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Review

Transgenic and knock-out mice for deciphering the roles of EGFR ligands

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Abstract. Generation of genetically engineered mice with either gain-of-function or loss-of-function mutations is the most popular technique for determining gene functions and the interrelationship between molecules in vivo. These models have provided a wealth of information about the developmental and physiological roles of oncogenes and growth factors. To date, transgenic techniques have been used extensively to study the functions of the epidermal growth factor (EGF) family. This review highlights some of the major recent findings pertinent to the EGF receptor (EGFR) and its ligands with special reference to elucidating how EGF and its related growth factors work together to regulate reproduction, growth and development. Finally, future investigations on ligand-ligand communications, EGFR and its ligands in neural stem cell research, and the mechanisms of EGFR signaling and trafficking in cells are also suggested.

Key words. EGF; EGFR; knock-out; transgenic; stem cell; growth factor; motor protein; transport.

Intracellular communication plays a pivotal role in establishing cell lineage diversity in multicellular organisms. At least two classes of molecules are considered to be crucial mediators of this communication. They are insoluble components, molecules forming the extracellular matrix or adhesion molecules on the cell surface, and soluble hormone-like messengers, often called growth factors. Some growth factors bind to cell surface receptors whose intracellular domain carries an enzymatic activity, a tyrosine-specific kinase or serine/tyrosine kinase [1–3].

Epidermal growth factor (EGF) is a classical growth factor which utilizes the receptor tyrosine kinase system. It was initially identified from mouse submaxillary gland extract as a stimulator of eyelid opening and incisor eruption when injected into newborn mice and rats. EGF, transforming growth-factor (TGF)-α, and amphiregulin only bind and activate EGF receptors (EGFRs) (also called erbB-1 and HER1), and they are referred to as group one of the EGF family. Group two members, which consist of two neuregulins, bind erbB-3 and -4. Group three consists of HB-EGF, betacellulin and epiregulin which bind both EGFR and erbB-4. ErbB-2 does not bind ligands directly but is the preferred heterodimerization partner for all other erbB members [4, 5] (table 1).

In recent years, information on the EGF family and erbB receptor family has expanded rapidly. Signal diversity is generated by ligand diversity and heterodimerization capacity. EGFR can also dimerize with erbB-3 and -4 [4–7]. EGFR is ubiquitously expressed in nonhematopoietic tissues and influences a wide range of responses, depending

Table 1. The binding partners of EGF family members.

	Member	Binding partners
Group I	EGF, TGF, amphiregulin	Only EGFR (erbB-1/HER1)
Group II	neuregulins	erbB-3 and erbB-4
Group III	HB-EGF, betacellulin and epiregulin	both EGFR and erbB-4

on the coordinate expression of the cognate ligands [8, 9]. EGF, TGF-α and amphiregulin are synthesized as a transmembrane precursor and translocated to the plasma membrane. Proteolytic cleavage of the ectodomain to produces a soluble growth factor receptor whose activiation is dependent on the processing of the precursor molecules [8]. Activation of EGFR mainly occurs through two pathways. The first is an autocrine mechanism whereby heterologous stimuli provoke the synthesis of EGF ligands, and was initially described in a landmark paper [10]. The other mechanism invokes an intracellular trans-activation signaling pathway [11]. It is proposed to involve phospholipids and the intracellular tyrosine kinases Pyk-2 and Src [8].

Transgenic mice have proven to be a powerful tool for studying the specificity, compensation and redundancy in the signaling cascades by altering the structure or expression of a single gene or a set of genes, from ligands to specific receptors and downstream signaling players [12]. On the other hand, the well-organized system of maintaining homeostasis prevents us from dissecting the functions of a single molecule *in vivo*, ironically due to compensation and redundancy of the fail-safe system. However, taking advantage of different approaches, an overall insight into the molecular mechanisms may be gained. For example, by selecting an appropriate promoter for transgene expression, we can analyze the functions of the target molecule in a more specific manner in the transgenic animals. On the other hand, the normal function of a gene may be deduced by expressing a mutated gene encoding a dominant negative protein. Mice carrying a homozygous null mutation ('knock-out') can be generated to examine the effects of the total lack of a specific gene product, whereas the effects of gene dosage can be studied in heterozygous mutants. Moreover, the conditional knock-out strategy developed recently enables us to study the function of genes in a spatiotemporally specific and even inducible manner. This review highlights the applications of these techniques for EGFR ligands. A summary is included, describing recent studies on increasing or decreasing the levels of EGFR and its ligands (TGF-α, EGF and amphiregulin). The discussion further extends to their interrelationship regarding specificity and redundancy in signaling. In addition, inherent limitations and future perspectives will also be considered.

Knock-out mice of EGFR and its ligands

To circumvent the complications of functional redundancy, individual null mutants of each of the three group one EGFR ligands were produced and the double and triple null mutants were generated by cross-breeding. Mice without amphiregulin had underdeveloped mammary glands. Following pregnancy, most amphiregulin-null female mutants could nurse their young, in comparison to only a few triple-null mutants. This finding implies a collaborative role for these ligands in mammopoiesis and lactogenesis. In triple-null glands, alveoli were poorly organized and differentiated, and milk protein gene expression was also decreased. Moreover, the loss of growth factors in pups further worsened their survival and growth. Triple-null pups could be rescued by nursing by wild-type foster mothers, but still showed growth retardation. This establishes the importance for both maternal- and neonatal-derived growth factors for the health and growth of neonates [13]. Furthermore, mice without TGF-α (*wa-1*) or expressing a partially functional EGFR (*wa-2*) show an identical phenotype of affected hair and eyelid development. The waviness of the whiskers and fur is due to derangement of hair follicles [14, 15]. Transgenic mice expressing TGF-α in type II cells under control of the lung-specific surfactant protein-C (SP-C) promoter develop pulmonary fibrosis and marked airspace hypoplasia. A mutant of EGFR lacking a portion of the intracytoplasmic domain (EGF-R-M) under control of the human SP-C promoter was made [16]. Transcripts of the SP-C-EGF-R-M transgene were detected in distal bronchiolar and type II cells by in situ hybridization. Lung fibrosis was not detectable and airspace hypoplasia was significantly corrected in bitransgenic mice derived by breeding SP-C-TGF-α and SP-C-EGF-R-M mice. Correction of lung pathology in the bitransgenic mice occurred without altering the level of hTGF-α mRNA in this rescue approach [16].

In comparison to single TGF-α null mice, the triple-null mutants showed an increased penetrance of eye defects (80–90% versus 40–50%), accelerated hair and weight loss, dermatitis and skin ulceration with aging [13]. Other than weight loss, the confinement of phenotypes to the skin, eye and mammary gland suggests that group one ligands have distinctive roles and tissue specificity when compared to other EGFR ligands. The cross-breeding experiments between various genotypes also reveal the importance of partial functional redundancy between these ligands.

Gene-targeting experiments which eliminated the expression of EGFR and therefore blocked the actions of all six known EGFR ligands have shown that the major ensuring effect is on epithelial cells located in skin, lung, gastrointestinal tract, tooth and eyelid. Moreover, the importance of EGFR in early embryonic and placental development is underscored by the fact that EGFR-null mice invariably died either at peri-implantation, mid-gestation or early in postnatal life [17–19]. Mouse mutants bred on a CD-1 or C57XMF1 background survived for up to 3 weeks, enabling more extensive phenotype analysis. These mutants had impaired gut proliferation with a reduced stem cell zone and disorganized mucosal architecture [18, 19]. In a C57 background, the mice survived up to 8 days after birth with phenotypes resembling necrotizing enterocolitis and

respiratory distress syndrome commonly associated with premature birth in humans [17]. Evidence shows that the strong strain dependence of the phenotype was not due to maternal rescue [19]. The genetic modifiers in EGFR signaling remain to be identified. The EGFR signaling network is intricate, raising the necessity to consider the participation of EGFR in the signaling of other EGFR ligands through receptor heterodimerization [9]. EGFR can also be transmodulated by other growth factors such as platelet-derived growth factor [20].

A second approach used for studying the function of EGFR is by targeted expression of a dominant negative EGFR in specific tissues. This approach has been used successfully to demonstrate the importance of EGFR in ductal branching and outgrowth of the mammary gland [21] and in differentiation and development of the hair follicles and epidermis [22]. On the other hand, these mice and the *wa-2* mice were resistant to skin tumor development induced by transgenic expression of Son of Sevenless due to the loss of the survival signal provided by normal EGFR [23].

Transgenic mice overexpressing EGFR ligands

Abnormal expression of EGFR, TGF-α and amphiregulin, but not EGF, is frequently found in epithelial cancers [4, 7]. In agreement with this observation, transgenic mice overexpressing TGF-α showed epithelial hyperplasia of several organs, pancreatic metaplasia and breast carcinoma [24, 25]. Using the metallothionein promoter, the authors have shown that TGF-α promoted uniform epithelial hyperplasia of several organs without otherwise causing major alterations in tissue architecture. In pancreas, it brought about overproliferation of both acinar cells and fibroblasts. The pancreas showed progressive interstitial fibrosis and a florid acinoductular metaplasia. TGF-α also caused dramatic hyperplasia of the coagulation gland epithelium, which displayed evidence of carcinoma, and in postlactational mammary gland it induced secretory mammary adenocarcinomas. Further analysis of these mice reveal the importance of TGF-α in regulating the differentiation and repair programs of the gastrointestinal tract [26]. Thus, TGF-α plays an important role in cellular proliferation, organogenesis and neoplastic transformation and displays characteristics of both a potent epithelial cell mitogen and an oncogenic protein in vivo. On the other hand, targeted expression of amphiregulin in basal keratinocytes induced a psoriasis-like phenotype in transgenic mice [27].

EGF transgenic mice with the β-actin promoter were infertile [28, 29] and born with only half the weight of normal littermates. These findings coincide with previous reports that injection of EGF induced growth retardation in newborn rats [28]. Another group has targeted the expression of full-length EGF precursor to the small intestine using rat intestinal fatty acid-binding protein promoter. Interestingly, their transgenic animals had improved postresection adaptation. Besides a shortened small intestine, no other abnormal phenotype was observed [30]. When EGF was expressed in pancreatic β-cells, the islets increased in size but the transgenic animals were healthy and normoglycemic [31].

To elucidate the possible mechanisms leading to growth retardation, previous studies focused on the insulin-like growth factor (IGF) system since it is a key player in growth regulation. Additionally, various in vitro studies have shown that EGF reduces synthesis of IGF-binding protein (IGFBP)-3 [32]. In vivo, IGF actions are influenced by IGFBPs. IGFBPs can potentiate activities of IGFs for cell proliferation. In addition, IGF-independent regulatory mechanisms of IGFBPs have been described. IGF-independent growth inhibition by IGFBP-3 is believed to occur through IGFBP-3-specific cell surface association proteins or receptors and involves nuclear translocation. Recent data indicate that low levels of IGFBP-3 are associated with stunted growth and an increased risk of at least several types of carcinoma that are common in economically developed countries [33]. The possible mechanisms leading to the growth problem and the relationship between EGF and IGFBP-3 were investigated in mice. The mean IGFBP-3 level of EGF transgenic mice was significantly lower than that of normal adult mice. The data suggested that EGF may change the production/secretion of IGFBP-3 in liver and kidney. Reducing serum IGFBP-3 is likely to be the result of EGF overexpression rather than a secondary effect of growth retardation [29]. EGF also acted prenatally because all transgenic mice identified at weaning were small from the day of birth. These data are in agreement with the hypothesis that EGF affects the production/secretion of IGFBP-3, hence decreasing the availability of IGFs and resulting in slower growth before and after birth.

In reproduction, EGF is involved in differentiation of the male reproductive system through modulation of androgen receptor activity. In adult mice, EGF precursor immunostaining was limited to pachytene spermatocytes and round spermatids, whereas mature EGF was found additionally in Sertoli cells. EGF transgenic males were sterile due to hypospermatogenesis [28]. These data provide the first in vivo evidence that EGF overexpression can adversely affect spermatogenesis. This is in sharp contrast to transgenic mice overexpressing TGF-α in the testis, which were reported to have normal testis morphology and spermatogenesis [22]. Although EGF seems to be the physiological ligand in germ cell development, mice with either single- or triple-null mutations in EGF, TGF and amphiregulin did not suffer from reduced fertility [13]. As a next step in delineating the functions of EGF in spermatogenesis, overexpression of EGF or a dominant

Table 2. Summary of major phenotypes of EGFR and its ligands.

Genetic alternation in mice	EGFR and its ligands	Major phenotypes	Reference
Knock-out	EGFR	invariably died either at peri-implantation, mid-gestation or early in postnatal life; defects in epithelial cells, including those involved in skin, lung, gastrointestinal tract, tooth and eyelid; impaired gut proliferation with a reduced stem cell zone and disorganized mucosal architecture; strong strain dependence of the phenotype	17 – 19
Knock-out	TGF-α	eye defects, accelerated hair and weight loss, dermatitis and skin ulceration with aging	13
Knock-out	TGF-α (*wa-1*) and EGFR (*wa-2*)	affected hair and eyelid development	14, 15
Knock-out	Amphiregulin	underdeveloped mammary glands	13
Triple knock-out	EGF, TGF-α, and amphiregulin	alveoli were poorly organized and differentiated; eye defects; accelerated hair and weight loss, dermatitis and skin ulceration with aging; milk protein gene expression was also decreased and showed growth retardation	13
Overexpression	EGF	infertile, induced growth retardation; shortened small intestine	28 – 30
Overexpression	TGF-α	epithelial hyperplasia of several organs, pancreatic metaplasia and breast carcinoma	24, 25
Overexpression	amphiregulin	induced a psoriasis-like phenotype	27
Overexpression mutant	EGFR intracyto-plasmic domain	rescue pulmonary fibrosis and marked airspace hypoplasia	16
Dominant negative	EGFR in specific tissues	demonstrating the importance of EGFR in ductal branching and outgrowth of the mammary gland, and in differentiation and development of the hair follicles and epidermis	21

negative EGFR in specific cell types, for example in pachytene spermatocytes, will help to establish the autocrine/paracrine role of EGF in spermatogenesis. These results are summarized in table 2.

Conclusions and perspectives

Despite the extensive investigations of EGF, especially in vitro, its precise role and relationship with the IGF system remain largely undefined. The study of EGFR and its ligands has reached an interesting point, even though the studies described here do not provide conclusive answers to many of the questions asked. The early death of EGFR null mice has precluded the elucidation of EGFR function in its entirety. What is now required is the production of conditional knock-out mice so that the contribution of EGFR both prenatally and postnatally and in diseases can be further assessed. Currently, the most powerful strategy is the use of the Cre/loxP system, in which mice expressing the enzyme Cre recombinase driven by a tissue-specific promoter are cross-bred with mutants expressing the gene of interest engineered with flanking loxP sites. Cre excises the sequence between two loxP sites. This means that the target gene can be deleted in a cell-type specific and inducible manner depending on the choice of promoters for Cre expression. For receptors which function as dimers or oligomers, the strategy of producing dominant negative mutant receptors is also fruitful.

Focusing on unraveling the specific functions of EGF/EGFR, the phenotypes of the transgenic and knock-out mice described might be the consequence of a tightly regulated interplay of many growth factors in vivo. A slight imbalance could either produce pathological phenotypes in one tissue or alter the activity of EGFR and IGFs in another. Of interest would be to find the docking proteins or secondary messengers which serve as a ‘postman’, delivering signals from EGF and other growth factors. For transgenic mouse models of the EGF family, knock-outs of the group three ligands have yet to be made. Comparing the mild phenotypes of the triple group one ligand null mutants to the EGFR knock-outs, does the adverse phenotype suggest distinctive roles of the group three ligands or further redundancy between group one and three ligands? Furthermore, recent findings suggest that EGF and fibroblast growth fctor-2 are essential for stem cell proliferation and survival. It would be intriguing to use EGFR ligand mutant mice to see what exact roles growth factors play in stem cells neurogenesis [34, 35].

Another aspect of future EGFR research is to combine signaling and trafficking. During the past several years our understanding of receptor trafficking and signaling led to the possibility that they are functionally intrerrelated [36, 37]. However, how EGFR signaling is linked to

EGFR trafficking is largely unknown [38]. There are an increasing number of trafficking proteins that are tyrosine phosphoylated in an EGF-dependent manner. Mapping these sites in conjunction with analysis of nonphosphorylated mutants should be the next focus [36]. Furthermore, endocytosis and lysosomal targeting of the EGFR is a normal consequence of receptor activation. Degradation will inevitably terminate receptor signaling and trafficking of EGFR has been viewed in the context of attenuation [39]. Indeed, inhibition of receptor internalization and degradation will enhance signaling. What kind of motor protein [40–42] (dynein, kinesin, myosin) is involved in trafficking EGFR to the plasma membrane and which ones transport it for the novel endosomal fusion process? Can we get in vivo evidence by using the gain- or loss-in-function approach in EGFR or EGFR ligand mutant mice? How EGFR ligands affect these signaling and trafficking processes should also be further investigated in vivo.

More knowledge about growth factors could provide vast opportunities for therapeutic applications in human diseases. Further elucidation of the complicated EGFR signaling network is awaited.

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