFα* (clones phTGF1–10–925 and phTGF1–10–3350), EGFR (clone pE7) and the control probe pYNH24 (detects the anonymous locus D2S44) were obtained from ATCC. Southern and Northern blots were hybridized using these probes labeled with [^{32}P]-dCTP by random priming. Hybridized membranes were exposed to phosphor storage screens (Molecular Dynamics, Sunnyvale, CA), scanned in a Molecular Dynamics PhosphorImager, and analyzed using the Molecular Dynamics ImageQuant software. Quantitative densitometric analysis of Southern blot hybridizations was performed by using the variable number of tandem repeats probe pYNH24 as reference.[14] A synthetic 50-base oligonucleotide probe complementary to bases 101–150 in the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (EMBL accession no. XO1677) was used to assess variations in loading of Northern blots. This probe was labeled with [^{32}P]-dCTP by 3′-tailing as described.[13]

Immunocytochemistry

Immunocytochemical stainings were performed on formalin-fixed paraffin sections of 51 capillary hemangioblastomas using the avidin-biotin peroxidase method as described.[15] The substrate for the peroxidase reaction was 3,3-diaminobenzidine (Sigma Chemical Co., Deisenhofen, Germany). As primary antibodies we used the mouse monoclonal IgG_1 antibody E30 (Merck, Darmstadt, Germany), which detects a formalin-resistent polypeptide epitope on the extracellular domain of the EGFR protein. The mouse monoclonal IgG_{2a} antibody 213–4.4 (Oncogene Science Inc., via Dianova, Hamburg, Germany) was used for the detection of TGF-α. This antibody recognizes a formalin-resistent epitope between residues 34 and 50 of the TGF-α polypeptide. Both antibodies were used at final dilutions of 1 μg/ml for an incubation period of 16 hours at room temperature. Negative controls were performed by omitting the primary antibodies and applying an irrelevant mouse monoclonal IgG antibody instead. As positive controls paraffin sections from selected glioblastomas with known overexpression of EGFR and/or TGF-α as well as normal skin were used.

Results

The expression of EGFR as well as the pre-pro form of TGF-α was studied by Northern blotting in 14 capillary hemangioblastomas. These experiments showed signals for EGFR and pre-pro-TGF-α transcripts in all cases studied (Figure 1). The signal intensities obtained for EGFR and the pre-pro-TGF-α mRNAs relative to the GAPDH control signals were stronger in the capillary hemangioblastomas than those seen in the four cerebellar control tumors (two pilocytic astrocytomas and two medulloblastomas) and also exceeded those obtained for nonneoplastic brain (Figure 1).

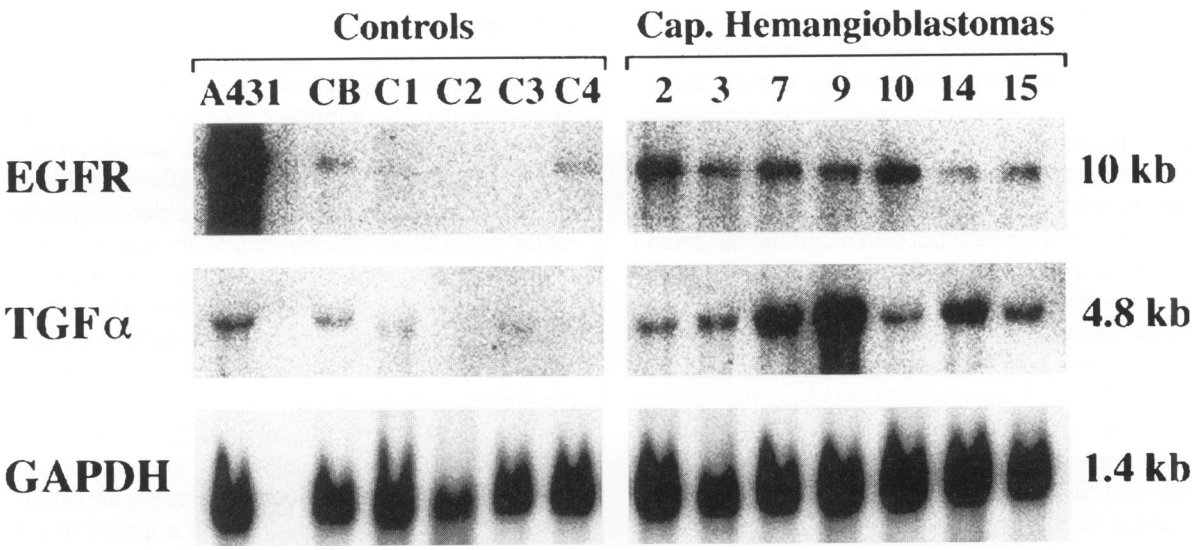

Figure 1. *Analysis of* EGFR *and* TGFα *precursor mRNA expression in capillary hemangioblastomas by Northern blot hybridizations. The controls include the A431 epidermoid carcinoma cell line, nonneoplastic control brain tissue (CB), two cerebellar pilocytic astrocytomas (C1, C2), and two medulloblastomas (C3, C4). Note the strong signals obtained for* EGFR *and* TGFα *precursor mRNAs in the capillary hemangioblastomas (cases 2, 3, 7, 9, 10, 14, and 15), while the control tumors (C1 to C4) demonstrate either no or very weak expression. A431 cells strongly overexpress* EGFR *because of gene amplification. The blot was stripped and rehybridized with an oligonucleotide probe for* GAPDH *to demonstrate approximately equal amounts of RNA loading. The approximate transcript sizes are indicated on the right side.*

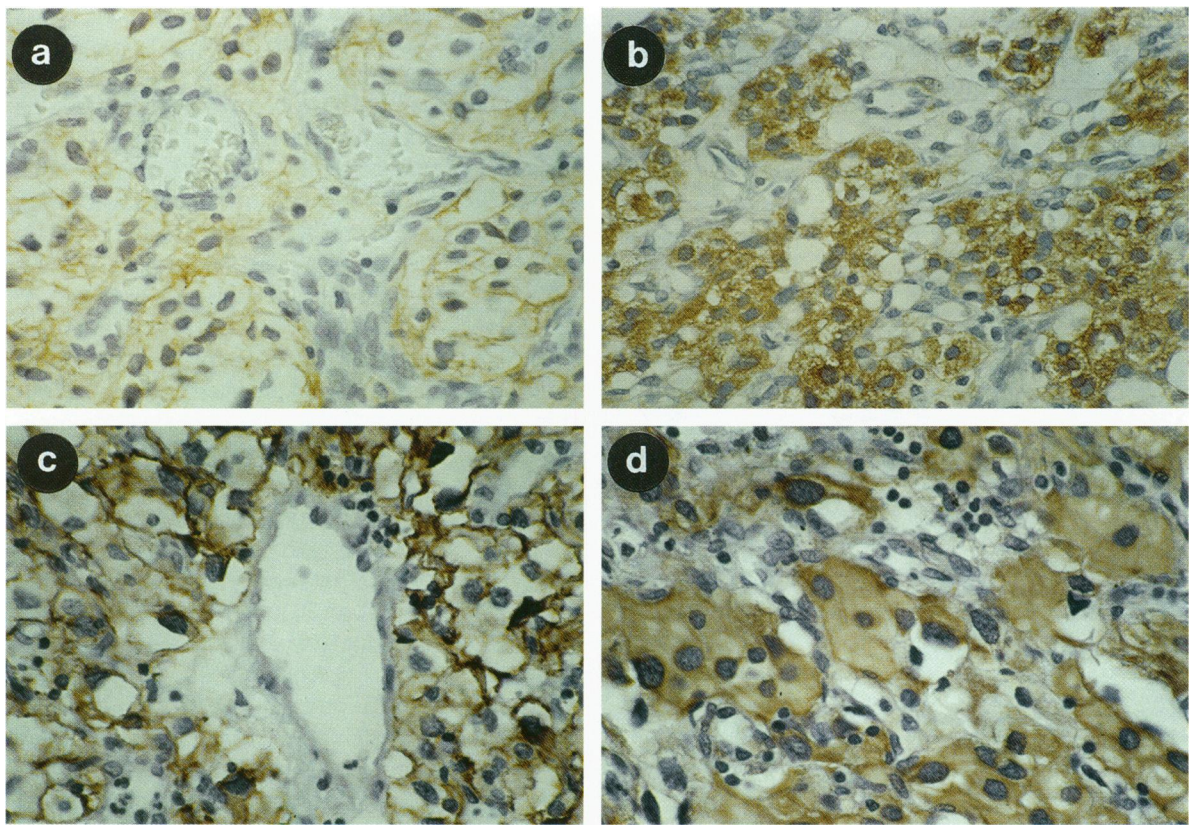

Figure 2. *Immunoreactivity for EGFR* (a, c) *and TGF-α* (b, d) *in two capillary hemangioblastomas (cases 28* (a *and* b) *and 16* (c *and* d)). *Note the moderate to strong immunostaining of stromal cells for both proteins. The sections are counterstained with hematoxylin. 350×.*

Immunocytochemistry revealed a moderate to strong immunoreactivity with the monoclonal antibody against TGF-α in stromal cells of all the capillary hemangioblastomas investigated (Figure 2, b and d). Some regional variations in the intensity of the immunostaining were present in most of the tumors. The immunoreactive products appeared to be mainly located in the cytoplasm but also along the cell membrane of the stromal cells. Occasional staining of capillary endothelial cells was observed. In contrast, immunoreactivity for EGFR was completely restricted to the stromal cells and was mainly located at the cell surface (Figure 2, a and c). The staining intensity for EGFR was generally weaker than for TGF-α and in some cases was restricted to focal areas. However, only 4/51 tumors were immunocytochemically totally negative for EGFR.

Densitometric analysis of *EGFR* and *TGFα* gene dosage in 20 capillary hemangioblastomas was performed on Southern blots. All tumors showed signal intensities corresponding to two copies of each gene and no evidence for loss of one allele or gene amplification could be detected (Figure 3).

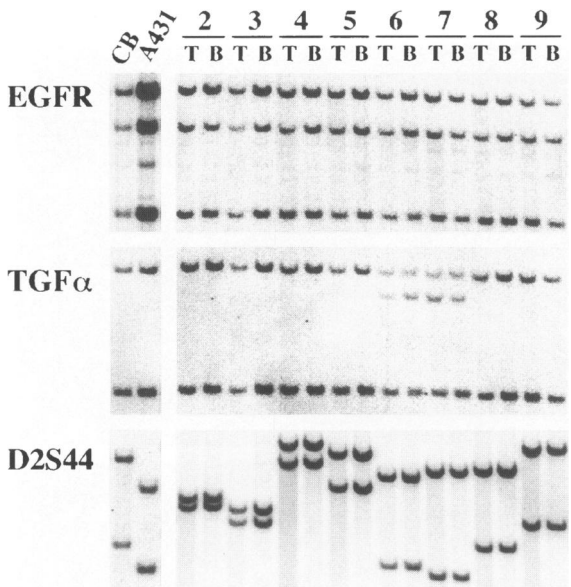

Figure 3. *Southern blot analysis of* EGFR *and* TGFα *gene dose in capillary hemangioblastomas (cases 2 to 9) as well as in control brain (CB) and A431 carcinoma cells. Tumor DNA (T) and blood DNA (B) of patients 2 to 9 was digested with* TaqI*, electrophoretically separated on 0.8% agarose gels, and blotted to nylon membranes. The membranes were probed with radiolabeled plasmid probes for* EGFR *(pE7),* TGFα *(phTGF1–10–925), and the control locus* D2S44 *(pYNH24). A431 shows an amplified signal for the* EGFR *gene, whereas none of the capillary hemangioblastomas demonstrates an increase in gene dosage. Densitometric analysis using the* D2S44 *signal as reference for the amount of DNA loaded in each lane revealed the presence of two alleles of both genes* (EGFR *and* TGFα) *in the tumor DNA of all capillary hemangioblastomas. Note that cases 6 and 7 are informative for the* TaqI *polymorphism detected by phTGF1–10–925 and both show retention of two alleles in the tumor DNA.*

Discussion

Growth factors and growth factor receptors have been shown to play important roles not only in normal development and differentiation but also in the abnormal growth of tumor cells.[16] Transforming growth factor-α (TGF-α) is a member of the epidermal growth factor superfamily of polypeptide mitogens.[17] The mature and secreted 50-amino acid polypeptide is derived from a 160-amino acid transmembrane precursor protein, which is transported to the cell surface.[17] The precise physiological functions of TGF-α are presently unknown, but it has been shown in various *in vitro* studies to regulate cellular proliferation, migration, and even differentiation.[17] *In vivo*, TGF-α is expressed by various normal developing and adult tissues.[18] However, its expression appears to be most abundant in certain pathological conditions including wound healing[19] and particularly neoplastic growth.[20] *In vitro* studies[21–23] as well as experiments using transgenic mice[24,25] have documented that overexpression of TGF-α can result in neoplastic transformation of various cell types. This growth-promoting activity of TGF-α is mediated by the EGFR.[26,27] It has been shown that not only the secreted mature TGF-α polypeptide but also the membrane-bound proTGF-α precursor is capable of EGFR activation.[28,29] EGFR activation resulting in neoplastic transformation *in vitro* may also be caused by overexpression of the receptor at high levels in the presence of one of its ligands.[30,31]

In vivo, coexpression of TGF-α and EGFR has been demonstrated in a variety of human tumors including different types of epithelial cancers as well as certain non-epithelial tumors.[20] Among the tumors of the nervous system transcripts for either TGF-α or EGF are consistently expressed in low- and high-grade gliomas, with the latter tumors frequently demonstrating amplification and overexpression of the *EGFR* gene.[13] In the present study we demonstrate a coexpression of TGF-α and EGFR by stromal cells in capillary hemangioblastomas of the central nervous system. In contrast to malignant gliomas, no evidence for EGFR gene amplification was found in these tumors. However, it is noteworthy that another tumor associated with the von Hippel-Lindau syndrome, ie, renal cell carcinoma, frequently demonstrates not only mutations in the *VHL* gene[9,10] but also enhanced expression of TGF-α and EGFR.[32,33] As in the capillary hemangioblastomas, overexpression of EGFR in renal cell carcinomas is usually not a consequence of gene amplification.[34] Thus, both tumor types, ie, renal cell carcinomas and capillary hemangioblastomas, may not only show morphological similarities but also

appear to share common molecular alterations. In conclusion, our results suggest that coexpression of EGFR and TGF-α may play a role in the growth of capillary hemangioblastomas by providing autocrine or juxtacrine growth stimuli for the stromal cells. It remains to be investigated whether and how the expression of TGF-α and EGFR can be related to alterations in the *VHL* gene.

Acknowledgments

The authors would like to thank their neurosurgical colleagues at the Department of Neurosurgery, Heinrich-Heine-University, Düsseldorf (Prof. Dr. W. J. Bock, Dr. J. C. W. Kiwit), the Neurosurgical Clinic, Evangelische und Johanniter Krankenanstalten, Duisburg-Nord/Oberhausen (Dr. G. Kremer), the Department of Neurosurgery, Sahlgrenska Hospital, Gothenburg, and the Department of Neurosurgery, Karolinska Hospital, Stockholm for providing the surgical specimens that enabled this study. We thank Ulla-Britt Strandlund, Ulla Karlsson, Kristina Müntzing, and Christa Mähler for excellent technical assistance.

References

1. Burger PC, Scheithauer BW: Tumors of the central nervous system. Atlas of Tumor Pathology, Third Series, Fascicle 10. Washington, D.C., Armed Forces Institute of Pathology, 1994, pp 239–243
2. Neumann HP, Eggert HR, Weigel K, Friedburg H, Wiestler OD, Schollmeyer P: Hemangioblastoma of the central nervous system. A 10-year study with special reference to von Hippel-Lindau syndrome. J Neurosurg 1989, 70:24–30
3. Frank TS, Trojanowski JQ, Roberts SA, Brooks JJ: A detailed immunohistochemical analysis of cerebellar hemangioblastoma: an undifferentiated mesenchymal tumor. Mod Pathol 1989, 2:638–651
4. Becker I, Paulus W, Roggendorf W: Histogenesis of stromal cells in cerebellar hemangioblastomas. An immunohistochemical study. Am J Pathol 1989, 134:271–275
5. Nemes Z: Fibrohistiocytic differentiation in capillary hemangioblastoma. Hum Pathol 1992, 23:805–810
6. Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, Schmidt L, Zhou F, Li H, Wei MH, Chen F, Glenn G, Choyke P, Walther MM, Weng Y, Duan DSR, Dean M, Glavac D, Richards FM, Crossey PA, Ferguson-Smith MA, Le Paslier D, Chumakov I, Cohen D, Chinault AC, Maher ER, Linehan WM, Zbar B, Lerman MI: Identification of the von Hippel-Lindau disease tumor suppressor gene. Science 1993, 260:1317–1320
7. Kanno H, Kondo K, Ito S, Yamamoto I, Fujii S, Torigoe S, Sakai N, Hosaka M, Shuin T, Yao M: Somatic mutations of the von Hippel-Lindau tumor suppressor gene in sporadic central nervous system hemangioblastomas. Cancer Res 1994, 54:4845–4847
8. Crossey PA, Foster K, Richards FM, Phipps ME, Latif F, Tory K, Jones MH, Bentley E, Kumar R, Lerman MI, Zbar B, Affara NA, Ferguson-Smith MA, Maher ER: Molecular genetic investigations of the mechanisms of tumourigenesis in von Hippel-Lindau disease: analysis of allele loss in VHL tumours. Hum Genet 1994, 93: 53–58
9. Shuin T, Kondo K, Torigoe S, Kishida T, Kubota Y, Hosaka M, Nagashima Y, Kitamura H, Latif F, Zbar B, Lerman MI, Yao M: Frequent somatic mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinomas. Cancer Res 1994, 54:2852–2855
10. Gnarra JR, Tory K, Weng Y, Schmidt L, Wei MH, Li H, Latif F, Liu S, Chen F, Duh FM, Lubenski I, Duan DR, Florence C, Pozzatti R, Walther MM, Bander NH, Grossman HB, Brauch H, Pomer S, Brooks JD, Isaacs WB, Lerman MI, Zbar B, Linehan WM: Mutations of the VHL tumour suppressor gene in renal carcinoma. Nature Genet 1994, 7:85–89
11. Morii K, Tanaka R, Washiyama K, Kumanishi T, Kuwano R: Expression of vascular endothelial growth factor in capillary hemangioblastoma. Biochem Biophys Res Commun 1993, 194:749–755
12. Plate K, Wizigmann-Voos S, Breier G, Risau W: Evidence for a paracrine growth regulatory mechanism in human hemangioblastoma. Clin Neuropathol 1994, 13:264 (Abstr.)
13. Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF, Collins VP: Genes for epidermal growth factor receptor, transforming growth factor-α, and epidermal growth factor and their expression in human gliomas *in vivo*. Cancer Res 1991, 51:2164–2172
14. Reifenberger G, Liu L, Ichimura K, Schmidt EE, Collins VP: Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. Cancer Res 1993, 53:2736–2739
15. Reifenberger G: Immunohistochemie der Tumoren des Nervensystems. Heidelberg, Springer-Verlag, 1991
16. Aaronson SA: Growth factors and cancer. Science 1991, 254:1146–1153
17. Derynck R: Transforming growth factor α. Cell 1988, 54:593–595
18. Luetteke NC, Lee DC: Transforming growth factor α: expression, regulation and biological action of its integral membrane precursor. Semin Cancer Biol 1990, 1:265–275
19. Schultz G, Rotatori DS, Clark W: EGF and TGF-α in wound healing and repair. J Cell Biochem 1991, 45: 346–352
20. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH: Synthesis of messenger RNAs for transforming growth factors α and β

and the epidermal growth factor receptor by human tumors. Cancer Res 1987, 47:707–712
21. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R: Expression in rat fibroblasts of a human transforming growth factor α cDNA results in transformation. Cell 1986, 46:301–309
22. Watanabe S, Lazar E, Sporn MB: Transformation of normal rat kidney (NRK) cells by an infectious retrovirus carrying a synthetic rat type α transforming growth factor gene. Proc Natl Acad Sci USA 1987, 84:1258–1262
23. McGeady ML, Kerby S, Shankar V, Ciardiello F, Salomon D, Seidman M: Infection with TGF-α retroviral vector transforms normal mouse mammary epithelial cells but not normal rat fibroblasts. Oncogene 1989, 4:1375–1382
24. Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH, Merlino GT: TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 1990, 61:1137–1146
25. Sandgren EP, Luetteke NC, Qiu TH, Palmitter RD, Brinster RL, Lee DC: Transforming growth factor α dramatically enhances oncogene-induced carcinogenesis in transgenic mouse pancreas and liver. Mol Cell Biol 1993, 13:320–330
26. Marquardt H, Hunkapillar MW, Hood LE, Twardzik DR, DeLarco JE, Stephenson JR, Todaro GJ: Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor. Proc Natl Acad Sci USA 1983, 80:4684–4688
27. Massagué J: Epidermal growth factor-like transforming growth factor II. Interaction with epidermal growth factor receptors on human placenta membrane and A431 cells. J Biol Chem 1983, 258:13614–13620
28. Brachman R, Lindquist PB, Nagashima M, Kohr W, Lipari T, Napier M, Derynck R: Transmembrane TGF-α precursors activate EGF/TGF-α receptors. Cell 1989, 56:691–700
29. Wong ST, Winchell LF, McCune BK, Earp HS, Teixidó J, Massagué J, Herman B, Lee DC: The TGF-α precursor expressed on the cell surface binds to the EGF receptor on adjacent cells leading to signal transduction. Cell 1989, 56:495–506
30. Di Fiore PP, Pierce JH, Fleming TP, Hazan R, Ullrich A, King CR, Schlessinger J, Aaronson SA: Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH3T3 cells. Cell 1987, 51:1063–1070
31. Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR: Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. Science 1987, 238:1408–1410
32. Mydlo JH, Michaeli J, Cordon-Carlo C, Goldenberg AS, Heston WDW, Fair WR: Expression of transforming growth factor α and epidermal growth factor receptor messenger RNA in neoplastic and nonneoplastic human kidney tissue. Cancer Res 1989, 49:3407–3411
33. Petrides PE, Bock S, Bovens J, Hofman R, Jakse G: Modulation of pro-epidermal growth factor, pro-transforming growth factor-α, and epidermal growth factor receptor gene expression in human renal carcinoma. Cancer Res 1990, 50:3934–3939
34. Gomella LG, Anglard P, Sargent ER, Robertson CN, Kasid A, Linehan WM: Epidermal growth factor receptor gene analysis in renal cell carcinoma. J Urol 1990, 143:191–193