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THE ROLE OF EGF-RELATED PEPTIDES IN TUMOR GROWTH

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1. ABSTRACT

The epidermal growth factor (EGF) family of peptides encodes several proteins that can function as growth factors. The EGF-like peptides, with the exception of proteins of the EGF-CFC subfamily, bind and activate tyrosine kinase receptors that belong to the *erb*B family. The EGF-like peptides are overexpressed in a majority of human carcinomas as compared with their nontransformed counterpart. By using different approaches, it has been shown that several different EGF-like peptides function as autocrine growth factors in carcinoma cell lines of different histological origin. Direct evidence that the EGF-like growth factors might function as transforming genes has been provided by *in vitro* and *in vivo* studies. In particular, the development of different transgenic mouse lines in which EGF-like growth factors have been overexpressed by means of tissue-specific or nonspecific promoters has provided invaluable information relating to their ability to function as dominantly transforming oncogenes. Cooperation of the EGF-like peptides with cellular protooncogenes in determining cell transformation has been demonstrated by using both *in vitro* and transgenic mice systems. Taken together, these data strongly suggest that the EGF-like peptides are involved in the pathogenesis of human carcinomas, and that they might represent suitable targets for novel therapeutic approaches.

2. INTRODUCTION

Cancer is the result of a series of somatic and germline mutations in several regulatory genes (1). In fact, genetic alterations in protooncogenes and/or tumor suppressor genes can eventually lead to the evolution of cells that possess a growth advantage over cells that do not carry such mutations (2,3). Protooncogenes can be activated by several different mechanisms such as point-mutations, amplification, chromosomal translocation or insertional mutagenesis, whereas inactivation of tumor suppressor genes is due to inactivating point mutations and/or deletions that determine loss of heterozygosity (2,3). Protooncogenes code for proteins that are important components of growth promoting pathways (2). Tumor suppressor genes generally function as negative effectors of cell growth by encoding proteins that are involved in signal transduction, growth inhibitors, transcription factors, or proteins that are involved in DNA repair and apoptosis (3). Cumulative genetic alterations of these proteins can ultimately contribute to the clonal outgrowth of a malignant population of tumor cells.

Peptide growth factors are locally derived hormones that are involved in regulating the proliferation, differentiation and survival of normal cells. However, growth factors are also involved in the pathogenesis of

Table 1. The EGF family of growth factors and their cognate receptors

Growth factor	Receptor
Epidermal growth factor (EGF)	EGFR
Transforming growth factor-alpha (TGF-alpha)	EGFR
Amphiregulin (AR)	EGFR
Epiregulin (EP)	EGFR
Betacellulin (BTC)	EGFR and *erb*B-4
Heparin-binding EGF-like growth factor (HB-EGF)	EGFR and *erb*B-4
Tomoregulin	*erb*B-4
Neuregulins (NRG) 1	*erb*B-3 and *erb*B-4
Heregulin (HRG) alpha and beta	*erb*B-3 and *erb*B-4
Glial cell growth factor (GGF)	*erb*B-3 and *erb*B-4
Sensory motor neuron-derived growth factor (SMDGF)	*erb*B-3 and *erb*B-4
Acetylcholine receptor-inducing activity (ARIA)	*erb*B-3 and *erb*B-4
NRG 2 alpha and beta	*erb*B-3 and *erb*B-4
NRG 3	*erb*B-3 and *erb*B-4
NRG 4	*erb*B-4
Cripto-1 (CR-1)	unknown

human cancer (4). In fact, one important feature of transformed cells is that they exhibit a reduced requirement for exogenously supplied growth factors to maintain a high rate of proliferation. This relaxation in growth factor dependency may be due in part to the ability of tumor cells to produce high levels of peptide growth factors. The tumor-derived growth factors may function through intracrine, paracrine, juxtacrine and/or autocrine pathways to control cell proliferation and survival in tumor cells that express the cognate receptors for these peptides (5). However, tumor growth factors are also involved in regulating angiogenesis and formation of metastasis through interaction with the surrounding stromal cells. Growth factor receptors are proteins that have an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular tyrosine-kinase or serine-threonine kinase domain that may be on the same molecule or on a distinct subunit. An additional mechanism through which peptide growth factors may contribute to the proliferation of tumor cells might be represented by the overexpression of growth factor receptors. In fact, expression of a high number of receptors on the surface of tumor cells can increase their sensitivity to low concentrations of host- or tumor-derived growth factors. Finally, it has long been established that a direct correlation exists between growth factors and cellular protooncogenes (4-6). In fact, several protooncogenes code for proteins that are either growth factors, or growth factor receptors, or proteins that are involved in the intracellular signal transduction pathway for growth factors. In addition, activated cellular protooncogenes may also control the endogenous production of and/or the response of tumor cells to peptide growth factors.

It has been demonstrated that tumor cells are able to synthesize and to respond to a number of different peptide growth factors (4). Among these, it has long been established that the epidermal growth factor (EGF)-related peptides might contribute to the growth of tumor cells. In fact, the EGF-like growth factors perform an important role in early embryonic development, in the renewal of stem cells in normal tissues such as the skin, liver and gut, and in the pathogenesis of cancer in a number of different tissues (7,8). The EGF superfamily includes several proteins of diverse function that are structurally and functionally related (9). Some of these proteins can function as peptide growth factors through binding specific membrane receptors (table 1). In particular, the EGF-like proteins bind and activate receptors of the *erb*B family, with the only exception of CR-1 and the other peptides of the EGF-CFC subfamily, whose receptors have not been identified yet.

Several review articles have been published on the role of EGF-like growth factors in the pathogenesis of human carcinomas. Therefore, we will focus our discussion on more recent advances on the biology of these peptides. In the first part of this review article, we will describe the biochemical and biological characteristics of the EGF-related growth factors, with particular regard to the newly identified subfamily of EGF-CFC proteins. We will next describe the biochemical characteristics of protein tyrosine kinases within the *erb*B family, and the complex interactions existing between the growth factors of the EGF-family, the *erb*B receptors and the signal transduction pathways that are activated by the ligand-receptor interactions. Then we will present evidence that suggests that the EGF-like growth factors possess an oncogenic potential, and that they are able to cooperate with cellular protooncogenes in determining cell transformation. Finally, we will discuss the significance of the co-expression of EGF-like growth factors in human tumors and the potential applications for therapeutic purposes.

3. THE EGF-RELATED GROWTH FACTORS

The epidermal growth factor family of proteins includes several growth factors structurally related by the presence of a common motif, the EGF-like domain (table 1) (9). The EGF-like domain is characterized by the presence of six conserved cysteine residues spaced at defined intervals within a region of approximately 40 amino acids (9). The cysteine residues within this region are able to form three intramolecular disulfide bonds that restrain the molecule in a three-loop secondary structure. The acquisition of this secondary structure is essential for the biological activity of these proteins and for the binding to their specific receptors. The epidermal growth factor family of peptides includes epidermal growth factor (EGF), Transforming Growth Factor-alpha (TGF-alpha), amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin (BTC), epiregulin (EPR), tomoregulin, and the neuregulin (NRG) and EGF-CFC (Cripto) subfamilies. Most of these growth factors are synthesized as cell membrane-associated precursors that are biologically active and can interact with extracellular proteins through a

juxtacrine pathway (10). In this regard, some of these cell-associated precursors can function as cell-cell adhesion molecules that regulate migration and colonization of specific organs during metastasis (8,10). Furthermore, cleavage of precursor forms of these growth factors is regulated by serine-protease and elastase-like enzymes that can regulate the release and diffusion of these peptides (10,11). The NRG subfamily, in addition to the EGF-like domain, is characterized by the presence of immunoglobulin and kringle-like motifs that probably are necessary for other functions (11). The EGF-like domain has also been identified in many different proteins with biological functions unrelated to cell proliferation, such as blood clotting and cell adhesion proteins (8). These findings suggest that EGF-related proteins may have evolved from molecules with different biological functions.

EGF is encoded by a gene of 110 kb that contains 24 exons and is located on chromosome 4. A 4.8 kb mRNA encodes for a protein of 53 amino acids with a molecular weight of 6 kDa (12). It is initially synthesized as a large precursor of 1217 amino acids and has biological activity since can bind and activate the EGF receptor (EGFR) expressed in the adjacent cells (13). This precursor form has 9 EGF-like domains and in the mature soluble form of EGF only the EGF motif closest to the transmembrane domain is preserved. EGF was originally identified in mouse submaxillary gland as a protein that induced incisor eruption and premature eyelid opening in newborn mice (14). The human EGF was purified from the urine of pregnant women and initially characterized as an inhibitor of gastric acid secretion (urogastrone) (15). EGF is expressed in several adult human tissues, such as the submandibular glands, the Brunner glands in the small intestine and the kidney (16).

TGF-alpha is a peptide of 50 amino acids with a molecular weight of 5.6 kDa that share 42 % homology with EGF (17). Like EGF, TGF-alpha derives from a large 1200 amino acid transmembrane precursor and cleaved from the precursor by an ADAM-like enzyme (18). Such as in the case of EGF, this precursor molecule is biologically active. TGF-alpha was identified in the conditioned media of virus-transformed cells and of human tumor cells (17). Together with TGF-beta, it was found to induce transformation in normal fibroblasts, promoting anchorage-independent growth of the cells. TGF-alpha is expressed in normal adult tissues, especially in regenerating epithelial cells (19).

AR is encoded by a gene of 10.2 kb located on chromosome 4 and organized in 6 exons (20). A membrane-bound precursor of 252 amino acids is cleaved into a mature form of 78 or 84 amino acids, with a molecular weight of 22-24 kDa (21). In fact, different isoforms of AR can be found in the conditioned media depending on the degree of glycosylation and the length of the core protein. AR was initially identified in the conditioned medium of MCF-7 human breast cancer cells treated with the phorbol ester tumor promoter TPA (22). AR is a heparin binding protein and can either stimulate or inhibit cell proliferation depending on the concentration and presence of other growth factors (23). AR has been detected in the nucleus of several human breast cancer cell lines and two nuclear targeting domains have been identified in the protein (24).

HB-EGF is closely related to AR and it can also bind to heparin (25). It is synthesized from a large glycosylated precursor of 208 amino acids that is cleaved in several secreted forms with molecular weight ranging from 19 to 23 kDa (26). The HB-EGF precursor can function as a receptor for the bacterial diphtheria toxin (27). The different isoforms of mature HB-EGF can be due to different degrees of truncation of amino acids at the amino terminus or various degrees of glycosylation (26). Like AR, HB-EGF contains two nuclear localization sequences in the amino terminal region (26). The human HB-EGF gene is composed of 6 exons spanning 14 kb of DNA and maps to chromosome 5 (28). A 2.5 kb HB-EGF mRNA can be detected in several adult human tissues (29).

BTC was initially identified and purified from the conditioned media of a mouse insulinoma cell line derived from a transgenic mouse expressing the SV40 large T gene under the control of the insulin promoter (30,31). BTC is proteolitically processed from larger membrane-anchored precusors of 177 and 178 amino acids, which are 79% homologous (32). The mature BTC protein consists of 80 amino acids with a molecular weight of 32 kDa. Similarly to AR and HB-EGF, BTC can bind to heparin (32). BTC is a potent mitogen for mouse fibroblast and for smooth muscle cells. BTC mRNA has been detected in several mouse tissues, with high levels of expression in the liver and kidney. In the human, BTC is highly expressed in the small intestine and in the pancreas (33).

EPR was purified from the conditioned media of the mouse fibroblast-derived tumor cell line NIH3T3/clone T7 (34). EPR consists of 46 amino acids and exhibits 25-50% amino acids sequence identity with the other EGF-related growth factors. Like AR, EPR has bifunctional regulatory properties. In fact, EPR can inhibit the growth of several epithelial tumor cells and stimulate the growth of fibroblast and other cell types (34). EPR mRNA is expressed in 7-day-old mouse embryo and then its expression decreases to low or undetectable levels (35).

Tomoregulin is a peptide that specifically binds to the type 1 *erb*B-4 tyrosine kinase receptor with low affinity and possesses two follistatin-related domains that have the potential to bind and neutralize activin (36).

The NRG family consists of four different genes: NRG-1, NRG-2, NRG-3 and NRG-4. NRG-1 family includes the rat neu differentiation factor (NDF) and the human homolog heregulin (HRG) as well as glial growth factors, acetylcholine receptor-inducing activity and sensory motor neuron-derived growth factor, reflecting the different biological systems where these growth factor have been identified (37,38). Different isoforms of alpha and beta HRG arise from alternative mRNA splicing of a single gene. NDF was initially identified in the conditioned media of Rat-1 fibroblast transformed with c-Ha-*ras* and HRG

Table 2. The EGF-CFC subfamily of proteins

Peptide	Species
Cripto-1 (CR-1)	human
Cryptin	human
Cripto –1 (Cr-1)	mouse
cryptic	mouse
cripto	chicken
FRL-1	xenopus
One-eyed pinhead (oep)	zebrafish

was purified from the conditioned media of the MDA-MB-231 human breast cancer cell line (39,40). HRG derives from a large transmembrane precursor. The mature form of 44 kDa is a membrane-associated glycoprotein containing in addition to the EGF-like domain an immunoglobulin-like domain in the amino terminus, a kringle-like domain and a long cytoplasmic tail (41). Like AR and HB-EGF, most of the HRG isoforms can bind heparin. Most variants of NRG-1 are widely expressed in numerous tissues including brain, heart, skeletal muscle, breast, liver and lung (42). NRG-2 has a structure similar to NRG-1 and an alternative splicing site in the EGF-like domain gives rise to alpha and beta isoforms (43). NRG-2 has a distinct expression pattern in the adult brain and in the developing heart with respect to that of NRG-1 (44). NRG-3 is 33% identical to HRG–beta 1 and lacks the immunoglobulin-like and kringle-like domains that are characteristic of many NRG-1 family members (45). The expression of NRG-3 is highly restricted to the developing and adult nervous system. Recently, a fourth NRG, denominated NRG-4, has been identified (46). Excluding the EGF-like domain, NRG-4 shares very little sequence homology to the known NRGs. Expression of NRG-4 mRNA has been detected in the pancreas and in the muscle, whereas other tissues have no detectable levels of NRG-4 mRNA.

4. THE EGF-CFC FAMILY

The EGF-CFC family includes a group of structurally related proteins that have been identified in man, mouse, chicken, frog and zebrafish (table 2). This family consists of human Cripto-1 (CR-1) (47) and criptin (unpublished Human Genome Science Inc., patent number S5981215), mouse cripto-1 and cryptic (48,49), chicken cripto (chick-cripto) (50), *Xenopus* FRL-1and zebrafish *one eyed pinhead* (*oep*) (51,52). CR-1, also known as teratocarcinoma-derived growth factor-1 (TDGF-1), was serendipitously isolated and sequenced from a human NTERA2/D1 embryonal carcinoma cDNA expression library (47). Thereafter, the mouse gene Cr-1 was cloned from an F-9 mouse embryonal carcinoma cDNA expression library (48). Mouse cryptic was identified from mouse ES-derived mesoderm cDNA library by differential display RT-PCR and human criptin from a human pancreatic carcinoma cDNA library (49). Chick-Cripto was cloned by subtractive hybridization between two explants of stage 7^{+} embryos (50). FRL1 was identified from a *Xenopus* embryonic mesoderm cDNA library and *oep* has been recently cloned in zebrafish (51,52). Human CR-1 maps to a region on chromosome 3p21.3 that is deleted or exhibits loss of heterozygosity in a subpopulation of renal, bladder, gastric, breast and small cell lung carcinomas (53,54). The mouse and human genes consist of six exons and five introns (48,53). The fourth exon contains the EGF-like domain and is well-conserved among the different genes. A different regulation of expression of the mouse and human cripto genes has been proposed, since the promoter region of the mouse genes contains TATA and CAAT boxes while these sequences are absent in the human promoter (55). Five human CR-1 related genes and two mouse Cr-1 genes have been identified (56). In this respect CR-3 in the human, which maps to the Xq21-q22 region, and Cr-2 in the mouse are intronless pseudogenes that can code for a functional protein five amino acids shorter that the protein encoded by CR-1 or Cr-1. Human CR-1 and mouse Cr-1 encode a major mRNA species of 2.2kb, but also minor transcripts of 1.7, 3.0, 3.2 and 3.7 kb have been detected in midgestation mouse embryos and in human colon and hepatic carcinomas (47,48,55). These shorter transcripts may arise by the use of a different polyadenylation sequence, by alternative splicing or by the use of an alternative initiation codon. The EGF-CFC gene family encodes for proteins that share a conventional amino terminus signal peptide, a modified epidermal growth factor (EGF)-like motif, a conserved cysteine rich domain (CFC region), and a short hydrophobic carboxy terminus that is essential for membrane anchorage by a glycosylphosphatydilinositol (GPI) mioety (47-52). Whereas the overall sequence identity between these proteins is only 22-32%, within the EGF-like domain the sequence similarity is nearly 60-70% and in the CFC domain is 35-48%. Unlike the canonical EGF-like domain that contains three loops (A, B and C loop), the modified EGF-like domain lacks the A loop and has a truncated B loop. The EGF-CFC proteins range from 171 to 202 amino acids with a core protein of 18-21 kDa. Native mouse and human cripto proteins range from 24 to 36 kDa in size but additional proteins ranging from 14 to 60 kDa have been identified by gel electophoresis (57-60). This variation in size could be due to the removal of the hydrophobic signal peptide and to post-translational modifications of the core protein. In fact, all the members of the EGF-CFC family, except for *oep*, are glycoproteins that contain a single N-glycosylation site and potential O-glycosylation sites (57-60). Recently, a single O-linked fucosylation site has been found between the second and third cysteines in the EGF-like domain of all the EGF-CFC proteins (61). This rare modification is also present in the EGF-module of secreted proteins involved in blood coagulation or clot dissolution such as blood coagulation factor VII and urokinase plasminogen activator (uPA) (62,63). In addition, an O-linked fucose modification has also been described for a membrane-associated protein, Notch I (64). Interestingly, disruption of this O-linked fucosylation site in Notch I has been found in one case of human disease CADASIL and may be important for receptor-ligand interaction (65). With the exception of FRL1, all the other members of the EGF-CFC family share a unique consensus sequence for GPI cleavage and attachment to the cell membrane that is adjacent to the carboxy terminus of the protein (60).

Phosphatidylinositol specific phospholipase C (PI-PLC) treatment can release Cr-1 from the cell membrane, suggesting that the EGF-CFC proteins can function as secreted or cell-associated molecules. EGF-CFC proteins also contain several potential myristylation sites and consensus sequence for potential phosphorylation on serine and threonine by protein kinase C (PKC), cyclic AMP-dependent protein kinase A (PKA) and casein kinase II (CK2).

During early mouse embryogenesis, low levels of Cr-1 mRNA expression can be detected by RT-PCR in the inner cell mass (ICM) of the postimplatation mouse blastocyst at day 4 of development (66-68). In this regard, CR-1 and Cr-1 are expressed in undifferentiated human NTERA2/D1 and mouse F-9 embryonal carcinoma cells, whereas its expression is lost in these cells when they differentiate in response to retinoic acid treatment (47,48). In contrast, cryptic exhibits a distinct expression pattern since it is found only in differentiated mesoderm cells and not in embryonal carcinoma cells (49). By *in situ* hybridization, Cr-1 mRNA expression is restricted to the epiblast cells of the primitive streak, to the developing mesoderm during gastrulation and later to the myocardium of the truncus arterious of the developing heart (48,66-68). After day 8 very little expression of Cr-1 can be detected in the embryo, with the exception of the developing heart. Cr-1 protein expression has also been detected in the developing embryo by Western blot analysis (60). In concordance with Cr-1 mRNA expression, Cr-1 protein was found in the developing mesoderm at day 6.5 and in the myocardium of the developing heart in later fetal stages. The presence of Cr-1 in the developing cardiac outflow suggests that it may play an essential role during embryonic cardiac development and differentiation. In this regard, Cr-1 deficient pluripotential embryonic stem (ES) cells loose their ability to differentiate *in vitro* in cadiomyocytes, whereas they maintain the ability to differentiate in other cell types (69).

Germline knockout of mouse Cr-1 is embryonically lethal with homozygous embryos lacking a primitive streak and embryonic mesoderm (67,68). In particular, homozygous embryos are mostly comprised of anterior neuroectoderm with a severe loss of mesoderm and endoderm. Furthermore, mouse Cr-1 is necessary for the correct orientation of the anterior-posterior (A-P) axis, since in homozygous embryos markers of the head organizer are localized in the distal visceral endoderm, while markers of the primitive streak are located in the proximal epiblast (67). Heterozygous embryos are normal, suggesting that Cr-1 produced by the wild type host is able to rescue the mutant phenotype. Genetic studies have also demonstrated that *oep* and cryptic are involved in the establishment of the left-right (L-R) embryonic axis (70,71). In this regard, mutation of *oep* in zebrafish and targeted disruption of cryptic in the mouse results in several L-R laterality defects including randomization of cardiac looping and atrial-ventricular septal defects, right pulmonary isomerization, inverted situs of the spleen, pancreas and stomach, hyposplenia and heterotaxia. Moreover, embryos that lack both maternal and zygotic activity of *oep* display a severe phenotype characterized by cyclopia, lack of head and trunk, mesoderm and endoderm and altered orientation of the A-P axis (52). Recently, human cryptic, isolated from a human fetal brain library, has been associated with human L-R laterality defects (72). Human Cryptic protein is 55% identical to mouse cryptic protein and its carboxy terminus is 29 amino acids longer. Four unique nucleotide changes have been identified in sporadic or familial cases of L-R axis abnormalities. The mutations identified were a 334 C→T transition in exon 4 leading to substitution of arginine 112 in the EGF-like domain with cysteine (R112C), a single nucleotide deletion 522 delC in exon 6 corresponding to deletion of glycine 174 (G174del1) at the beginning of the membrane-associating domain with generation of a protein that is not cell-membrane associated, a 562 C→T transition in exon 6 corresponding with a change of arginine 189 with cysteine (R189C), and a 232 C→T transition in exon 3 corresponding with a change of arginine 78 with tryptophan (R78W). The R112C and G174del1 mutant proteins were completely inactive in rescuing zebrafish maternal-zygotic *oep* mutant embryos, suggesting a loss of function of these proteins. In contrast, R78W and R189C were functional proteins since they rescued the phenotype of mutant embryos. Interestingly, all the patients with mutations of cryptic gene were heterozygous, whereas heterozygous cryptic mice or *oep* zebrafish are phenotypically wild type. This can be due to differences in gene dosage together with environmental and/or genetic factors.

It has previously been demonstrated that CR-1 can activate the *ras*/*raf*/MEK/MAPK pathway in several nontransformed and malignant human mammary epithelial cells (73). In particular, CR-1 full-length protein or a refolded peptide corresponding to the EGF-like domain can enhance a rapid and transient increase in the tyrosyne phosphorylation of p66, p52 and p46 isoforms of the adaptor protein Shc, thereby facilitating its association with Grb2, and induce activation of p42 and p44 isoforms of MAPK. Although CR-1 is a member of the EGF-family of peptides, it is not able to bind directly to any of the four known members of the type I receptor tyrosine kinase family when they are ectopically expressed in Ba/F3 mouse pro-B lymphocytes or mouse 32D myeloid cells that do not normally express this family of receptor tyrosine kinases (73). Furthermore, a specific, high affinity and saturable binding site was identified for CR-1 in several different human mammary epithelial cell lines and other EGF-related peptides were unable to compete for binding, suggesting that this may represent a novel receptor (73). Interestingly, CR-1 can indirectly enhance the specific tyrosine phosphorylation of *erb*B-4 in mouse and human mammary epithelial cells, and *erb*B-4 activity is essential for CR-1 ability to activate MAPK (74). Finally, chemical cross-linking of ^{131}I-CR-1 to mouse and human mammary epithelial cells identifies two bands of 60 and 120 kDa, suggesting the presence of a multicomponent receptor for CR-1 (74).

Although these results implicate CR-1 in the *ras*/*raf*/ MAPK pathway, recent genetic evidence has shown a role for EGF-CFC family in nodal signaling (75). Nodal is a member of the TGF-beta superfamily and

perfoms an essential role during mesoderm development in the mouse, zebrafish and frog (75). Recent data have shown that EGF-CFC proteins are important co-factors of Nodal signaling (76). In fact, embryos that lack both maternal and zygotic *oep* display the same phenotype of zebrafish *cyclops* and *squint* mutants, which are nodal-related genes. In addition, the phenotype induced in wild type embryos by overexpression of *cyclops* and *squint* is blocked by *oep* mutation. It has been proposed that the combined activity of *oep* and nodal correspond to the activity of Activin, another TGF-beta family member. In this respect, mRNA injection of a costitutive activated form of Activin receptor RIB or the transcription factor Smad 2 is able to rescue the *oep* mutant phenotype (52). Also injection of Activin can rescue the *oep* mutants, confirming that the combined activity of *oep* and nodal is equivalent to that Activin activity (52). Further studies will be necessary to understand the role of EGF-CFC molecules in the nodal signaling pathway and elucidate the link to the *ras*/*raf*/MAPK pathway.

5. THE *erb*B RECEPTOR FAMILY

The *erb*B receptors belong to the Type I receptor tyrosine kinase family (77). This family includes four members: EGF receptor (also known as *erb*B1/HER1), *erb*B-2/Neu/HER2, *erb*B-3/HER3 and *erb*B-4/HER4. EGFR is a 170 kDa transmembrane glycoprotein that exhibits considerable homology with the avian erythroblastosis virus-transforming protein v-*erb*B (78). The EGFR protein consists of an extracellular domain, a hydrophobic transmembrane domain, and an intracellular region containing the tyrosine kinase domain (78). The extracellular domain is organized into four subdomains (I-IV). Subdomain III is primarily involved in ligand binding, whereas subdomains II and IV are rich in cysteine residues. The intracellular region can be divided into three domains: the juxtamembrane region, the tyrosine kinase domain and a long carboxy terminal tail containing five autophosphorylation sites which link to proteins containing Src homology 2 (SH2) and phosphotyrosine binding domain (PTB) motifs (79). The other three members of the *erb*B family have a similar structure with a high degree of homology in the tyrosine kinase domain (77,79). This homology is less pronounced for *erb*B-3 and in fact it has been shown that this receptor has an impaired tyrosine kinase domain (80). In contrast, the extracellular domains are less conserved among the four receptors, which is indicative of different specificity in ligand binding.

The are three classes of ligands that can bind and activate distinct sets of individual receptors (81). The first group consists of EGF, AR and TGF-alpha that can exclusively bind to EGFR. HB-EGF and BTC can efficiently interact with the EGFR and *erb*B-4, whereas epiregulin appears to be a broad spectrum receptor ligand (82-84). Finally the heregulins can bind to *erb*B-3 and *erb*B-4. In this respect, NRG-1 and NRG-2 bind both *erb*B-3 and *erb*B-4, whereas NRG-3 and NRG-4 bind *erb*B-4 but not *erb*B-3 (45,46,85,86). Thus far, no ligand that binds to *erb*B-2 has been identified.

Binding of a peptide growth factor to its receptor is accompanied by dimerization of its receptor, which is essential for the subsequent generation of intracellular second messengers and biological responses. Upon ligand binding to *erb*B receptors, not only homodimers but also heterodimers receptor complexes can be formed. All possible homo- and heterodimeric receptor complexes between members of the *erb*B family have been identified in different systems (81). Since *erb*B-2 is an orphan receptor, it can only take part in signal transduction by forming heterodimers with other members of the *erb* B family (87). In addition, *erb*B-3 lacks an intrinsic tyrosine kinase activity and its homodimers are inactive (87). Therefore, *erb*B-3 can function only when complexed with another *erb*B receptor. In this regard, *erb*B-2 and *erb*B-3 can complement each other's deficiencies: a heterodimeric complex containing an orphan receptor and an inactive tyrosine kinase receptor can form the most potent *erb*B signaling complex in terms of cell proliferation and transformation (88). Recently, several findings have elucidated the rules that drive the formation of receptor association upon ligand binding with a strict hierarchical principle followed by the four *erb*B receptors (81). *erb*B-2 seems to be the preferred dimerization partner for all the other *erb*B receptors (89,90). In fact, *erb*B-2-containing heterodimers have a decelerated off rate with a subsequent increased affinity for the ligand and prolonged activation of intracellular signaling pathways (91). The three heterodimers that are formed most frequently are *erb*B-2/*erb*B-3, *erb*B-2/*erb*B-4 and *erb*B1/*erb*B-4. In addition, the different ability to form homo- and heterodimers is dictated also by the levels of expression of these receptors in the cells (92). Even if the formation of a particular receptor complex is not favored according to the above theory, it can become important if the particular proteins are expressed at high levels.

Since high-quality crystal structures of EGF-like growth factors and their receptors are not yet available, very little is known about the requirements for *erb*B-receptor-ligand interaction (93). It is also uncertain if homodimeric receptor complexes require one or two molecules of ligand. Calorimetric analysis and gel permeation chromatography has demonstrated that a 2:2 receptor ligand is formed for EGF/*erb*B1 receptor complex (94,95). For heterodimeric complexes containing *erb*B-2, a single molecule of ligand should be sufficient for receptor heterodimerization, since no ligands are known for *erb*B-2. This has led to a “bivalent ligand” model in which a single ligand after binding with high affinity to its specific receptor can form a bridge with another *erb*B receptor (96). For example, in the case of NRG-beta 1, hydrophobic amino acids in the N-terminal region and the B-loop form the high affinity *erb*B-3 receptor binding domain, and hydrophobic residues in the C-terminal tail form the low affinity binding domain for *erb*B-2.

The four *erb*B receptors share most of the intracellular second messengers generated when they are activated (81). These include adaptor proteins such as Shc and Grb2, which couple the *erb*B receptors to the mitogen-activated protein kinase (MAPK) pathway, kinases such as

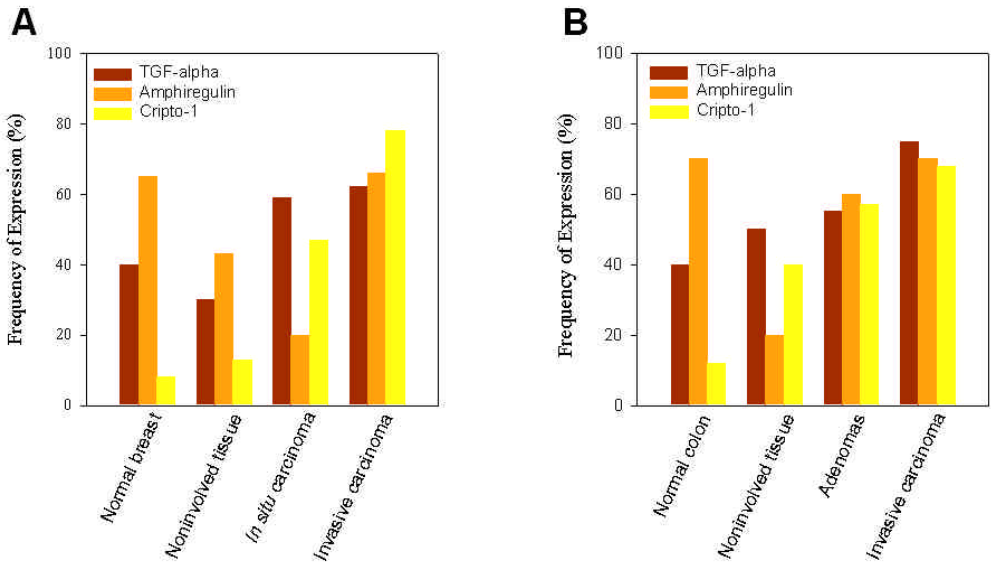

Figure 1. Expression of immunoreactive TGF-alpha, amphiregulin and Cripto-1 in normal, pre-malignant and neoplastic breast (A) and colon (B) tissues.

c-*src* and phosphatidylinositol 3-kinase (PI3K), and protein tyrosine phosphatases such as SHP1 and SHP2 (81). However, there are also examples of preferential activation of specific pathways. Eps 15 and Cbl are specifically activated by *erb*B-1, SHP-2 is specifically activated by *erb*B-2 and PI3-K is activated in particular by *erb*B-3 for the presence of multiple binding sites for p85 (79,97-99). Furthermore, Eps 15 and Cbl are involved in downregulation of EGFR. Eps 15 binds the clathrin-coated adaptor protein AP-2 and mediates rapid internalization of EGFR via clathrin-mediated endocytosis (100). In contrast, the other three *erb*B receptors do not show enhanced endocytosis following ligand binding and they remain mainly localized to the cell surface (101). Cbl is involved in receptor degradation by promoting the specific attachment of ubiquitin and by targeting of EGFR to the lysosomal compartment (102). Although internalization of EGFR is induced upon binding with all the known EGFR ligands, this process is different between EGF or TGF-alpha-bound receptors (103). EGF interaction with the EGFR is stable at different pH in the endocytotic vesicles, resulting in degradation of both ligand and receptor in the lysosomes. In contrast, TGF-alpha dissociates from EGFR at the low pH in the lysosomes, resulting in the recycling of the receptor to the plasma membrane. This can account for the higher activity showed by TGF-alpha in some biological systems, since EGFR-activated receptors can be used several times.

6. EGF-LIKE GROWTH FACTORS IN CELL PROLIFERATION AND ONCOGENESIS

6.1. The EGF-like growth factors are transforming genes

In the following paragraphs we will summarize the experimental evidence demonstrating that the EGF-related growth factors are overexpressed in human primary carcinomas, and that they can mediate at least in part the autonomous proliferation of cells derived from human carcinomas. We will also describe several data that have been generated by using *in vitro* and *in vivo* systems, and that suggest that some EGF-like growth factors might act as transforming genes. As described above, a cellular protooncogene is a gene that can determine cell transformation following qualitative or quantitative activation. Generally speaking, the mechanism of activation of EGF-like peptides is represented by overexpression, which is not usually due to genetic modifications, such as gene amplification, but rather to deregulated gene expression. Overexpression of *erb*B receptors has also been demonstrated to occur in human primary carcinomas. In this regard, it has been shown that overexpression of *erb*B-2 might be associated with amplification of the *erb*B-2 gene (104). However, overexpression without amplification has also been described (104). Mutational activation of these receptors is not a frequent event in human carcinogenesis (105). We will focus our discussion on the transforming properties of the EGF-related peptides.

6.1.1. TGF-alpha

Several pieces of evidence have suggested that TGF-alpha might be involved in neoplastic transformation. In fact, TGF-alpha expression can be induced in cultured cells by transformation with several viral and cellular oncogenes or by treatment with carcinogens or tumor-promoting agents (106,107). Furthermore, TGF-alpha expression has been detected in neoplastic tissues or cell lines derived from human tumors (9). In fact, expression of TGF-alpha mRNA or protein has been virtually described in carcinoma cells of every histological origin (9). Although expression of TGF-alpha occurs in regenerating tissues, such as the basal layers of the epidermis or in the cells that are at the bottom of gastrointestinal crypts, the expression of TGF-alpha in the tumor tissue is usually

Table 3 . Summary of transgenic studies

Author	Transgene	Tumor type
Jhappan *et al.* (125)	MT-TGF-alpha	Liver carcinoma
Sandgren *et al.* (127)	MT-TGF-alpha	Mammary carcinoma in multiparous females
Matsui *et al.* (126), Halter *et al.* (131)	MMTV-TGF-alpha	Mammary carcinoma in virgin and multiparous females
Sandgren *et al.* (130)	WAP-TGF-alpha	Mammary carcinoma in multiparous females
Vassar *et al.* (133)	K14-TGF-alpha	None
Dominey *et al.* (134)	K1-TGF-alpha	None
Cook *et al.* (137)	K14-AR	None
Krane *et al.* (154)	MMTV-NDF	Mammary carcinoma in multiparous females
Murakami *et al.* (173)	Alb-c-myc x MT-TGF-alpha	Liver carcinoma
Sandgren *et al.* (174)	Alb-c-myc x MT-TGF-alpha	Liver and pancreatic carcinoma
Sandgren *et al.* (130)	WAP-c-myc x WAP-TGF-alpha	Mammary carcinomas in virgin and multiparous females, and in males
Amundadottir *et al.* (175)	MMTV-c-myc x MT-TGF-alpha	Mammary carcinomas in virgin females and in males
Muller *et al.* (179)	MMTV-c-neu x MMTV-TGF-alpha	Mammary carcinomas in virgin and multiparous females

higher when compared to corresponding normal tissues (figure 1) (9,19). These data suggest that activation of TGF-alpha-EGF-receptor autocrine circuits is a frequent phenomenon in human carcinogenesis. Although overexpression of TGF-alpha has been demonstrated in a majority of human primary carcinomas, no correlation between TGF-alpha levels of expression and prognosis in cancer patients has been demonstrated. However, the presence of TGF-alpha activity in the pleural effusions was directly correlated with tumor burden, patient performance status and reduced survival (108).

The involvement of TGF-alpha in the autonomous proliferation of human carcinoma cells has been formally demonstrated by using either retroviral antisense expression vectors, or antisense oligonucloetides or neutralizing antibodies directed against TGF-alpha. In fact, it has been shown that blockade of endogenous TGF-alpha results in a significant inhibition of the *in vitro* growth in several human carcinoma cell lines (109-115). Furthermore, blockade of TGF-alpha expression has been shown to significantly inhibit the *in vivo* growth of human colon carcinoma xenografts (116). Similar results have also been obtained by using EGF receptor blocking antibodies in human colon, breast and lung tumor xenografts (117-119). However, in the latter case this effect might be due to the blockade of different growth factors that bind and activate the EGF receptor.

A potential direct role of TGF-alpha in carcinogenesis has been suggested by several *in vitro* studies. In fact, overexpression of TGF-alpha transformed Rat-1 and NRK fibroblasts, as assayed by colony formation in soft agar and tumorigenicity in nude mice (120,121). NIH3T3 cells have also been transformed by expression of TGF-alpha, but only with concomitant expression of the EGF receptor (122). Finally, transformation by overexpression of TGF-alpha of human and mouse mammary epithelial cells that coexpress a sufficient complement of functional EGF receptors has been demonstrated (123,124). In particular, NOG-8 mouse mammary epithelial cells that overexpress the human TGF-alpha cDNA become able to grow as colonies in soft agar and to form tumors in immunodeprssed mice (124). Human mammary epithelial MCF-10A cells bearing a TGF-alpha retroviral expression vector were able to grow in serum free medium and to form colonies in agar, but they did not generate tumors in nude mice (123).

More importantly, several studies in transgenic mice have shown the transforming properties of TGF-alpha (table 3). The oncogenic potential of TGF-alpha was first shown in 1990 when three different studies showed that TGF-alpha is a potent *in vivo* epithelial cell mitogen and an oncogenic protein in several tissues (125-127). In particular, transgenic mice have been generated by using either non-tissue-specific promoters such as the mouse metallothionein 1 promoter, or tissue-specific promoters. Metallothionein-directed TGF-alpha expression in transgenic mice determined changes in the growth and differentiation of several adult tissues (125,127). In fact, TGF-alpha overexpression determined a uniform epithelial hyperplasia of theliver, pancreas and gastrointestinal tract. In addition, the occurrence of hepatocellular and breast carcinoma has been reported in metallothionein-TGF-alpha (MT-TGF-alpha) transgenic mice (125,127). In particular, development of different neoplasia in MT-TGF-alpha mice seems to be related to the genetic background. In fact, MT-driven overexpression of the human TGF-alpha cDNA leads to development of mammary adenocarcinoma when operating on a FVB/N genetic background, whereas it induces liver tumors on a CD-1 genetic background (125,127). Liver carcinoma was not observed in MT-TGF-alpha mice before 10 months of age (125). In these mice, adenomas and carcinomas appeared stochastically and at multiple sites. Therefore, TGF-alpha transgenic mice require a much longer time period for the development of liver tumors, as compared with transgenic mice expressing

viral and some activated oncogenes. These data suggest that chronic exposure to TGF-alpha is not a sufficient step in liver cancer. Tumor incidence appeared to be elevated in male transgenic mice (44). In this respect, it has been shown that castration reduced about 7-fold the occurrence of liver tumors in TGF-alpha transgenic mice, whereas the incidence of liver tumors was increased about 6-fold in ovariectomized transgenic animals (128). More recently, it has been shown that administration of dihydotestosterone to castrated TGF-alpha transgenic mice restored the number of liver tumors (129). These data clearly suggest that sex hormones may collaborate with TGF-alpha in inducing liver carcinogenesis. However, genetic background upon which the transgene operates, and expression of endogenous c-*myc* and insulin-like growth factor II have been suggested to be involved in TGF-alpha-induced hepatocarcinogenesis (128).

Overexpression of TGF-alpha under the control of either the MT, the mouse mammary tumor virus (MMTV) or the mouse whey acidic protein (WAP) promoter determined the occurrence of mammary adenocarcinomas in transgenic female mice (126,127,130). In MT-TGF-alpha mice, mammary adenocarcinomas were observed in the postlactational mammary gland (127). These tumors contain a prominent secretory alveolar component that does not appear to be present in transgenic mouse mammary tumors induced by overexpression of oncogenes such as *myc*, *ras* or *neu*. Furthermore, transformation of mammary epithelium in MT-TGF-alpha transgenic mice occurs despite a low level of TGF-alpha expression in this organ, suggesting that the mammary epithelium is particularly sensitive to the effects of TGF-alpha *in vivo*. Higher levels of expression of TGF-alpha were observed in MMTV-TGF-alpha transgenic mice (126). In these mice, overexpression of TGF-alpha in the mammary epithelium of virgin females leads to widespread hyperplasia of the terminal ducts and alveolar glands. Mammary adenocarcinomas were mainly observed in multiparous female mice, suggesting that other events, such as hormonal influences, are required for neoplastic transformation of mammary epithelium by TGF-alpha. Occurrence of mammary adenocarcinomas in virgin MMTV-TGF-alpha has also been described (131). However, tumors appeared in virgin mice at a much older age as compared with multiparous transgenic mice (325 days vs. 210 days). WAP-TGF-alpha females accumulated large numbers of hyperplastic alveolar nodules within the mammary gland, that could represent precursors to mammary tumors in TGF-alpha transgenic mice (130). In fact, nonvirgin female WAP-TGF-alpha transgenic mice developed relatively well-differentiated adenomas and adenocarcinomas. These tumors displayed induction of cyclin D1 mRNA, suggesting that expression of this gene may complement that of TGF-alpha during mammary tumor development (130). Expression of TGF-alpha inhibited mammary involution in TGF-alpha transgenic mice (130). This phenomenon in the mouse is characterized by apoptosis. This observation suggests that expression of TGF-alpha might be involved in regulating cell survival of mammary epithelial cells. Finally, no mammary gland tumors were observed in male MMTV-TGF-alpha or WAP-TGF-alpha mice, despite the high levels of the transgene (126,130). Taken together, these findings clearly suggest that transgenic mice expressing TGF-alpha under the control of tissue-specific and nonspecific promoters stochastically developed focal mammary tumors with an incidence and latency that was markedly affected by pregnancy. These conclusions have been recently confirmed in a transgenic rat model. In fact, MMTV-TGF-alpha transgenic rats were found to stochastically develop mammary tumors (132).

TGF-alpha is also thought to be the major autocrine growth factor controlling growth in epidermal cells. Targeted expression of TGF-alpha in the epidermis of transgenic mice has been obtained by using either the human keratin 1 or keratin 14 promoter (133,134). No carcinomas were detected in these mice. However, spontaneous papillomas frequently developed in regions of mechanical irritation or wounding. These data suggest that overexpression of TGF-alpha might affect proliferation of epidermal cells. In this regard, it has been also demonstrated that treatment of skin of MT-TGF-alpha transgenic mice with dimethylbenzanthracene (DMBA), but not treated control animals, caused the occurrence of papillomas, sebaceous adenomas, and more infrequently sebaceous and squamous cell carcinomas (135). High expression of TGF-alpha was found in tumors with no mutations in *ras*, while tumor bearing a mutated *ras* showed low levels of TGF-alpha expression. Taken together, these data suggest that overexpression of TGF-alpha might functionally substitute mutational activation of *ras* in skin tumorigenesis. Similar findings were described by Vassar and co-workers (136).

6.1.2. AR

A direct role of AR in carcinogenesis has not yet been demonstrated. In fact, unlike TGF-alpha and EGF, AR was not able to induce colony formation in soft agar of NRK cells (21). Furthermore, it has not been shown that overexpression of AR might lead to transformation of mouse or human epithelial cells. Finally, overexpression of AR in the basal keratinocytes of transgenic mice by using a K14 promoter-driven AR gene, caused the occurrence of psoriatic lesions, but it did not induce the occurrence of papillomas or carcinomas (table 3) (137).

However, a potential involvement of AR in human carcinogenesis is suggested by the finding that AR is expressed in several human carcinoma cell lines as well as in approximately 40 to 80% of human breast, lung, colon, gastric and ovarian carcinomas (23,24,138-145). It has been shown that AR is preferentially expressed in well-differentiated colon carcinomas when compared with poorly differentiated tumors (140). Analogously, expression of AR is more frequent in human primary breast carcinomas and in human breast carcinoma cell lines that express the estrogen receptor (ER), and that therefore are more differentiated when compared with ER-negative breast cancer cells (24,143). AR is frequently expressed in normal tissue (figure 1). For example, AR expression has been found in normal colonic epithelial cells that are in the

upper third of the intestinal crypts, and its expression seems to be correlated with the state of differentiation of the intestinal cells (139). Loss of expression of AR might represent an early event in human carcinogenesis. In fact, we found that in both colon and breast carcinomas the noninvolved mucosa that surrounds the tumor may not express AR, whereas the specimens of normal tissue that is far from the tumor is frequently positive for AR expression (140,142) (figure 1). Furthermore, AR expression was lost in colon mucosa specimens from high-risk individuals with high proliferative activity (146). Taken together, these data suggest that a potential correlation between AR expression, differentiation and low proliferative activity in epithelial cells might exist.

Several pieces of evidence also suggest that AR functions as an autocrine growth factor in human carcinoma cells. In fact, the autonomous proliferation of colon carcinoma cells was inhibited by immunological removal of AR from the culture medium (139). Furthermore, the *in vitro* growth of breast, colon, gastric, pancreatic and ovarian carcinoma cells was significantly inhibited by treatment with antisense oligonucleotides directed against the AR mRNA (114,115,147-149). It has also been demonstrated that transfection with an antisense AR expression vector was able to suppress the tumorigenicity of a transformed human breast epithelial cell line (150). More recently, we have shown that the growth of human colon carcinoma xenografts in nude mice was inhibited by administration of anti-AR mixed backbone oligonucleotides (MBOs) (116).

Finally, AR has been retrovirally transduced into primary cultures of mouse mammary epithelial cells *in vitro*, which have been subsequently implanted in the cleared mammary fat pad of virgin syngeneic mice (151). AR overexpressing cells developed into hyperplastic tertiary structures and hyperplastic lobules, suggesting that AR might be an important intermediary in early malignant progression (151).

6.1.3. HRGs

The potential role of HRGs in regulating normal and oncogenic signals through its receptor has been particularly investigated in breast cancer. In fact, expression HRG mRNA has been described to occur in a small number of human breast carcinoma cell lines and in 25-30% of human primary breast carcinomas (24,143). HRG expression is more restricted in its distribution as compared with TGF-alpha and AR. In fact, HRG is only expressed in ER-negative, estrogen-independent cell lines (24). In this regard, it has also been shown that overexpression of HRG induces a more aggressive, hormone-independent phenotype in ER-positive, MCF-7 human breast cancer cells, that is most likely directly related to the constitutive activation of the *erb*B-2, *erb*B-3, and *erb*B-4 receptor signaling cascade (152). The effect of HRG on breast cancer cell lines can be either mitogenic or growth inhibitory, and it appears to be a function of the receptor types that are expressed by the target cells and of the concentrations of HRG that are used to stimulate tumor cells (40,41,153). A formal demonstration of the oncogenic potential of HRG comes from transgenic mice studies (table 3). In fact, it has been shown that overexpression of HRG in the mammary gland of transgenic mice by using the MMTV promoter induces a persistence of the terminal end buds in the transgenic females (154). More importantly, multiparous female mice develop mammary adenocarcinomas at a median age of 12 months (154). Since these tumors arise in a solitary fashion, it is conceivable that HRG overexpression is not sufficient for their formation. In this regard, it has also been shown that overexpression of HRG-beta 1c lacking the cytoplasmic domain is sufficient to promote mammary gland tumorigenesis (155). Tumors from transgenic mice that overexpress the extracellular region of HRG showed levels of apoptosis that were lower as compared with tumors from mice that overexpress the full length HRG, suggesting that the cytoplasmic domain of HRG can induce apoptosis in tumor cells (155).

6.1.4. CR-1

Direct evidence of the involvement of CR-1 in carcinogenesis has been provided by two studies that have demonstrated that both mouse NIH-3T3 fibroblasts and nontransformed NOG-8 mouse mammary epithelial cells transfected with the human CR-1 cDNA exhibit the ability to form colonies in soft agar, although these cells fail to form tumors in nude mice (47,156). In addition, CR-1 protein and/or mRNA have been detected in 40-80% of human breast, colon, gastric, lung and ovarian carcinomas (138,140-145,157). Unlike TGF-alpha and AR, CR-1 is not usually expressed in normal tissues (140,142) (figure 1). However, expression of CR-1 has been described in noninvolved tissue surrounding colon carcinomas (140) (figure 1). In this context, Gagliardi and co-workers (158) found that cripto immunorectivity in noninvolved mucosa adjacent to rectal carcinomas was correlated with higher recurrence rate. We also found that colon mucosa specimens from high-risk individuals with high proliferative rate express with higher frequency CR-1 when compared with low kinetic specimens, or with samples from non-high risk individuals (146). Expression of CR-1 has been found in pre-neoplastic lesions. In fact, immunoreactive CR-1 has been found in approximately 50% of human colon adenomas and of human ductal breast carcinoma *in situ* (140,144) (figure 1). In particular, expression of CR-1 is more frequent in tubulo-villous adenomas as compared with tubular adenomas (140). Furthermore, expression of CR-1 has been found in 16/30 (53%) of noninvolved gastric mucosa samples that were adjacent to carcinoma but that exhibited intestinal metaplasia, which is a potential precursor for gastric carcinoma (141). Taken together, these data suggest that expression of CR-1 might represent a common early event in human tumorigenesis and that it is associated with altered proliferation and differentiation of epithelial cells.

Several data suggest that CR-1 functions as an autocrine growth factor in human carcinoma cells of different histological origin. In fact, the *in vitro* growth of human colon, breast, ovarian and teratocarcinoma derived cells is significantly inhibited following treatment with specific antisense oligodeoxynucleotides or transfection

with antisense expression vectors directed against CR-1 (113-115,159,160). Furthermore, it has also been demonstrated that blockade of CR-1 expression significantly reduces the growth of human colon carcinoma xenografts in immunodepressed mice (116,159). CR-1 might also function as a survival factor in human carcinoma cells. In particular, it has been shown that CR-1 can enhance the tyrosine phosphorylation of the 85 regulatory subunit of PI3K and transiently induce the phosphorylation of AKT in a time- and dose-dependent manner (161). Furthermore, CR-1 was able to rescue the apoptotic effect of a PI3K inhibitor in human cervical carcinoma cells, suggesting that CR-1 may function as a survival factor through a PI3K-dependent signalling pathway involving AKT (161).

6.2. Functional correlations between EGF-like growth factors and other cellular protooncogenes

Cancerogenesis is a multistep process that involves inactivation of different tumor suppressor genes and activation of several cellular protooncogenes (1). In this regard, it is conceivable that EGF-like growth factors might cooperate with other cellular genes in determining transformation of human epithelial cells. Such cooperation might occur through two different mechanisms. In fact, expression of EGF-like growth factors is induced by activation of cellular protooncogenes, and might mediate at least in part the effects of activation of protooncogenes on cell proliferation, differentiantion and survival (162,163). Alternatively, overexpression of EGF-like growth factors and/or their receptors might represent a distinct event in cell transformation, which might cooperate with different pathways that are involved in cell transformation and that are activated by mutational activation or overexpression of cellular protooncogenes. Examples of both mechanisms will be described in this paragraph.

6.2.1. EGF-like peptides and chemical carcinogens

It has been demonstrated that TGF-alpha cooperates with chemical carcinogens in inducing tumorigenesis in transgenic mice models. In this regard, several studies described above have shown that treatment of skin of MT-TGF-alpha transgenic mice with DMBA, caused the occurrence of papillomas and, more infrequently, sebaceous and squamous cell carcinomas (135). Furthermore, Coffey and co-workers (164) have shown that administration of DMBA to virgin MMTV-TGF-alpha mice dramatically accelerated tumor formation in the transgenic mice compared to controls. The authors administered DMBA to the transgenic animals at two different time points, 21 and 56 days of age. Interestingly, mammary tumors appeared in greater number and with shorter latency in TGF-alpha transgenic mice that were treated at day 56 as compared with day 21. These data suggest that a preliminary, TGF-alpha-induced expansion of a target cell population occurs in transgenic animals. Finally, cooperation of TGF-alpha with both N-nitosodiethylamine (DEN) and Phenobarbital (PB) in inducing liver carcinogenesis has been demonstrated (165). In fact, hepatocellular carcinomas have been described to occur in higher frequency and with earlier onset in MT-TGF-alpha mice treated with DEN and/or PB as compared with non-transgenic mice (165). The occurrence in the MT-TGF-alpha mice of preneoplastic lesions, such as adenomas and foci of hepatocytes, with high proliferative index might be responsible for the increased sensitivity of TGF-alpha transgenic mice to the development of liver carcinomas following administration of the tumor initiator DEN and/or the tumor promoter PB.

6.2.2. EGF-like peptides and *ras*

A functional correlation exists between cellular *ras* protooncogene and the EGF-like peptides. In fact, it was first established that *ras* transfected cells do not mitogenically respond to exogenous EGF, and that they secrete elevated levels of EGF-like factors (166). More importantly, it has been demonstrated that *ras* transformation of mouse and human mammary epithelial cells, as well as of rat intestinal cells induces an increase in the endogenous production of TGF-alpha (123,167,168). Furthermore, blockade of TGF-alpha by neutralizing antibodies or antisense expression vectors significantly reduced the growth of *ras* transformed rat intestinal and human mammary epithelial cells (123,168). The increase in TGF-alpha production in *ras* transformed cells seems to be specific for this protooncogene rather than due to a nonspecific process involved in cell transformation. In fact, no increase in the levels of TGF-alpha production was observed in both mouse and human mammary epithelial cells following transformation induced by overexpression of the *neu* protooncogene (123,169). An increase in the endogenous levels of AR was observed in *ras* transformed MCF-10A human mammary epithelial cells (170). Both the anchorage dependent and independent growth of MCF-10A-*ras* cells were significantly inhibited by antisense oligonucleotides directed against AR, suggesting that AR might function as autocrine growth factor in *ras* transformed MCF-10A cells. This observation was confirmed by the finding that activated *ras* was able to transform keratinocytes derived from mice with genetic defects in TGF-alpha expression (171). In these cells activated *ras* induced a significant up-regulation of transcripts encoding for AR, HB-EGF and BTC (171). This observation confirms that *ras* transformation requires the expression of EGF-like growth factors, and that several different growth factors might mediate the proliferative effects induced by activation of *ras*. In this regard, we have also shown that *ras* transformed mouse and human mammary epithelial cells produce higher levels of heregulin mRNA and protein as compared with their non-transformed counterpart (172).

6.2.3. EGF-like peptides and *myc*

A potential cooperation between c-*myc* and EGF-like growth factors in human tumorigenesis has been hypothesized following early studies that showed induction of colony formation in *myc* transfected cells by exogenously added EGF (166). Several studies in transgenic mice have demonstrated that such cooperation occurs in liver and mammary carcinogenesis (table 3). In fact, two different groups have demonstrated that coexpression of TGF-alpha and c-*myc* as transgenes in mouse liver accelerated the development of neoplastic lesions as compared with expression of either transgene

alone (173,174). Expression of TGF-alpha and *myc* in liver tumors was much higher relative to expression in surrounding non-neoplastic liver, suggesting that high level of expression of both proteins is necessary for tumor development (174). Pre-neoplastic lesions represented by foci of dysplastic cells displaying mitotic figures were evident in the liver lobule of MT-TGF-alpha/Alb-c-*myc* transgenic mice following 10 weeks of treatment with zinc (173). Furthermore, co-expression of TGF-alpha and c-*myc* produced the occurrence of less differentiated tumors than did expression of c-*myc* alone (174).

The cooperation of c-*myc* and TGF-alpha in inducing mammary carcinogenesis has been also evaluated, by using double transgenic mice. Bitransgenic WAP-c-*myc* – WAP-TGF-alpha mice were generated by Sandgren and co-workers (130). All multiparous bitransgenic females developed multiple large, transplantable tumors. These mice had an average life span of <4 months, compared to the average 8 and 9.5-month lifespans of the parental transgenic lines. Multiple tumors were found in the mammary gland, and lung metastases were occasionally observed. Furthermore, tumors were observed also in female mice in absence of pregnancy, as well as in bitransgenic males. A dramatic synergy between TGF-alpha and c-*myc* transgenes in virgin mice was also described by Amundadottir and co-workers (175). In fact, they found that double transgenic TGF-alpha/c-*myc* animals developed synchronous mammary tumors at a mean age of 66 days. Unexpectedly, a similar tumor latency and frequency in virgin female and male mice was found, whereas only 50% of virgin c-*myc* females developed mammary tumors with a much longer latency, and no tumors were detected in virgin TGF-alpha animals. The molecular mechanisms involved in such cooperation have been also investigated. In fact, the occurrence of apoptosis in mammary tumors from double transgenic and single transgenic mice was compared (176). Tumors derived from mice that express c-*myc* in the absence of TGF-alpha exhibited high levels of apoptosis, as compared with tumors from double transgenic mice. These data indicate that TGF-alpha might protect c-*myc* overexpressing cells from programmed cell death. Tumors from c-*myc* animals show areas with high proliferation that express low levels of c-*myc* and high levels of cyclin D1 (177). In TGF-alpha/c-*myc* tumors, cells positive for cyclin D1 were randomly spread, without any association with c-*myc* levels (177). Furthermore, in contrast with c-*myc* induced tumors, the majority of the tumors from double transgenic animals showed undetectable levels of retinoblastoma protein (pRB), and in some cases mRNA (177). This observation suggests that TGF-alpha might play its synergistic role with c-*myc*, at least in part, by inducing cyclin D1 expression and by facilitating loss of pRB.

6.2.4. EGF-like peptides and *erb*B-2

A functional correlation exists between *erb*B-2 and the EGF-like growth factors. In fact, it has been shown that *erb*B-2 transformed mouse and human mammary epithelial cells express levels of TGF-alpha that are comparable to parental cells (123,169). However, an increase of both AR and HRG expression has been described to occur in *erb*B-2 transformed human mammary epithelial cells (170,172). Furthermore, the growth of MCF-10A human mammary epithelial cells that were transformed by overexpression of a normal c-*neu* protooncogene (the rat homologue of c-*erb*B-2) was significantly inhibited by treatment with specific antisense oligonucloetides directed against the AR transcript (170). In this regard, an increase in both AR and CR expression was found in mammary tumors from MMTV-activated c-*neu* transgenic mice (50,178).

Studies in transgenic mice have shown that a cooperation in mammary carcinogenesis occurs between TGF-alpha and *erb*B-2 (179). Transgenic strains harboring either an MMTV-TGF-alpha or MMTV-c-*neu* transgene were interbred in order to generate bitransgenic TGF-alpha/*neu* mice (table 3). Female mice carrying the double transgene developed mammary tumors after a significantly shorter latency period than either parental strain alone. In particular, 95% of the bitransgenic mice developed palpable tumors by 250 days, whereas only 6% of MMTV-TGF-alpha and 35% of MMTV-c-*neu* mice did. Furthermore, tumors in the bitransgenic were multifocal and involved the entire mammary epithelium, while tumors arising in either strain were generally focal in origin.

7. CO-EXPRESSION OF EGF-LIKE GROWTH FACTORS IN HUMAN CANCER: THE NETWORK HYPOTHESIS

We and other groups have demonstrated that co-expression of two or more EGF-like peptides frequently occurs in human carcinomas. In fact, we found that co-expression of TGF-alpha, AR and/or CR-1 occurs in human colon, breast, lung, ovarian and gastric carcinomas, suggesting that co-expression of different EGF-like growth factors is a common phenomenon in human carcinogenesis (138,140-145).

Each growth factor of the EGF family might regulate distinct cellular functions. This might be due to the ability of the EGF-like growth factors to activate different receptors, as discussed above. However, differences in biological activity have been delineated for some family members that bind to the same receptor. For example, TGF-alpha is more potent than EGF in stimulating calcium release from fetal bones and angiogenesis (180,181). In this regard, it has been suggested that EGF receptor downregulation and heterodimerization are more efficiently induced by EGF as compared with TGF-alpha (103,182). It is also conceivable that the different members of the EGF-family might regulate cellular functions distinct from cell proliferation in normal tissues. For example, TGF-alpha is highly expressed in proliferating cells of the intestinal crypts that express high levels of EGF receptor, whereas AR is expressed in differentiated cells that are at the top of the intestinal villi, and that express very low levels of EGF receptor (19,139).

However, several pieces of evidence suggest that EGF-related peptides such as TGF-alpha, AR and CR-1 promote cell proliferation and survival of human carcinoma

cells. Since the majority of human carcinomas express several different EGF-like peptides, we have hypothesized that a network consisting of multiple peptides is sustaining the autonomous proliferation of carcinoma cells. In this respect, several studies suggest that a functional correlation exists between the different members of the EGF-like family of growth factors. In fact, it has been demonstrated that auto- and cross-induction among several growth factors of the EGF family occurs in several cell types, such as keratinocytes, colon carcinoma cells, and both transformed and non-transformed human mammary epithelial cells (169,183). Both transcriptional and posttranscriptional mechanisms are involved in this process (183,184). Furthermore, we have formally demonstrated that different EGF-like growth factors cooperate in sustaining the proliferation of human carcinoma cells. In fact, we have shown that treatment of human colon, breast and ovarian carcinoma cells with combinations of antisense oligonucleotides directed against the AR, CR-1 and TGF-alpha mRNAs results in a more efficient growth inhibition as compared with treatment with a single antisense oligonucleotide (113-115). Autocrine EGF-like growth factors probably cooperate in the regulation of cell survival as well. In fact, a protective role of the EGF-like growth factors against apoptosis has been demonstrated in several different non-transformed and tumor cell types (185-189). However, we found that treatment of human breast carcinoma cells with AR, CR-1 or TGF-alpha antisense oligonucloetides did not induce programmed cell death, whereas a significant increase in the levels of fragmented DNA was observed following treatment of carcinoma cells with a combination of the three antisense oligonucleotides (114). These findings have been recently confirmed in an *in vivo* setting. In fact, we have demonstrated that simultaneous blockade of different EGF-like growth factors results in an efficient growth inhibition of human colon carcinoma xenografts in nude mice (116). Treatment of mice with a combination of AR, CR-1 and TGF-alpha antisense oligonucleotides also produced a more significant reduction in microvessel count as compared to treatment with a single antisense oligonucleotide (116). In this respect, it has been previously demonstrated that both TGF-alpha and AR can induce neo-angiogenesis (150,181). Therefore, the EGF-related peptides AR, CR-1 and TGF-alpha probably cooperate in stimulating tumor growth through two different mechanisms: 1) a direct stimulation of tumor cell proliferation, that is sustained by autocrine circuits involving the EGF-like peptides and their receptors; 2) an induction of neoangiogenesis, either directly or indirectly through other factors such as VEGF or bFGF.

8. CONCLUSIONS AND PERSPECTIVES

In this review article we have described the complex interactions existing between the members of the EGF family of peptides and the receptors of the *erb*B family of receptors. These peptides might bind and activate different receptor tyrosine kinases belonging to the *erb*B family. However, heterodimers are usually formed, with transactivation of several different receptors (81). Furthermore, following activation of the cognate receptors, the EGF-like peptides can induce the synthesis of other members of the same family (170,183). Therefore, it is evident that an autocrine or paracrine EGF-like peptide might start a cascade of events with amplification of signals that are driven by different receptors and peptides. The response of a normal or transformed cell to an EGF-like growth factor will be clearly dependent on the number and type of receptors that are expressed on its membrane, and on its ability to synthesize other growth factors that might continue to boost these circuits. In fact, although all the *erb*B receptors are tyrosine kinases, they show qualitative and quantitative differences in activating intracellular transducers, such as different affinity for PI3K (99). Furthermore, the rate of internalization of the receptor, and therefore the length of its activation, is also dependent on the growth factor that binds to it (103,182). The complexity of the EGF-like family has been further increased by the discovery of the sub-family of EGF-CFC proteins. These peptides do not bind any receptor within the *erb*B family. Actually, it is possible that the EGF-CFC peptides might be functioning as co-receptors for nodal, a member of the TGF-beta superfamily (75). In particular, the EGF-CFC proteins might be a part of a receptor complex in which they facilitate the presentation of nodal to the type IIB receptor. Therefore, the EGF-CFC proteins might functionally link two different families of growth factors, the EGF-like and the TGF-beta like families, which have been shown to possess completely different growth controlling effects in several cell systems. Cell survival, proliferation and differentiation might depend on the balance between the stimuli that are driven by peptides of these families. The EGF-CFC peptides might regulate such a balance.

From the foregoing review it is also evident that several different EGF-like growth factors are involved in the pathogenesis of human carcinomas, and that they can act as transforming genes. In particular, the development of different transgenic mouse lines in which EGF-like growth factors have been overexpressed by means of tissue-specific or nonspecific promoters has provided invaluable information relating to their ability to function as dominantly transforming oncogenes in different tissues in an *in vivo* setting, and can serve a potential *in vivo* models for testing new therapeutic modalities. The transgenic mice studies have shown that overexpression of EGF-related peptides induces an hyperproliferative state that can favor the occurrence of additional events (activation of protooncogenes or inactivation of tumor suppressor genes) that are necessary for cell transformation. In this regard, it is widely accepted that carcinogenesis is a multistep process in which different events must occur to induce a fully transformed phenotype in a normal cell. These observations are consistent with a role of EGF-like growth factors in early phases of carcinogenesis. In this context, these proteins may serve as potential biological markers that might have some significant diagnostic and prognostic value. For example, loss of expression of AR and increased synthesis of CR-1 are correlated with increased proliferation of colon mucosa, that might favor the occurrence of mutational inactivation of tumor suppressor genes or activation of cellular protooncogenes, thus leading

to cell transformation (146). In this respect, detection of such changes in the mucosa of high-risk patients might be useful to assess the risk of developing a neoplasia. Furthermore, CR-1 expression might be used as a tumor marker for the early detection of breast lesions such as atypical ductal hyperplasias that might have a high propensity to convert to carcinoma *in situ* (144). In this regard, novel therapies aimed to inhibit the expression and/or the activity of EGF-related peptides might be employed in high-risk patients.

Several studies have demonstrated that the EGF-related growth factors and/or their receptors are overexpressed in human carcinomas as compared with their normal counterparts (9). These peptides might therefore represent suitable targets for experimental therapy approaches with human carcinomas. In fact, there are several approaches that have been assessed for their ability to interfere with the function of growth factors and their receptors and that might be useful for therapeutic intervention. These approaches include the use of monoclonal antibodies that block or prevent ligand binding or the utilization of monoclonal antibodies that are directed against the extracellular domain of receptors, and that might be conjugated with toxins or radionuclides. In addition, the tyrosine kinase domain of growth factor receptors represents another potential target for therapy by using specific and selective tyrosine kinase inhibitors, some of which are orally active *in vivo*. Finally, modified (phosphorothioate or mixed backbone) antisense oligonucloetides or antisense expression vectors that block growth factor or growth factor receptor gene expression are also of potential therapeutic utility. All these approaches have been proven to produce a significant inhibition of carcinoma cell growth in both *in vitro* and *in vivo* settings (116,190,191). Interestingly, the *in vivo* results appear even more promising than the *in vitro* preliminary findings. In fact, the anti-angiogenic effects that are elicited by molecules that block the pathways driven by the EGF-like peptides increase their anti-tumor activity, which is not due to simple inhibition of cancer cell proliferation (192-194). The use of molecules directed against the signal transduction pathways driven by the EGF-related peptides and their cognate receptors in the therapy of carcinoma patients is imminent. In fact, clinical trials have shown clinical efficacy of humanized anti-*erb*B-2 (e.g., Herceptin^{TM}) and anti-EGFR monoclonal antibodies (e.g., Imclone 225), and of tyrosine kinase inhibitors such the anilinoquinazoline ZD1839 ('Iressa') in patients affected by different carcinoma types (190,191,195,196). Phase III trials in which patients are being treated with combinations of these novel compounds and conventional anti-tumor drugs are ongoing. The aim of these studies is to obtain a control of tumor growth by using low, non-myelotoxic doses of cytotoxic drugs. Therefore, the use of such combinations might represent the standard treatment of cancer patients whose tumors express the target receptors in the very near future.

Co-expression of several, different EGF-like peptides frequently occurs in human carcinomas, suggesting that a network of EGF-like peptides regulates cell proliferation of human carcinoma cells. This observation might be important for future clinical applications. In fact, it is conceivable that the contemporary blockade of different EGF-like peptides might be necessary to significantly inhibit carcinoma cell growth, or to induce programmed cell death in tumor cells. In fact, preliminary evidence suggests that an extremely efficient inhibition of tumor cell growth might be obtained by using combinations of antisense oligonucleotides directed against different EGF-like peptides (113-116). In this respect, we have recently proposed a novel therapeutic approach of human carcinomas that is based on the use of a combination of different inhibitory molecules directed against the EGF-like peptides, their receptors and/or downstream effectors such as MAPK or PI-3 kinase in the therapy of human carcinomas (197). The use of combinations of these molecules might allow an improvement of clinical efficacy as compared with the use of a single molecule through additional mechanisms. In fact, the use of combinations of molecules that possess different toxicities might allow to obtain a significant tumor growth inhibition by using lower concentrations of each molecule, and therefore with a lower incidence of toxic effects. Furthermore, heterogeneous expression of the different EGF-like peptides and *erb*B receptors might occur within the tumor mass. Therefore, the use of combinations of different molecules might allow to simultaneously target a higher number of tumor cells. Finally, synergistic effects might occur following the blockade of different signal transduction pathways. Studies are ongoing in our laboratories to assess the feasibility of this approach.

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