Animal Model

Effects of *in Vivo* Heregulin β 1 Treatment in Wild-Type and ErbB Gene-Targeted Mice Depend on Receptor Levels and Pregnancy

Sue O'Shea,* Kent Johnson,[†] Ross Clark,[‡]
Mark X. Sliwkowski,[§] and Sharon L. Erickson[‡]

From the Departments of Cell and Developmental Biology* and Pathology,[†] University of Michigan, Ann Arbor, Michigan; and the Departments of Endocrinology[‡] and Molecular Oncology,[§] Genentech, Incorporated, South San Francisco, California

Mice heterozygous (+/–) for either heregulin (HRG), ErbB2, or ErbB3 were created by gene targeting, resulting in the loss of one functional gene copy and an associated decrease in targeted protein. We examined the *in vivo* activity of recombinant HRG peptide, rHRG β 1 (amino acids 177 to 241), in the three heterozygous mouse lines and in wild-type (WT) mice, both pregnant and nonpregnant. Nonpregnant WT and HRG(+/–) mice of both sexes were sensitive to rHRG β 1 treatment as evidenced by a high mortality rate associated with abdominal enlargement and parietal cell loss. However, pregnant WT mice and ErbB2 and ErbB3 heterozygous mice treated with rHRG β 1 were less affected, with significantly lower mortality rates and a less severe abdominal phenotype. Histological analysis revealed extensive breast ductal hyperplasia in females of all genotypes after rHRG β 1 treatment. Hyperplasia of other epithelial tissues such as the pancreas and intestine and the growth of cardiac nerve bundles were also observed, independent of sex. (*Am J Pathol* 2001, 158:1871–1880)

Amplification and overexpression of ErbB2 (HER2), a type-1 receptor tyrosine kinase of the epidermal growth factor receptor (EGFR) (ErbB) family, is found in ~30% of human breast cancers and is associated with poor prognosis.^{1,2} Overexpression of ErbB2 is thought to lead to ligand-independent ErbB2:ErbB2 dimer formation, resulting in constitutive activation and signaling through the potent ErbB2 tyrosine kinase pathway.³ ErbB2 overexpression is also implicated in other human cancers of epithelial origin such as ovarian, gastric, pancreatic, and non-small cell lung cancer.⁴

The heregulins (HRGs), a family of structurally diverse glycoproteins with at least 15 spliced isoforms, indirectly activate ErbB2 signaling by binding to co-receptors (ErbB3, ErbB4) that can in turn dimerize with ErbB2 allowing HRG-induced signaling to proceed by receptor tyrosine phosphorylation.^{5–7} Interestingly, ErbB4 is an active tyrosine kinase receptor,^{7–10} whereas ErbB3^{8,11} is catalytically impaired.^{12,13} Therefore, although tyrosine kinase activation of both ErbB2 and ErbB4 can occur in the ErbB2:ErbB4 heterodimer, only ErbB2 tyrosine kinase activation is possible in the ErbB2:ErbB3 heterodimer. ErbB3, however, preferentially signals through the SH2-containing PI-3-kinase pathway.^{14–17} Thus, the heterodimers ErbB2:ErbB3 and ErbB2:ErbB4 are the preferred HRG signaling complexes.^{18–20}

The absence of either the entire HRG ligand family or the ErbB2, ErbB3, or ErbB4 receptor in gene-targeted mice resulted in embryonic lethality because of cardiac defects.^{21–25} The ErbB3 null mutant illustrated additional roles of HRG in cerebellar development, in the development of neural crest-derived lineages, and in epithelial-mesenchymal interactions critical for organogenesis.²³

Given the pleiotropic activity that is attributed to the HRG family, it was of interest to determine the dominant biological effects observed in mice with different ErbB and HRG background when treated with rHRG β 1. Therefore, heterozygous (+/–) mice, with only one functional copy of their targeted gene, were created for HRG, ErbB2, and ErbB3 by homologous recombination. These mice had decreased levels of their targeted gene product compared to wild-type (WT) mice,^{23,26} establishing a unique model to study the interactions of HRG and its receptors, with the expectation that reduced levels of receptors or endogenous ligand would lead to changes in the response to exogenous HRG. In this study, we investigate the activity of rHRG β 1, administered *in vivo* by ALZA pump (ALZA Corp., Mountain View, CA) through-

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Address reprint requests to Sharon L. Erickson, Department of Endocrinology, Genentech, Inc., 1 DNA Way, S. San Francisco, CA 94080. E-mail: sle@gene.com.

out 14 days, in these gene-targeted mouse lines, as well as in pregnant and nonpregnant female WT mice. We demonstrate that in WT and HRG(+/-) mice, the effects of rHRG β 1 treatment are severe, as evidenced by a high mortality rate. The decrease in receptor levels in ErbB2(+/-) and ErbB3(+/-) mice and the pregnant state of WT females seems to diminish the effects of rHRG β 1 treatment, resulting in a significantly lower mortality rate. rHRG β 1-induced mortality seems to involve the gastrointestinal system, where a loss of parietal and chief cells is seen, as well as possible motility defects. Histological analysis reveals hyperplasia in epithelial target tissues, along with the growth of cardiac peripheral nerve bundles in all genotypes. Interestingly, breast hyperplasia was extensive in all genotypes but was confined to the female. This finding, along with the protective effect of pregnancy, suggests that the female hormonal environment can influence the activity of HRG and the ErbB family.

Materials and Methods

Experimental Animals

Chimeric mice were generated by conventional gene targeting.²³ Mice were bred on C57BL/6J and BALB/c mouse strains with no differences noted in rHRG β 1 response because of background strain or backcross level. All animal care was in accordance with NIH guidelines.

Experimental Design/Test Compound

Adult 8- to 12-week-old mice of each genotype, with an average weight of 20 g, were treated with a sustained 14-day systemic delivery of rHRG β 1 (amino acids 177 to 244), a soluble form of the EGF-like domain common to all HRG isoforms, using ALZA pumps.⁵ The EGF-like domain is a critical part of the HRG molecule, as it alone can stimulate tyrosine phosphorylation of ErbB2, resulting in such downstream signaling effects as acetylcholine receptor synthesis in cultured myotubes.^{5,27} The rHRG β 1 form of the EGF-like domain, used in this study, seems to exhibit higher binding affinity in ErbB2-expressing cells lines than the α form and has increased potency in activating PI-3-kinase in nontransformed human mammary epithelial cells.^{5,17}

Each group that received rHRG β 1 consisted of six female and six male mice. Control groups for each genotype (two females and three males) received phosphate-buffered saline (PBS) (Gibco catalog no. 14190-144; Gibco, Gaithersburg, MD). ALZA miniosmotic pumps, model 2002 (pumping rate, 0.5 μ l/hr; duration, 14 days; reservoir volume, 200 μ l), were filled, as per ALZA instructions, with rHRG β 1 (amino acids 177 to 244), formulated, and diluted in PBS for a delivered dose of 0.75 mg/kg/day or 1.0 mg/kg/day. Pumps were stored at 4°C overnight in PBS, before sterile implantation. Animals were anesthetized with ketamine 75 to 80 mg/kg, xylazine, 7.5 to 15 mg/kg and acepromazine (all from Fort Dodge Laboratories, Fort Dodge, IA), 0.75 mg/kg, delivered intraperitoneally. The filled pump, delivery portal

first, was inserted into a subcutaneous pocket along the back. Animals were individually housed and observed daily. Any moribund animals were immediately sacrificed and necropsied.

Histopathology

Surviving animals were sacrificed and necropsied at day 14. Serum was collected, centrifuged, and frozen for clinical chemistry determinations and kinase receptor assay (KIRA) rHRG β 1 analysis;²⁸ bone marrow smears were also prepared. Organs were fixed in 10% neutral-buffered formalin (Richard Allen Scientific, Kalamazoo, MI) at 22°C overnight, followed by storage in 70% ethanol. For paraffin embedding, tissues were dehydrated through graded alcohols, followed by methyl salicylate, and overnight infiltration in Paraplast at 57°C. Serial 6- μ m sections were cut and affixed to polylysine-coated slides before hematoxylin and eosin staining and histological analysis.

Statistical Methods

Mortality was compared among the four genotypes and between the pregnant and nonpregnant WT females using the Pearson chi-square test and Kaplan-Meier cumulative survival plots. After log-transformation (log10 pmol/L) of the raw serum rHRG β 1 concentrations to reduce variance heterogeneity, concentrations were compared by genotype and sex, or by pregnancy and nonpregnancy, using analysis of variance. If an interaction between genotype and sex was observed, the relationship between rHRG β 1 concentration and genotype was assessed separately for males and females. Pairwise comparisons between genotypic groups was done using the Fisher protected least significant difference procedure for multiple comparisons.

Results

Using gene targeting, heterozygous (+/-) mice were created that lacked one of the functional copies of the *HRG-1*, *ErbB2*, or the *ErbB3* gene.²³ These mice were viable and fertile, yet had altered levels of their respective targeted gene product, providing a model system to study the interactions of HRG, ErbB2, and ErbB3. In the current study, we challenged these mice with 0.75 mg/kg/day rHRG β 1 throughout a 14-day period to investigate effects of receptor and endogenous HRG levels on *in vivo* rHRG β 1 activity.

Overall Effects of rHRG β 1 Treatment Based on Genotype (Morbidity and Mortality)

Tolerance for rHRG β 1 treatment varied depending on genotype. Mortality differed significantly between the genotypes ($P < 0.001$) with the HRG(+/-) group having the highest mortality (12 of 12; 100%), the ErbB2 and ErbB3(+/-) groups having the lowest mortality (1 of 12;

Figure 1. Cumulative survival plot analysis; rHRG β 1 serum levels from females of the four genotypic groups. **A:** Male and female wild-type (WT), HRG(+/-), ErbB2(+/-), and ErbB3(+/-) animals were treated with 0.75 mg/kg/day rHRG β 1. Mortality varied significantly between the genotypic groups using the Pearson chi-square test ($P < 0.001$); no difference in mortality was noted based on sex. In the WT group receiving rHRG β 1, 7 of the 12 animals (58%) died by days 5 to 6; the remaining five animals all survived the 14-day length of the study. In the HRG(+/-) group receiving rHRG β 1, four animals were found dead at days 5 to 6; the remaining eight HRG(+/-) animals in this group were sacrificed on days 5 to 6 because of severe clinical signs. In contrast, 92% of the ErbB2(+/-) and ErbB3(+/-) animals survived the study. **B:** No relationship was noted between the concentration of rHRG β 1 and genotype in males; using analysis of variance, in females rHRG β 1 levels in ErbB2(+/-) animals were significantly lower than in the other genotypic groups ($P = 0.001$). rHRG β 1 concentrations were log-transformed ($\log_{10}$ pmol/L) to reduce variance heterogeneity. The scatter plot of log-serum rHRG β 1 concentrations in female mice treated with 0.75 mg/kg/day rHRG β 1 illustrates comparisons by genotype. The mean for each group is shown as a horizontal line within each plot. All control mice had less than detectable rHRG β 1 levels.

8%) and the WT group having intermediate mortality (7 of 12; 58%), (Figure 1A). There was no apparent difference in mortality by sex. Both WT and HRG(+/-) animals receiving rHRG β 1 exhibited lacrimation, dehydration, hunching, ruffled fur, a cool body temperature, and were noticeably hypoactive. The WT and HRG(+/-) animals also seemed to have enlarged abdominal regions. All control animals received PBS and survived the full 14 days with no clinical signs.

Serum samples were collected at necropsy and a KIRA was run to determine levels of circulating rHRG β 1.²⁸ Although virtually all control mice had no detectable rHRG β 1, all animals treated with rHRG β 1 had detectable rHRG β 1 serum levels. Interestingly, serum concentrations of rHRG β 1 were found to differ by sex and genotype ($P < 0.05$). In females, there was a significant relationship ($P < 0.02$) between genotype and HRG concentration; rHRG β 1 levels in the ErbB2(+/-) group

were significantly lower than those in the ErbB3(+/-), HRG(+/-), and WT groups (Figure 1B). No relationship was noted between rHRG β 1 concentration and genotype in males.

Clinical chemistries were also performed on serum collected at necropsy. Although there were trends toward higher values in the clinical chemistry profiles of rHRG β 1-treated animals (cell/liver injury (aspartate aminotransferase, alanine aminotransferase), pancreatic injury (amylase), liver injury (alkaline phosphatase), muscle injury (creatinine kinase), hepatobiliary system (γ -glutamyl transpeptidase), kidney injury (blood urea nitrogen and creatinine), hepatobiliary system (total bilirubin), electrolytes (Ca, Na, K, Cl), and phosphorus), most were within established normal ranges. Sporadic elevations of alanine aminotransferase, aspartate aminotransferase, creatine kinase, and amylase were noted in some rHRG β 1-treated WT and HRG(+/-) animals, as well as lowered glucose levels relative to controls, probably reflective of their poor condition.

Hematology and urine evaluations were not performed because of the small sample sizes available for analysis from the ill mice. The brain, heart, kidney, and liver were weighed in selected mice with no significant differences found because of rHRG β 1 treatment. Bone marrow smears showed evidence of trilinear cellularity indicating no obvious abnormalities and no differences were noted between the groups in this study.

Breast Tissue

Differential expression of the four ErbB receptor family members and distinctive patterns of expression of ErbB ligands have been reported for the different stages of mammary gland development, with ErbB2: ErbB3 being the predominant HRG signaling heterodimer.²⁹ Although ErbB2 overexpression has been implicated in 30% of human breast cancers,^{1,2} the role of HRG in cancer is poorly understood. Interestingly, in 19 of 20 female mice examined that were treated with rHRG β 1, extensive breast ductal hyperplasia was present regardless of genotype (Figure 2). Because of early mortality, HRG(+/-) females were only exposed to rHRG β 1 for 5 to 6 days in this study, yet they consistently showed breast hyperplasia, illustrating the rapidity of the response (Figure 2c). Surprisingly, breast ductal hyperplasia was observed only in female mice, with male mice primarily unaffected. In fact, except for two males, one ErbB2(+/-) and one ErbB3(+/-), which showed a very unusual focal pattern of acinar hyperplasia and squamous metaplasia of the ducts, quite different from the females, there was only slight sporadic ductal proliferation noted in any of the other male mice. Therefore, although there was no effect in breast tissue based on genotype, rHRG β 1 activity was profoundly influenced by the sex of the animal. These observations suggest an association between rHRG β 1 activity and sex or possibly hormonal status.

WT + PBS control

WT + rHRG

HRG (+/-) + rHRG

ErbB2 (+/-) + rHRG

ErbB3 (+/-) + rHRG

Figure 2. Female breast. Sections through the breast tissue from female mice; a representative control WT animal (a) and a WT (b), HRG(+/-) (c), ErbB2(+/-) (d), and ErbB3(+/-) animal (e) that received rHRG β 1. Sections are from mice that received rHRG β 1 treatment for 14 days except in the case of the HRG(+/-) that was only dosed for 5 to 6 days. rHRG β 1 exposure induced a generalized ductal hyperplasia with papillomatosis (arrows) in all groups that received rHRG β 1, regardless of genotype. Males did not show hyperplasia. H&E; original magnification, $\times 20$.

Gastric Tissue

Overexpression of ErbB2 is also known to play a role in gastric cancer⁴ and there is evidence that HRG α may play a role in the proliferation of gastric epithelial cells through mesenchymal-epithelial interaction in the gastric mucosa.³⁰ Treatment of WT and HRG(+/-) mice of both sexes with rHRG β 1 produced animals with grossly distended abdomens; this abdominal phenotype was associated with early mortality. In contrast, the abdomens in the ErbB2(+/-) and ErbB3(+/-) mice and the five sur-

living WT mice appeared normal on external examination. At necropsy of nonsurviving rHRG β 1-treated WT and HRG(+/-) mice, visible abdominal distention correlated with stomachs filled with undigested food and little fecal formation, suggesting reduced digestion/motility, rather than starvation because of a lack of feeding. On histological examination, these severely affected WT and HRG(+/-) animals exhibited a marked dilation of the stomach with extensive muscle thinning (Figure 3, b and c). No mucosal ulceration was present, however. The

WT + PBS control

WT + rHRG

HRG (+/-) + rHRG

ErbB2 (+/-) + rHRG

ErbB3 (+/-) + rHRG

Figure 3. Stomach. Transverse sections through the stomach of WT animals with and without rHRG $\beta 1$ treatment (**a** and **b**) as well as HRG (**c**), ErbB2 (**d**), and ErbB3 (**e**) heterozygous (+/-) animals exposed to rHRG $\beta 1$. Both the nonsurviving WT and HRG (+/-) animals exposed to rHRG $\beta 1$ for 5 to 6 days exhibited a thinning of the muscular layers presumably because of distention of the stomach (**b** and **c**). Those animals receiving rHRG $\beta 1$ that survived to 14 days showed a hyperplasia of the gastric mucosa associated with localized apoptosis (**d** and **e**). H&E; original magnification, $\times 20$.

chief cells, gastric zymogen-producing cells, and parietal cells, gastric acid producers in the stomach were examined in detail. A striking depletion of both lineages was noted in these rHRG $\beta 1$ -treated WT and HRG (+/-) mice, with an increase in the number of surface and undifferentiated epithelial cells moving down into the gastric crypts (Figure 3, **b** and **c**).³¹

In contrast, ErbB2 and ErbB3 (+/-) mice exhibited far less severe abdominal abnormalities in response to rHRG $\beta 1$ (Figure 3, **d** and **e**). There was histological evidence of only a mild gastric mucosal hyperplasia and parietal cell

and chief cell loss was not as severe as that observed in the nonsurviving WT or HRG (+/-) animals. Additionally, the digestive system in the ErbB2 (+/-), ErbB3 (+/-), and surviving WT mice seemed to be functional. Fecal material was present in the intestine, suggesting that factors other than just parietal and chief cell loss, such as gut motility, were also implicated in the severely affected WT and HRG (+/-) animals. rHRG $\beta 1$ treatment also induced a generalized mucosal hypertrophy and hyperplasia in the small and large intestines in all genotypes that was more extensive with prolonged exposure.

WT + PBS control

WT + rHRG

Figure 4. Heart. Representative sections of epicardial and muscle tissue from a WT control mouse (**a**) and a rHRG $\beta 1$ -treated WT mouse (**b**) illustrating the numerous enlarged peripheral nerve bundles (**arrow**) located just under the epicardial surface after the 14 days of rHRG $\beta 1$ treatment. Cardiac nerve bundles were present in those animals receiving rHRG $\beta 1$ regardless of genotype; none were noted in the control groups. H&E; original magnification, $\times 20$.

Heart

During development, HRG, acting through the ErbB2:ErbB4 heterodimer, is required for the morphogenesis of cardiac myocyte trabeculae as shown by gene-targeting experiments.^{21–25} In contrast, ErbB3 expression is limited to the cardiac cushions and ErbB3 null mice die in mid-gestation likely because of abnormal cardiac cushion formation.²³ Interestingly, recent studies have shown that whereas ErbB3 expression is dramatically down-regulated, HRG, ErbB2, and ErbB4 continue to be expressed in adult rat myocardium and play a role in cardiac myocyte proliferation, hypertrophic growth and survival *in vitro*.³² In this study, heart tissue was sporadically affected by rHRG $\beta 1$ treatment in all four genotypes with focal subendocardial areas of necrosis and with macrophage accumulation. These lesions were not present in any control animals. Interestingly, mice treated with rHRG $\beta 1$ also showed changes in the heart valves, characterized by an increase in the amount of extracellular matrix proteoglycans in the valve leaflets in sporadic animals of all four genotypes as compared to controls (data not shown). However, the most striking effect of rHRG $\beta 1$ treatment in the heart was the presence of numerous peripheral nerve bundles just under the epicardial mesothelium in all four genotypes (Figure 4). Because none was noted in the control groups, the growth and enlargement of these nerve bundles seem to be a direct response to the action of rHRG $\beta 1$. Further analysis is ongoing to determine the origin of these nerve bundles and associated ErbB expression, because peripheral nerves are known to produce HRG and Schwann cells of developing peripheral nerves are a target for HRG.^{33–37}

Pancreas

The pancreas appeared primarily normal in the treated nonsurviving WT and HRG(+/-) mice at necropsy, with limited ductal ectasia and minimal hyperplasia, probably reflecting the short exposure of the animals to rHRG $\beta 1$ (5 to 6 days) (Figure 5, b and c). In contrast, in the ErbB2 and ErbB3(+/-) animals exposed for the full 14 days, there was pronounced ductal hyperplasia and proliferation in the main pancreatic ducts at necropsy with inflammatory cells present in the lumen of the ducts (Figure 5, d and e). Acinar cell injury was not widespread, however, although amylase levels were elevated in many of the animals.

Other Tissues

Epithelial proliferation was noted in the genitourinary system (primarily the urinary bladder), the liver bile ducts, and the salivary glands in all groups receiving rHRG $\beta 1$ treatment. Some animals also had a slight degree of hyperplasia of the epithelium lining the reproductive tract. WT, HRG(+/-), and ErbB3(+/-) mice, treated with rHRG $\beta 1$, exhibited extensive thymic atrophy and slight splenic atrophy with lymphoid depletion; rHRG $\beta 1$ -treated ErbB2(+/-) mice were the least affected with most thymus tissue appearing normal. No effect of rHRG $\beta 1$ treatment was seen in the brain, adrenal glands, skin, lungs, or kidneys in this study and no necrosis or inflammation was noted in sciatic nerve of any animals. No significant organ weight changes in the brain, heart, kidney, or liver were noted in the rHRG $\beta 1$ versus control animals.

Figure 5. Pancreas. Representative sections through pancreas taken from an untreated WT animal (a), and a WT (b), HRG(+/-) (c), ErbB2(+/-) (d), and ErbB3(+/-) animal (e) that received rHRG β 1 treatment. Sections are from animals that received rHRG β 1 treatment for 14 days except in the case of the HRG(+/-) (c), that was only dosed for 5 to 6 days. Note the presence of ductal hyperplasia (arrows) most evident in the WT and ErbB2(+/-) and ErbB3(+/-) groups, probably reflective of their longer exposure to rHRG β 1. H&E; original magnification, $\times 20$.

Overall Effects of rHRG β 1 Treatment Associated with Pregnancy (Morbidity and Mortality)

Based on the sensitivity of the female breast to rHRG β 1, we decided to examine whether hormonal influences could play a role in the differential sensitivity of the breast tissue to rHRG β 1. Two groups of WT female mice, non-pregnant and pregnant, were treated with 1 mg/kg/day rHRG β 1 by ALZA pump for 14 days, beginning on day 7.5 of pregnancy. A slightly higher dose of rHRG β 1 was used in this experiment to better discern differences be-

tween these two WT groups. Surprisingly, mortality was significantly higher in the nonpregnant mice (14 of 14) than the pregnant mice (2 of 8) ($P < 0.001$) (Figure 6A). Some pregnant mice delivered viable pups that, on histological examination, showed no anomalies, indicating pregnancy somehow lessened the severity of effects with rHRG β 1 treatment. Serum was again collected at necropsy and analyzed by KIRA to determine rHRG β 1 levels.²⁸ Although all control mice had undetectable levels of rHRG β 1, serum rHRG β 1 was significantly lower in pregnant mice than in nonpregnant mice with a P value of 0.011 (Figure 6B). Of the two pregnant mice that died

Figure 6. Cumulative survival plot analysis; rHRG β 1 serum levels from WT pregnant and nonpregnant females. **A:** Female WT animals, pregnant and nonpregnant, were treated with 1 mg/kg/day rHRG β 1 beginning on day 7.5 of pregnancy. Mortality was significantly higher in the nonpregnant group ($P < 0.001$); 13 of the 14 animals (mortality = 93%) were dead by days 6 to 7; the remaining animal survived until day 9. In the pregnant group receiving rHRG β 1, six of the eight animals survived the length of the study (mortality = 25%). **B:** Scatter plot of the log-serum concentration of rHRG β 1 of WT pregnant and nonpregnant mice treated with 1 mg/kg/day rHRG β 1. rHRG β 1 concentrations were log-transformed (log₁₀ pmol/L) to reduce variance heterogeneity. The mean for each group is shown as a horizontal line within each plot. By analysis of variance, rHRG β 1 levels in the pregnant females were significantly lower than in the nonpregnant females ($P = 0.011$). All control mice had less than detectable levels of rHRG β 1.

early, one had the highest level of serum rHRG β 1 in this study, suggesting that the generally lower levels of rHRG β 1 in the pregnant mice correlated with their enhanced survival.

Discussion

These ErbB2, ErbB3, or HRG heterozygous mouse lines provide a unique experimental system to assess the activity of exogenous rHRG β 1 treatment. In the ErbB2(+/-) mice, the reduction in ErbB2 should negatively compromise the formation of both rHRG β 1-signaling heterodimers (ErbB2:ErbB3 or ErbB2:ErbB4), with the ex-

pectation of an associated compromise in rHRG β 1 activity. Similarly, the decrease in ErbB3 level should result in both a reduction in the formation of ErbB2:ErbB3 heterodimers, as well as a reduced potential for rHRG β 1 binding to occur. Our results indeed suggest that lower levels of signaling receptors in these animals heterozygous for the ErbB2 or ErbB3 receptor does correlate with reduced rHRG β 1 activity as reflected in the lower mortality observed on rHRG β 1 challenge.

Surprisingly, the HRG(+/-) mice, already possessing subnormal levels of endogenous HRG, showed an enhanced sensitivity to the exogenous administration of rHRG β 1, as evidenced by mortality data. A possible explanation is that ErbB receptors in these mice could become hypersensitized to HRG as a compensatory mechanism. Interestingly, HRG(+/-) mice demonstrate hyperactivity in multiple behavioral tasks whereas ErbB2 and ErbB3(+/-) mice do not, suggesting that, in the central nervous system, diminished levels of HRG could not be adequately compensated for.²⁶ Studies to further characterize these heterozygous animals are ongoing.

In this study, female mice were found to be uniquely responsive to rHRG β 1 treatment, with a significant relationship between circulating levels of rHRG β 1 and genotype; rHRG β 1 levels in the female ErbB2(+/-) group were significantly lower than in the other genotypes. In addition, the mammary gland exhibited an extensive hyperproliferation in response to rHRG β 1 regardless of genotype, but again only in female mice. This dramatic effect in the mammary gland correlates with previous reports where HRG was targeted to the breast via the murine mammary tumor virus promoter promoter, resulting in terminal end-bud structures persisting in the gland of the virgin female transgenics; when bred, these mice later developed mammary adenocarcinomas.³⁸ Additionally, when HRG was introduced in the breast of mice via Elvax pellets, induction of epithelial ductal branching occurred with the overall response increased by exogenous estradiol and progesterone.³⁹

We also determined that rHRG β 1 effects in WT female mice were likely influenced by pregnancy, as circulating levels of rHRG β 1 were lower than in nonpregnant females, correlating with their reduced mortality. During pregnancy, this reduction in circulating rHRG β 1 may be because of expansion of its distribution volume,⁴⁰ an increased clearance rate of rHRG β 1, or the hormonal status of the animal *per se*.⁴¹ Both estrogen and the estrogen receptor have been implicated in the regulation of ErbB2 signaling in numerous studies; ErbB2 overexpression is associated clinically with estrogen receptor-negative status and thus resistance to hormonal treatment with antiestrogens such as tamoxifen.^{42,43} Studies of the ErbB2 promoter have identified a 409-bp site in intron-1 that confers estrogen suppression on the ErbB2 promoter in transfection assays⁴⁴ and it has also been recently reported that transcription of ErbB2 is negatively regulated by estrogen in breast cancer cells *in vitro*.⁴⁵ Accordingly, the elevated estrogen levels in the pregnant females could potentially contribute to the lessened effects noted with rHRG β 1 treatment. Response of ovariectomized females would add significantly to our under-

standing of the mechanism, as would studies of the pharmacokinetics of rHRG β 1 clearance.

It has recently been reported that transcripts of all four ErbB receptor family members are expressed in the murine breast during early, mid, and late pregnancy, with HRG-1 transcripts paralleling those of ErbB3 and ErbB4.²⁹ Although endogenous phosphorylation of all four receptors was detectable at late pregnancy and during lactation, ErbB3 and ErbB4 were specifically unresponsive to ectopic EGF or HRG during late pregnancy, suggesting a correlation with the lessened effects noted with rHRG β 1 treatment during pregnancy in our study.²⁹

A profound effect of rHRG β 1 treatment on gastric tissue was noted in this study, particularly in the non-surviving WT and HRG(+/-) mice, that exhibited enlarged abdomens filled with undigested food. And, although hyperplasia of the stomach/intestinal epithelium and destruction of the parietal cells was observed in all four genotypes after rHRG β 1 treatment, the effects were considerably less severe in surviving WT mice and ErbB2(+/-) and ErbB3(+/-) animals. In accordance with our observations, ErbB2 expression is reported in the epithelium of the intestine and colon³⁰ and low level expression of ErbB2 is also present in both the stomach epithelium⁴⁶ and in the parietal cells in adulthood. Similarly, ErbB3 is localized in the glandular epithelial cells throughout the gastrointestinal tract³⁰ as well as in the parietal cells.⁴⁷ EGFR and its associated ligands, including EGF and transforming growth factor (TGF)- α , are also expressed throughout the gastrointestinal epithelium.³⁰ In addition, HRG expression has been reported in the mesenchyme of the embryonic stomach^{33,48} and HRG α was found by reverse transcriptase-polymerase chain reaction in human gastric fibroblasts and by immunohistochemistry in fibroblasts in the lamina propria in human gastric tissue.³⁰ When HRG α signaling was examined in MKN-28 gastric cancer cells, HRG α was found to induce heterodimerization of ErbB2 and ErbB3 with EGFR, as well as of ErbB2 with ErbB3.³⁰ Therefore, a decrease in either ErbB2 or ErbB3 could lead to less efficient rHRG β 1 signaling in the parietal cells, resulting in the less severe parietal cell pathology in the ErbB2 and ErbB3(+/-) mice compared to WT and HRG(+/-) mice.

Similarly, overexpression of TGF- α caused aberrant gastric mucosal growth as well as parietal cell and chief cell depletion without loss of precursor cells or significant cell death.⁴⁹ When the effects of rHRG α , TGF- α , and EGF on tyrosine phosphorylation were compared in MKN-28 gastric cancer cells, TGF- α and EGF both stimulated tyrosine phosphorylation of EGFR and ErbB2, whereas HRG α stimulated EGFR, ErbB2, and ErbB3 with the expected association of PI-3 kinase with ErbB3.³⁰ These data suggest that, in the parietal cell phenotype, ErbB2 signaling can be associated with both TGF- α and rHRG β 1 overexpression.

Gene targeting experiments first revealed that HRG, signaling through ErbB2 and ErbB4, was required for the formation of ventricular trabeculae during cardiac development.²¹⁻²⁵ The continued adult expression of HRG, ErbB2, and ErbB4 in adult rat myocardium may play a role in the response of the myocardium to stress.³² Nu-

merous large peripheral nerve bundles that appeared under the epicardial mesothelium were found in most mice treated with rHRG β 1 in all four genotypes. Although HRG is a known mitogen and survival factor for Schwann cells,^{34,35,50} it is intriguing that no similar histological changes were noted in sciatic nerve in these mice.

This investigation describes results of a careful examination of the *in vivo* activity of rHRG β 1 treatment in mice heterozygous for HRG, ErbB2, or ErbB3 allowing the discrimination of differences in the activity of rHRG β 1 based on endogenous receptor and HRG levels. rHRG β 1 treatment effects were particularly interesting in female animals. Because of the reported cross-talk between the estrogen receptor and the ErbB2 receptor pathways, we also examined the interactions of pregnancy and rHRG β 1 activity in WT female mice and found that pregnancy played a protective role in our studies. Also, animals treated with rHRG β 1, regardless of genotype, exhibited hyperplasia in many epithelial target tissues where ErbB2 overexpression has been implicated in adulthood cancers such as breast, pancreatic, and gastric cancer. Our findings indicate that HRG might also prove to play a role in the development of such cancers and suggest a possible future therapeutic potential for HRG antagonists.

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References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987, 235:177-182
2. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989, 244:707-712
3. Weiner DB, Liu J, Cohen JA, Williams WV, Greene MI: A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. *Nature* 1989, 339:230-231
4. Pinkas-Kramarski R, Alroy I, Yarden Y: ErbB receptors and EGF-like ligands: cell lineage determination and oncogenesis through combinatorial signaling. *J Mammary Gland Biol Neoplasia* 1997, 2:97-107
5. Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, Shepard HM, Kuang W, Wood WI, Goeddel DV, Vanden RL: Identification of heregulin, a specific activator of p185ErbB2. *Science* 1992, 256:1205-1210
6. Sliwkowski MX, Schaefer G, Akita RW, Lofgren JA, Fitzpatrick VD, Nuijens A, Fendly BM, Cerione RA, Carraway III KL: Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J Biol Chem* 1994, 269:14661-14665
7. Plowman GD, Culouscou JM, Whitney GS, Green JM, Carlton GW,

- Foy L, Neubauer MG, Shoyab M: Ligand-specific activation of HER4/p180ErbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci USA* 1993, 90:1746–1750
8. Plowman GD, Whitney GS, Neubauer MG, Green JM, McDonald VL, Todaro GJ, Shoyab M: Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc Natl Acad Sci USA* 1990, 87:4905–4909
9. Plowman GD, Green JM, Culouscou JM, Carlton GW, Rothwell VM, Buckley S: Heregulin induces tyrosine phosphorylation of HER4/p180ErbB4. *Nature* 1993, 366:473–475
10. Culouscou JG, Plowman G, Carlton GW, Green J, Shoyab M: Characterization of a breast cancer cell differentiation factor that specifically activates the HER4/p180ErbB4 receptor. *J Biol Chem* 1993, 268:18407–18410
11. Kraus MH, Issing W, Miki T, Popescu NC, Aaronson S: Isolation and characterization of ErbB3, a third member of the ErbB/epidermal growth factor family: evidence of overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci USA* 1989, 86:9193–9197
12. Guy P, Platko J, Cantley L, Cerione R, Carraway K: Insect cell-expressed p180 ErbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci USA* 1994, 91:8132–8136
13. Pinkas-Kramarski RL, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, Lavi S, Seger R, Ratzkin BJ, Sela M, Yarden Y: Diversification of neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J* 1996, 15:2452–2467
14. Fedi P, Pierce JH, Di Fiore PP, Krause MH: Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase C or GTPase-activation protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. *Mol Cell Biol* 1994, 14:492–500
15. Soltoff SP, Carraway III KL, Prigent A, Gullick WG, Cantley LC: ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. *Mol Cell Biol* 1994, 14:3550–3558
16. Kim H, Sierke SL, Koland JG: Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the ErbB3 gene product. *J Biol Chem* 1994, 269:24747–24755
17. Ram TG, Hosick HL, Ethier SP: Heregulin- β is especially potent in activating phosphatidylinositol 3-kinase in nontransformed human mammary epithelial cells. *J Cell Physiol* 2000, 183:301–313
18. Carraway K, Sliwkowski M, Akita R, Platko J, Guy P, Nuijens A, Diamonti J, Vanden R, Cantley L, Cerione R: The ErbB3 gene product is a receptor for heregulin. *J Biol Chem* 1994, 269:14303–14306
19. Carraway KLR, Cantley LC: A neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signaling. *Cell* 1994, 78:5–8
20. Tzahar E, Levkowitz G, Karunagaran D, Yi L, Peles E, Lavi S, Chang D, Liu N, Yayon A: ErbB3 and erbB4 function as the respective low and high affinity receptors of all neu differentiation factor/hergulin isoforms. *J Biol Chem* 1994, 269:25226–25233
21. Meyer D, Birchmeier C: Multiple essential functions of neuregulin in development. *Nature* 1995, 378:386–390
22. Gassmann M, Casagrande F, Orioli D, Simon H, Lai C, Klein R, Lemke G: Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* 1995, 378:390–394
23. Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frantz G, Bauer M, Lu LH, Moore MW: ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2 and heregulin deficient mice. *Development* 1997, 124:4999–5011
24. Lee K, Simon H, Chen H, Bates B, Hung M, Hauser C: Requirement for neuregulin receptor ErbB2 in neural and cardiac development. *Nature* 1995, 387:394–398
25. Lemke G: Neuregulins in development. *Mol Cell Neurol* 1996, 7:247–262
26. Gerlai R, Pisacane P, Erickson S: Heregulin, but not ErbB2 or ErbB3, heterozygous mutant mice exhibit hyperactivity in multiple behavioral tasks. *Behav Brain Res* 2000, 109:219–227
27. Corfas G, Rosen KM, Aratake H, Krauss R, Fischbach GD: Differential expression of ARIA isoforms in the rat brain. *Neuron* 1995, 14:103–115
28. Sadick MD, Sliwkowski MX, Nuijens A, Bald L, Chiang N, Lofgren JA, Wong WL: Analysis of heregulin-induced ErbB2 phosphorylation with a high-throughput kinase receptor activation enzyme-linked immunosorbent assay. *Anal Biochem* 1996, 235:207–214
29. Schroeder JA, Lee DC: Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. *Cell Growth Differ* 1998, 9:451–464
30. Noguchi H, Sakamoto C, Wada K, Akamatsu T, Uchida T, Tatsuguchi A, Matsui H, Fukui H, Fujimori T, Kasuga M: Expression of heregulin α , erbB2 and erbB3 and their influences on proliferation of gastric epithelial cells. *Gastroenterology* 1999, 117:1119–1127
31. Goldenring JR, Ray GS, Soroka CJ, Smith J, Modlin IM, Meise KS, Coffey Jr RJ: Overexpression of transforming growth factor- α alters differentiation of gastric cell lineages. *Dig Dis Sci* 1996, 41:773–784
32. Zhao Y, Sawyer DR, Baliga RR, Opel DJ, Han X, Marchionni MA, Kelly RA: Neuregulins promote survival and growth of cardiac myocytes. *J Biol Chem* 1998, 273:10261–10269
33. Meyer D, Birchmeier C: Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. *Proc Natl Acad Sci USA* 1994, 91:1064–1068
34. Dong Z, Brennan A, Liu N, Yarden Y, Lefkowitz G, Mirsky R, Jessen KR: Neu differentiation factor is a neuron-glial signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* 1995, 15:585–596
35. Syroid DE, Maycox PR, Burrola PG, Liu N, Wen D, Lee K, Lemke G, Kilpatrick TJ: Cell death in the Schwann cell lineage and its regulation by neuregulin. *Proc Natl Acad Sci USA* 1996, 93:9229–9234
36. Garratt A, Voiculescu O, Topilko P, Charnay P, Birchmeier C: A dual role of erbB2 in myelination and in expansion of the Schwann cell precursor pool. *J Cell Biol* 2000, 148:1035–1046
37. Maurel P, Salzer JL: Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI 3-kinase activity. *J Neuroscience* 2000, 20:4635–4645
38. Krane IM, Leder P: NDF/hergulin induces persistence of terminal end buds and adenocarcinomas in the mammary glands of transgenic mice. *Oncogene* 1996, 12:1781–1788
39. Jones FE, Jerry DJ, Guarino BC, Andrews GC, Stern DF: Heregulin induces in vivo proliferation and differentiation of mammary epithelium into secretory lobuloalveoli. *Cell Growth Differ* 1996, 7:1031–1038
40. Reynolds F, Knott C: Pharmacokinetics in pregnancy and placental drug transfer. *Oxf Rev Reprod Biol* 1989, 11:389–449
41. Pepe GJ, Albrecht ED: Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr Rev* 1995, 16:608–648
42. LeRoy X, Escot C, Brouillet JP, Theillet C, Maudelonde T, Simony-Lafontaine J, Pujol H, Rochefort H: Decrease of c-ErbB2 and c-myc RNA levels in tamoxifen-treated breast cancer. *Oncogene* 1991, 6:431–437
43. Leitzel K, Teramoto Y, Konrad K, Chinchilli VM, Volas G, Grossberg H, Harvey H, Demers L, Lipton A: Elevated serum c-ErbB-2 antigen levels and decreased response to hormone therapy of breast cancer. *J Clin Oncol* 1995, 13:1129–1135
44. Bates NP, Hurst H: An intron 1 enhancer element mediates oestrogen-induced suppression of ErbB2 expression. *Oncogene* 1997, 15:473–481
45. Perissi V, Menini N, Cottone E, Capello D, Sacco M, Montaldo F, DeBortoli M: AP-2 transcription factors in the regulation of ERBB2 gene transcription by oestrogen. *Oncogene* 2000, 19:280–288
46. Kokai Y, Cohen JA, Drebin JA, Greene MI: Stage- and tissue-specific expression of the neu oncogene in rat development. *Proc Natl Acad Sci USA* 1987, 84:8498–8501
47. Prigent SA, Lemoine NR, Hughes CM, Plowman GD, Selden C, Gullick WJ: Expression of the c-erbB3 protein in normal human adult and fetal tissues. *Oncogene* 1992, 7:1273–1278
48. Orr-Urtreger A, Trakhtenbrot L, Ben-Levy R, Wen D, Rechavi G, Lironi P, Yarden Y: Neural expression and chromosomal mapping of neu differentiation factor to 8p12–p21. *Proc Natl Acad Sci USA* 1993, 90:1867–1871
49. Sharp R, Babyatsky MW, Takagi H, Tagerud S, Wang TC, Bockman DE, Brand SJ, Merlino G: Transforming growth factor α disrupts the normal program of cellular differentiation in the gastric mucosa of transgenic mice. *Development* 1995, 121:149–161
50. Shah NM, Marchionni MA, Isaacs I, Stroobant P, Anderson DJ: Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* 1994, 77:349–360