

A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation

(*wa-2* mice/mutant mouse/epidermal growth factor receptor signaling/postnatal mortality)

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ABSTRACT The mutant mouse waved-2 (*wa-2*) is strikingly similar to transforming growth factor α -deficient mice generated by gene targeting in embryonic stem cells. We confirm that *wa-2* is a point mutation (T \rightarrow G resulting in a valine \rightarrow glycine substitution at residue 743) in the gene encoding the epidermal growth factor (EGF) receptor. *wa-2* fibroblastic cells lack high-affinity binding sites for EGF, and the rate of internalization of EGF is retarded. Although the tyrosine kinase activity of *wa-2* EGF receptors is significantly impaired, NIH 3T3 cells lacking endogenous EGF receptors but overexpressing recombinant *wa-2* EGF receptor cDNA are mitogenically responsive to EGF. While young and adult *wa-2* mice are healthy and fertile, 35% of *wa-2* mice born of homozygous *wa-2* mothers die of malnutrition because of impaired maternal lactation.

The epidermal growth factor receptor (EGFR) is expressed in a wide range of adult tissues and cell types, in blastocysts (1–3), and in all three germ layers of the developing embryo (4). The broad tissue distribution of the EGFR and its ligands, epidermal growth factor (EGF; ref. 5) and transforming growth factor α (TGF- α ; ref. 6), has led to the belief that activation of the EGFR signal-transduction pathway contributes to the regulation of numerous cellular processes in both embryonic development and in the adult. To explore the physiological processes regulated by activation of the EGFR, we (7) and others (8) generated mice homozygous for a disruptive mutation in the TGF- α gene. Surprisingly, the major phenotype of TGF- α -/- mice was a pronounced waviness of the hair and whiskers. The TGF- α -deficient mice resembled *wa-1* mice (9), and the genes were shown to be allelic (7, 8). While surveying other mouse mutants that display wavy hair, we (K.J.F. and A.R.D., unpublished observations) and others (8) noticed that the mutation in the spontaneous mutant *wa-2* (10) had been mapped to chromosome 11 (11) close to the gene (*c-erbB*) encoding the EGFR (12). This suggested that an EGFR mutation resulting in defective signaling may account for the *wa-2* phenotype. Here we, like Luetkeke *et al.* (13), describe a mutation in the *wa-2* EGFR that has a profound effect on receptor biochemistry and biology. We also observed that while young and adult mice are healthy and fertile, 35% of mice born to mothers homozygous for the *wa-2* mutation die soon after birth due to impaired maternal milk production. Thus, while defective EGFR signaling appears not to adversely affect the health of young or adult *wa-2* mice, normal EGFR signaling is critical for lactation.

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MATERIALS AND METHODS

Breeding of Mice. Breeding pairs of B6C3-*a/A-wa-2/wa-2-vt/vt* homozygous waved-2 (*wa-2*) mice, derived from a cross-intercross breeding system, and B6C3-*a/A* nonmutant control mice were purchased from The Jackson Laboratory.

Infection of Fibroblastic Cells with Recombinant EGFR Retrovirus. Viral supernatant recovered from ψ 2 cells expressing the human EGFR (14), was applied to subconfluent monolayers of *wa-2* and nonmutant fibroblastic cells (15). Cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum and G418 at 100 μ g/ml for 12 days. Drug-resistant colonies were pooled to generate *wa-2*EGFr1 and nonmutant EGFr1 cell lines.

¹²⁵I-Labeled EGF (¹²⁵I-EGF) Binding and Internalization. Iodination of murine EGF and equilibrium binding studies were performed as described (16). The data were plotted and equilibrium binding constants were derived using the LIGAND program (17). Receptor-mediated internalization of ¹²⁵I-EGF was monitored by acid washing (16, 18).

In Vitro and in Vivo Protein Kinase Assays. Single-cell suspensions of livers depleted of red blood cells were solubilized with extraction buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 200 kallikrein inactivator units of Trasylol (Bayer, Wuppertal, Germany)] for 30 min. Ligand-receptor complexes were isolated with 10 μ l of EGF-Affi-Gel beads (19) at 4°C for 2 h. *In vitro* kinase assays were carried out in 50 μ l of kinase buffer containing 10 μ Ci (1 Ci = 37 GBq) of [γ -³²P]ATP (4000 Ci/mmol; Bresatec, Adelaide, Australia) in the presence or absence of recombinant human lipocortin 1 (5 μ g per lane). Products were separated by SDS/PAGE and analyzed with a PhosphorImager (Molecular Dynamics). Immunoblotting was carried out on the same samples using an EGFR antiserum (no. E-3138; Sigma) and visualized by ECL (Amersham). For analysis of *in vivo* phosphorylation, confluent cultures of NIH 3T3 cell lines expressing EGFR (see below) were transferred to serum-free DMEM for 16 h, when cultures were supplemented with sodium orthovanadate (300 μ M) with or without mouse EGF (300 ng/ml) for a further 10 min. Cells were rinsed in phosphate-buffered saline and lysed for 1 h at 4°C in extraction buffer containing sodium orthovanadate. EGFRs

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF- α , transforming growth factor α .

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were immunoprecipitated from extracts using anti-human EGFR monoclonal antibody 528 bound to protein A-Sepharose. Proteins were separated on SDS/7.5% PAGE and transferred to poly(vinylidene difluoride) membrane for immunoblotting with anti-EGFR antibody 1005 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology), respectively.

cDNA Cloning, PCR, and Nucleotide Sequence Analysis. DNA reverse transcribed from RNA prepared from livers of nonmutant and *wa-2* mice was used as templates for PCR. The coding region of the *wa-2* EGFR cDNA was amplified in segments using four pairs of oligonucleotides [nucleotides 107–1217, 5'-d(CGCTGTCTCGGATTAATCC)-3' and 5'-d(GCATTTATGGAGAGTGTGTC)-3'; nucleotides 1117–2142, 5'-d(GAAGATGGCATCCGCAAGTG)-3' and 5'-d(CACCACTATGAAGAGGAGGC)-3'; nucleotides 2059–3018, 5'-d(CAAGGATGTGAAGTGTGGCC)-3' and 5'-d(CCAGCACTTGACCATGATCA)-3'; nucleotides 2960–3849, 5'-d(GCCTTCCACAGCCACCTATC)-3' and 5'-d(GGTCCTGGGATTCTAGAAAG)-3']; nucleotide numbers are consistent with (ref. 20; M.L.H., A.R.D., and W.A., GenBank accession no. X78987). PCR reactions employing *Pfu* DNA polymerase were cycled 40 times through sequential incubation at 95°C for 60 s, 55°C for 60 s, and 72°C for 120 s. The nucleotide sequence of each segment was determined using an automated DNA sequencer (Applied Biosystems) and compared to the sequence of the mouse EGFR using the DNASTAR program. (M.L.H., A.R.D., and W.A., GenBank accession no. X78987).

Mutagenesis and Generation of NIH 3T3 Cells Expressing EGFRs. The *wa-2* mutation was introduced into human EGFR cDNA by oligonucleotide-directed mutagenesis in pcDNA1-Amp (Invitrogen) (32). Wild-type and V743G EGFR cDNAs including the polyadenylation signal sequence from pcDNA1Amp were subcloned into APTag-1 (22). NIH 3T3 cells were cotransfected with EGFR cDNAs and pGKNeo (or pGKNeo alone) using calcium phosphate (23). G418-resistant clones were picked and assayed for EGFR expression by binding of radiolabeled EGF.

[³H]Thymidine Incorporation. Cells were seeded in duplicate at a density of 5×10^4 cells per well in 96-well plates in DMEM/10% (vol/vol) newborn calf serum, allowed to adhere for 3 h, and then starved for 18 h in DMEM/0.5% newborn calf serum. Various concentrations of human EGF were added, and the cells were incubated for 20 h prior to the addition of [³H]thymidine (5 μ Ci/ml; 6.7 Ci/mmol; DuPont). After 2 h, cells were lysed in 30 mM Tris, pH 8.0/100 mM EDTA/20% sarcosyl, harvested onto filter mats (Wallac, Finland), treated with Betaplate scintillation liquid, and assayed for radioactivity (16).

Cell Proliferation Assay. Cells were plated in quadruplicate at 4×10^4 cells per well in 24-well plates, grown to confluency, and starved for 18 h in DMEM/0.125% fetal calf serum. Human EGF (10 ng/ml) was added, and cells were counted 3 days later (24).

RESULTS

EGFRs in fibroblastic cell lines derived from *wa-2* and nonmutant mice were examined. Scatchard analysis of [¹²⁵I]-EGF binding data showed that nonmutant mouse fibroblasts displayed two discrete EGFR populations with high and low affinities, whereas *wa-2* cells displayed only low-affinity EGFRs (Fig. 1A and C). To confirm that the *wa-2* defect was an intrinsic property of the EGFR, we infected cultured *wa-2* cells with a retrovirus encoding the human EGFR and the neomycin gene (14). G418-resistant cells were pooled (*wa-2*EGFr1), and Scatchard analysis (Fig. 1B and C) showed that *wa-2*EGFr1 cells displayed both high- and low-affinity receptors.

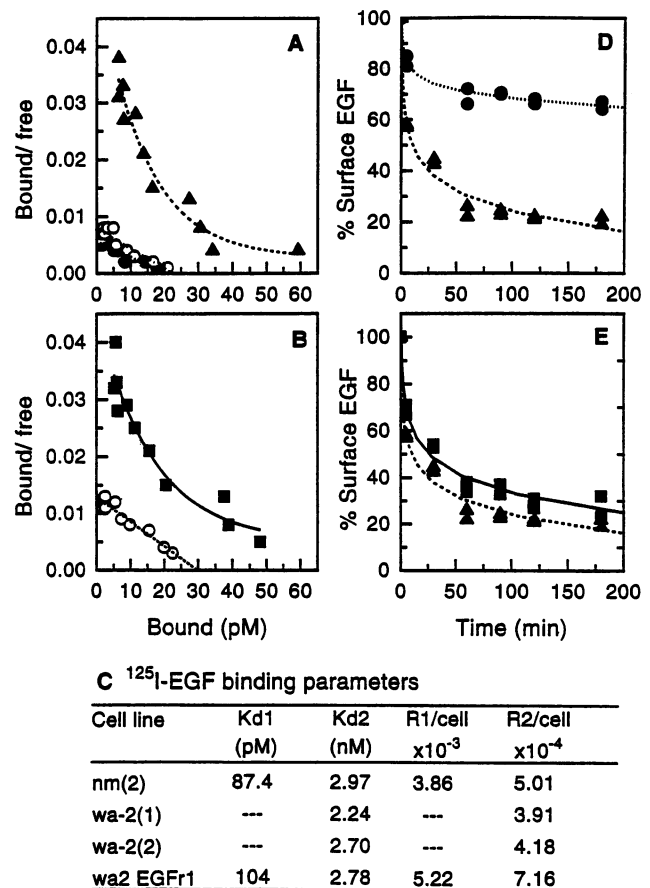


FIG. 1. [¹²⁵I]-EGF binding and internalization by nonmutant (nm) and *wa-2* cells. (A and B) Scatchard analysis of [¹²⁵I]-EGF binding to fibroblastic cell lines derived from a nonmutant mouse [nm(2) (\blacktriangle)] and *wa-2* mice [*wa-2*(1) (\circ) and *wa-2*(2) (\bullet)] and to *wa-2* fibroblastic cells expressing the human EGFR [*wa-2*EGFr1 (\blacksquare)]. (C) [¹²⁵I]-EGF binding characteristics and affinities in nm(2), *wa-2*(1), *wa-2*(2), and *wa-2*EGFr1 cells (R1 and R2, number of high- and low-affinity EGFRs per cell). (D and E) Kinetics of internalization of [¹²⁵I]-EGF in nm(2) (\blacktriangle), *wa-2*(2) (\bullet), and *wa-2*EGFr1 (\blacksquare) cells; double symbols represent duplicate determinations.

The rate of internalization of EGFRs in *wa-2* and nonmutant fibroblasts was compared by measuring the proportion of [¹²⁵I]-EGF present on the cell surface after binding. Internalization of occupied *wa-2* EGFRs occurred more slowly than that of nonmutant EGFRs (Fig. 1D). Comparable rates of internalization were observed in nonmutant fibroblasts and *wa-2*EGFr1 cells expressing the human EGFR (Fig. 1E). Thus, the mutation in the *wa-2* EGFR does not alter its biosynthesis or display, but formation of the high-affinity complex and the kinetics of ligand-dependent internalization are defective.

To identify the presumed *wa-2* mutation, we synthesized cDNAs from mRNA isolated from livers of *wa-2* and nonmutant mice and compared the nucleotide sequence of the corresponding EGFR clones. A single-nucleotide difference (T \rightarrow G) was identified, which results in a valine \rightarrow glycine substitution at residue 743 in subdomain III of the kinase domain (25) of the *wa-2* EGFR (Fig. 2). This substitution lies 20 residues C-terminal of the lysine residue that defines the ATP-binding site of the EGFR. Because a valine, or other hydrophobic amino acid at residue 743 is a common feature of tyrosine kinase receptors (25), it seems likely that the V743G mutation accounts for the defects associated with the *wa-2* EGFR.

To establish whether EGFRs from *wa-2* mice were capable of auto- and/or transphosphorylation, EGFRs were purified

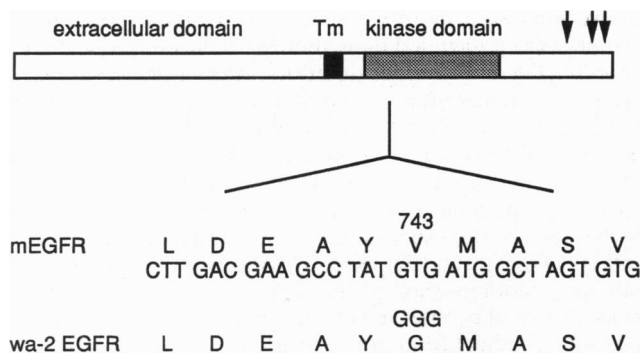


FIG. 2. Nucleotide sequence of part of subdomain III of the wild-type and *wa-2* EGFR. Nucleotide and deduced amino acid sequence of part of subdomain III of the catalytic domain (25) of the wild-type mouse (mEGFR) and *wa-2* mouse (*wa-2* EGFR) EGFRs. Tm, transmembrane domain. Numbering is based on the first residue being that of the mature mouse EGFR protein. Arrows at the C terminus of the figure represent major autophosphorylation sites.

from livers of nonmutant and *wa-2* mice and assayed for *in vitro* protein kinase activity. Autophosphorylation of the EGFR was observed in ligand-receptor complexes prepared from nonmutant mice but not *wa-2* mice (Fig. 3A). Western blot analysis using an EGFR antiserum revealed the 180-kDa EGFR in liver extracts of both nonmutant and *wa-2* mice (Fig. 3A). To determine whether *wa-2* EGFRs were capable of transphosphorylation, protein kinase assays were set up in the presence of lipocortin. EGFRs from *wa-2* livers, unlike those from nonmutant mice, failed to phosphorylate lipocortin (Fig. 3B). The kinetics of ATP binding to the *wa-2* EGFR indicated normal affinity and stoichiometry (data not shown). Determination of the effect of EGF on receptor phosphorylation in

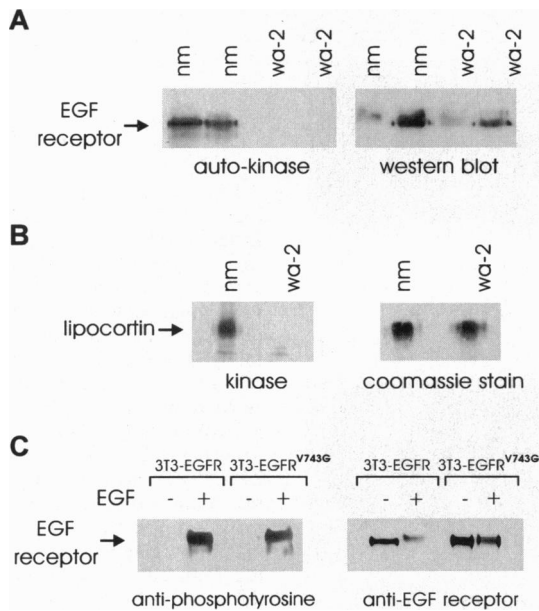


FIG. 3. Auto- and transphosphorylation of nonmutant and *wa-2* EGFRs. (A) *In vitro* protein kinase/Western blot assay of EGFRs extracted and purified from the livers of two nonmutant (nm) and two *wa-2* mice by EGF-Affi-Gel affinity chromatography. (B) *In vitro* protein kinase/Western blot assay carried out in the presence of lipocortin (and corresponding Coomassie-stained gel) from similar extracts. (C) Phosphorylation of the EGFR in whole cells determined by Western blotting with anti-phosphotyrosine antibodies and with anti-EGFR antibodies. No other bands were detected in the autoradiographs. 3T3-EGFR, NIH 3T3 cells transfected with the wild-type human EGFR; 3T3-EGFR^{V743G}, NIH 3T3 cells transfected with the V743G EGFR mutant.

whole cells was hampered by the low number of EGFRs and high endogenous phosphotyrosine levels. We therefore examined EGF-dependent *in vivo* phosphorylation in NIH 3T3 cell lines expressing either wild-type human EGFRs (3T3-EGFR) or V743G EGFRs (3T3-EGFR^{V743G}). Expression of EGFR on transfected cells and its affinity for the ligand were determined by ¹²⁵I-EGF binding and Scatchard analysis. Cloned cell lines expressing EGFR^{V743G} displayed only low-affinity binding (≈ 1 nM), and receptor number varied between 10^5 and 2×10^6 per cell. Clonal cell lines expressing wild-type EGFRs displayed high- and low-affinity binding with $1-2 \times 10^5$ receptors per cell (data not shown). While both wild-type and *wa-2* EGFRs showed enhanced tyrosine phosphorylation after stimulation with EGF (Fig. 3C), the specific tyrosine phosphorylation was 5-fold lower for the mutant receptor.

To determine whether the *wa-2* receptor could transmit a mitogenic signal, we assayed [³H]thymidine incorporation and the kinetics of cellular proliferation in 3T3-EGFR and 3T3-EGFR^{V743G} cells. EGF stimulated [³H]thymidine uptake and cellular proliferation in both cell types (Fig. 4). However, the concentration of EGF required for half-maximal stimulation of 3T3-EGFR^{V743G} cells ($EC_{50} = 1.5$ nM) was considerably higher than the concentration required for 3T3-EGFR cells ($EC_{50} = 80$ pM). These differences most likely reflect differences in both the affinity and activity of the *wa-2* EGFR. The 3T3-EGFR^{V743G} cell line displays ≈ 10 -fold more EGFRs (2×10^6 receptors per cell) than 3T3-EGFR cells (1.3×10^5 receptors per cell). Therefore, the number of receptors that are occupied at EC_{50} in wild-type cells is 20,000, while in *wa-2* cells it is close to 10^6 (data not shown). Thus, the impaired mitogenic response in these cells is not solely a result of the lower affinity for EGF but

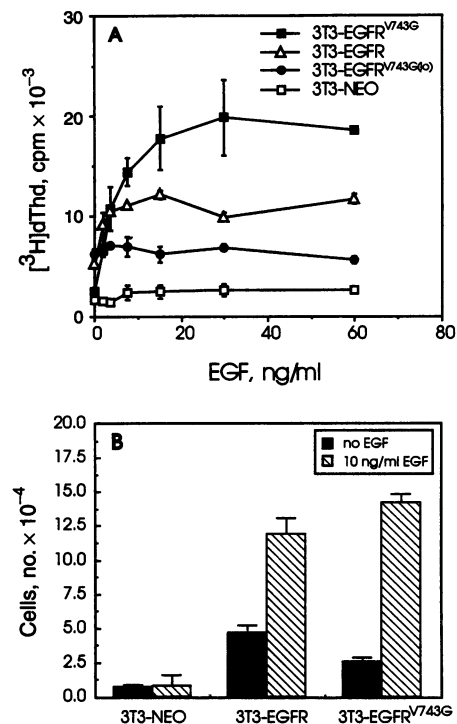


FIG. 4. EGF stimulation of transfected cells. (A) DNA synthesis measured by [³H]thymidine incorporation. (B) Cell proliferation assays. Data are means \pm SD of quadruplicate wells from one representative experiment of three. 3T3-NEO, NIH 3T3 cells transfected with pGKNeo; 3T3-EGFR, NIH 3T3 cells transfected with the human EGFR; 3T3-EGFR^{V743G} and EGFR^{V743G(lo)}, NIH 3T3 cells transfected with the V743G EGFR mutant expressing high and low numbers of receptors, respectively. To avoid confusion, we have designated the mutation in the recombinant human EGFR (EGFR^{V743G}) in line with the corresponding residue in the mouse; the corresponding valine residue in the human EGFR occurs at position 741 (21).

Table 1. Mortality and weight of weaned pups born to parents of different *wa-2* genotypes

Parents Male × female	Total pups born	Mortality by 3.5 days, n (%)	Weight of weaned pups, g
<i>wa-2/+</i> × <i>wa-2</i>	55	19 (35%)	7.4 ± 1.6
<i>wa-2</i> × <i>wa-2/+</i>	27	1 (4%)	11.1 ± 2.4*

There is no significant difference in mortality or weight of *wa-2* and *wa-2/+* pups of the same crosses; therefore, data from *wa-2* and *wa-2/+* pups in each mating group have been pooled. Pups were genotyped as *wa-2* or *wa-2/+* on the basis of whisker morphology (11, 12). Pups were weaned at 21.5 days.

* $P < 0.001$, Student's *t* test.

probably reflects a defect in the signaling capacity of the mutant EGFR. This is consistent with the observation that 3T3-EGFR^{V743G(10)} cell lines expressing relatively few receptors ($1-2 \times 10^5$), do not respond even to high concentrations of EGF (Fig. 4).

While maintaining a colony of *wa-2* mice, we observed a striking mortality (35%) in the first few days of postnatal life in litters of mixed genotypes born to homozygous *wa-2* but not to heterozygous (*wa-2/+*) mothers (Table 1). We suspected that the basis for this mortality would be nutritional, since both *wa-2* and *wa-2/+* pups (from *wa-2* mothers) that died had less gastric milk than littermates (Fig. 5A); indeed, the presence or absence of gastric milk on the day of birth (0.5 day) proved a reliable prognostic indicator of death within the first day or two postpartum. While most pups suckled by homozygous *wa-2* mothers survived beyond day 3, they showed significant evidence of runting compared with pups suckled by heterozygous mothers (Table 1). To determine whether the mortality was intrinsic to live-born pups or reflected impaired lactation by

homozygous *wa-2* mothers, mice born to homozygous *wa-2* mothers, which included pups showing reduced levels of gastric milk (Fig. 5A), were fostered to lactating BALB/c mice. Twenty-four hours after cross-fostering, all pups had approximately normal levels of gastric milk (Fig. 5B), and no significant neonatal mortality was observed. By contrast, pups born from matings with *wa-2/+* mothers showed no evidence of reduced gastric milk, and these animals flourished regardless of whether they were suckled by their natural mothers or foster mothers (Fig. 5C and D). BALB/c pups suckled by homozygous *wa-2* mothers showed the same nutritionally based increase in mortality observed for *wa-2* pups suckled by homozygous *wa-2* mothers (data not shown).

Histologically, the mammary glands of *wa-2* mice were small, and the ratio of gland to adipose tissue was dramatically reduced compared to that of nonmutant mice. There was a marked reduction of milk within ducts of *wa-2* mammary glands, and secretory vacuolation within lobules was less pronounced than in corresponding sections from nonmutant mice (Fig. 5E and F).

DISCUSSION

In this study we have shown that the striking phenotypic similarities between *wa-2* and TGF- α knockout/*wa-1* mice are related by impaired signaling through the EGFR. Our results, and those of Luetkeke *et al.* (13), show that the *wa-2* mutation is a single nucleotide alteration that results in the substitution of glycine for valine in a highly conserved region of the EGFR. While the V743G mutation has a profound impact on receptor kinase activity (ref. 13 and Fig. 3) and biology (Fig. 5), it does not constitute a null mutation since NIH 3T3 cells overexpressing transfected human EGFRs carrying the V743G mu-

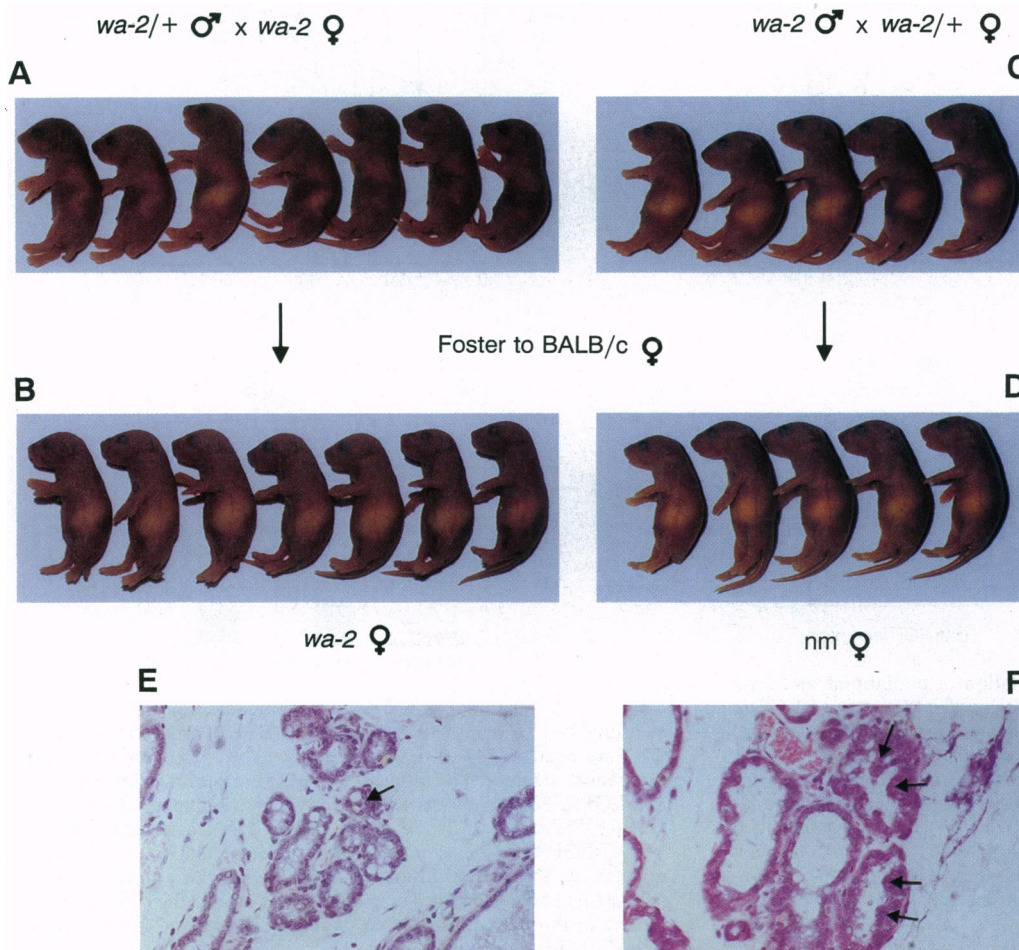


FIG. 5. Lactation and histopathology of *wa-2* mice. (A) Litter of 0.5-day-old pups, with various amounts of milk in their stomachs, suckled by *wa-2* (homozygous) mother. (B) Rescue of pups in A by fostering to lactating BALB/c mother for 24 h. (C) Litter of 0.5-day-old pups suckled by *wa-2/+* (heterozygous) mother. (D) Pups in C after fostering to BALB/c mother for 24 h. (E) Transverse section (hematoxylin/eosin; $\times 375$) through *wa-2* postpartum (1.5 days) mammary gland tissue illustrating reduced size of mammary ducts and lobules. The precipitate located in the lumen of some ducts resembles early secretory activity typically seen in proliferating mammary gland during puberty. (F) Normal lactating mammary gland tissue (nonmutant; 1.5-days postpartum) at the same magnification as *wa-2* breast tissue showing typical size of mammary gland ducts and lobules. Lobules undergoing active holocrine secretion of milk are indicated by arrows.

tation are mitogenically responsive to EGF (Fig. 44). While Luetkeke *et al.* (13) report no difference in the affinity of EGF binding in wild-type and *wa-2* cells, we have demonstrated by Scatchard analysis that the high-affinity EGF binding site is absent in cells expressing *wa-2* EGFRs. Interestingly, responsiveness to EGF and EGF-dependent phosphorylation were observed only after stimulation with high levels of EGF (1–10 nM) and in cells expressing large numbers ($>10^6$ per cell) of transfected *wa-2* EGFRs, suggesting a severe impairment of receptor function. These observations are consistent with the report by Luetkeke *et al.* (13) that phosphorylation of *wa-2* EGFR in liver cells or cell membranes occurs at very high concentrations of EGF (30 nM). However, we were unable to demonstrate autophosphorylation in an *in vitro* kinase assay on EGFR prepared directly from tissues of *wa-2* mice (Fig. 3) or from cultured *wa-2* fibroblastic cells using EGF-Affi-Gel (data not shown). The profound defect in *in vitro* kinase activity of *wa-2* receptors might be exacerbated by low stability during extraction, although even in intact cells the phosphorylation of *wa-2* EGFR receptors was detectable only at high receptor density. It is still unclear whether this is autophosphorylation or phosphorylation by another cellular protein kinase (26).

In light of the wide distribution of the EGFR in embryonic development and in adult tissues, why is the pathology in *wa-2* mice not more profound? It is possible that a normal physiological response to EGF (or related ligands) occurs only in tissues expressing large numbers of EGFRs with a high local concentration of ligand. It is also possible that responsiveness to EGF is modulated by phosphorylation of the EGFR by other cell surface protein kinases such as c-erb-B2. In this regard it has been shown that coexpression of c-erb-B and c-erb-B2 leads to the formation of active heterodimers and reciprocal transphosphorylation (27, 28).

The defect in lactation associated with homozygous *wa-2* mice is interesting in light of the observation that the development of mammary glands of pregnant mice in which the submandibular glands (a rich source of EGF) had been surgically removed was retarded (29), leading to decreased milk production and increased infant mortality. EGF replacement therapy effectively reversed the effects of pregestational sialoadenectomy (29). While impaired lactation in the sialoadenectomized mice reflects reduced levels of circulating EGF, the same outcome in *wa-2* mice is a manifestation of defective signaling through the EGFR as a result of the V743G mutation.

It is unclear precisely how signaling through the EGFR contributes to the regulation of lactation. Perhaps one or more hormones such as estrogen, growth hormone, adrenocorticotropic hormone, progesterone, or prolactin, which regulate mammary and ductal growth, lobo-alveola growth, or lactogenesis (for review see ref. 30), is under the control of EGF or another member of the EGF family of ligands; recent evidence implicates TGF- α as an important mediator of mammary development (31). Notwithstanding the various phenotypic similarities shared by *wa-2* and TGF- α -deficient mice, the notion that TGF- α , like EGF, might contribute to the regulation of lactation must be tempered by the fact that TGF- α -/- females give rise to normal sized litters that suckle normally, are weaned without difficulty, and are not subject to neonatal mortality.

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