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Placental and Embryonic Growth Restriction in Mice With Reduced Function Epidermal Growth Factor Receptor Alleles

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ABSTRACT

Embryos lacking an epidermal growth factor receptor (EGFR) exhibit strain-specific defects in placental development that can result in mid-gestational embryonic lethality. To determine the level of EGFR signaling required for normal placental development, we characterized congenic strains homozygous for the hypomorphic *Egfr*^{wa2} allele or heterozygous for the antimorphic *Egfr*^{Wa5} allele. *Egfr*^{wa2} homozygous embryos and placentas exhibit strain-dependent growth restriction at 15.5 days post-coitus while *Egfr*^{Wa5} heterozygous placentas are only slightly reduced in size with no effect on embryonic growth. *Egfr*^{wa2} homozygous placentas have a reduced spongiotrophoblast layer in some strains, while spongiotrophoblasts and glycogen cells are almost completely absent in others. Our results demonstrate that more EGFR signaling occurs in *Egfr*^{Wa5} heterozygotes than in *Egfr*^{wa2} homozygotes and suggest that *Egfr*^{wa2} homozygous embryos model EGFR-mediated intrauterine growth restriction in humans. We also consistently observed differences between strains in wild-type placenta and embryo size as well as in the cellular composition and expression of trophoblast cell subtype markers and propose that differential expression in the placenta of *Glut3*, a glucose transporter essential for normal embryonic growth, may contribute to strain-dependent differences in intrauterine growth restriction caused by reduced EGFR activity.

EPIDERMAL growth factor receptor (EGFR) is the prototypical member of the ERBB family of receptor tyrosine kinases and is known to regulate many aspects of cellular biology including cell proliferation, survival, differentiation, and migration (reviewed in Yarden and Sliwkowski 2001). Eleven known ligands bind the extracellular region of ERBB-family receptors, and activation of the tyrosine kinase domain occurs following receptor homo- or heterodimerization. The resulting biological responses are dependent upon specific signaling cascades initiated by ERBBs and can be influenced by the particular ligand–ERBB combination (Yarden and Sliwkowski 2001). Studies using cultured cells have underscored the importance of EGFR in modulating various cellular processes, while animal models have been able to demonstrate that EGFR is required for numerous developmental and physiological processes (Casalini *et al.* 2004). *In vivo* studies have shown that EGFR is particularly important for normal placental development in mice; placentas from *Egfr* nullizygous (*Egfr*$^{tm1Mag/tm1Mag}$) embryos exhibit strain-specific defects that result in differential embryonic lethality (Sibilia and Wagner 1995; Threadgill *et al.* 1995). Two additional *Egfr* alleles result in reduced EGFR signaling in mice: the recessive hypomorphic *Egfr*^{wa2} and dominant antimorphic *Egfr*^{Wa5} alleles (Luetteke *et al.* 1994; Fowler *et al.* 1995; Du *et al.* 2004; Lee *et al.* 2004). These alleles can provide insight into the level of EGFR signaling required for normal placental development.

Egfr^{wa2} is a classical spontaneous mutation that arose in 1935 that causes a distinct wavy coat phenotype in the homozygote (Figure 1; Keeler 1935). This recessive mutation was subsequently found to be a single nucleotide transversion resulting in a valine → glycine substitution in the highly conserved kinase domain of EGFR (Luetteke *et al.* 1994; Fowler *et al.* 1995). Since mice homozygous for the *Egfr*^{tm1Mag} null allele die before or shortly after birth depending on genetic background, the hypomorphic *Egfr*^{wa2} allele has been the primary model used to study the effect of attenuated EGFR signaling in a variety of adult physiological and disease states. In addition to eye and hair phenotypes, the adult *Egfr*^{wa2} homozygous mouse exhibits delayed onset of puberty, abnormal ovulation, enlarged aortic valves and cardiac hypertrophy, decreased body size, defects in mammary gland development and lactation, increased susceptibility to colitis, and impaired intestinal adaptation following small bowel resection (Fowler *et al.* 1995; Helmrath *et al.* 1997; Chen *et al.* 2000; Egger *et al.* 2000; O'Brien *et al.* 2002; Prevot *et al.* 2005; Hsieh *et al.*

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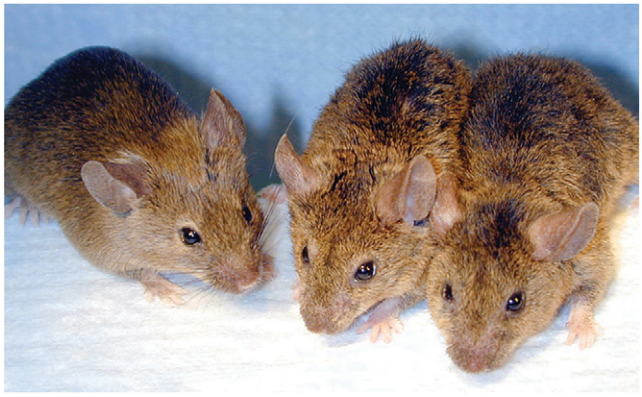

Figure 1.—Congenic 129 *Egfr* allelic series. Wild-type (left), *Egfr*^{wa2} homozygote (middle), and *Egfr*^{wa5} heterozygote (right) mice. As weanlings and adults, the *Egfr*^{wa2} homozygotes and *Egfr*^{wa5} heterozygotes are grossly indistinguishable.

2007). Despite the widespread use of the *Egfr*^{wa2} allele, there are limitations in using *Egfr*^{wa2} homozygous mice to clearly define the physiological roles of EGFR. *Egfr*^{wa2} has traditionally been maintained in *cis*, tightly linked with a hypomorphic *Wnt3a* allele, *Wnt3a*^{vt} (*vestigal tail*), making phenotypic analysis of reduced EGFR signaling by itself difficult. Furthermore, *Egfr*^{wa2} has also typically been maintained on a mixed genetic background and since the *Egfr* nullizygous phenotype is similarly influenced by genetic modifiers, a mixed background could mask phenotypes that become evident when *Egfr*^{wa2} mice are inbred.

The *Egfr*^{Wa5} allele arose in a large, genomewide *N*-ethyl-*N*-nitrosourea mutagenesis screen for dominant visible mutations in the mouse. *Egfr*^{Wa5} heterozygous mice were first identified by their open eyelids at birth and by development of a wavy coat, similar to the phenotype of *Egfr*^{wa2} homozygous mice (Figure 1). *Egfr*^{Wa5} failed to complement the *Egfr*^{tm1Mag} null allele and was shown to function as an antimorph since *Egfr*^{Wa5}, but not *Egfr*^{tm1Mag}, heterozygotes exhibit eyelid and coat phenotypes (Lee *et al.* 2004). A single nucleotide missense mutation was found in the *Egfr*^{Wa5} allele that results in an Asp → Gly substitution in the highly conserved DFG domain of the EGFR kinase catalytic loop (Du *et al.* 2004; Lee *et al.* 2004). Although *Egfr*^{Wa5} heterozygotes are viable, *Egfr*^{Wa5} homozygotes die prenatally and exhibit placental defects identical to those from *Egfr*^{tm1Mag} homozygous null embryos. Placentas from *Egfr*^{Wa5} heterozygotes on a mixed background show variable reduction in the spongiotrophoblast layer and minor abnormalities in the labyrinth region, but no effects on embryo survival have been reported.

In vitro studies with *Egfr*^{Wa5} suggest that it encodes a kinase-dead EGFR since no phosphorylation of EGFR^{Wa5} is detected following stimulation with ligands. In agreement with the genetic data showing that *Egfr*^{Wa5} is an antimorph, *in vitro* studies have demonstrated that the EGFR^{Wa5} receptor can inhibit phosphorylation of EGFR and MAPK in a dose-dependent manner (Lee *et al.* 2004). In Chinese hamster ovary cells expressing an equimolar ratio of EGFR and EGFR^{Wa5} receptors, <10% of wild-type phosphorylation levels were observed by Western blot analysis.

The *Egfr* allelic series available in the mouse has high utility for studying gene function since EGFR is involved in a multitude of developmental processes and human diseases. Although both *Egfr*^{wa2} and *Egfr*^{Wa5} alleles result in reduced EGFR signaling, the activity and phenotypic consequences of *Egfr*^{wa2} homozygosity has not been compared to that of *Egfr*^{Wa5} heterozygosity when both are on the same genetic backgrounds. Adult *Egfr*^{Wa5} heterozygous mice appear highly similar to *Egfr*^{wa2} homozygotes, but crosses with the *Apc*^{Min} intestinal tumor model have shown that a more substantial reduction in tumor number occurs when the *Apc*^{Min} mutation is bred onto the *Egfr*^{wa2} homozygous *vs.* *Egfr*^{Wa5} heterozygous background (Roberts *et al.* 2002; Lee *et al.* 2004). These results suggest that *Egfr*^{Wa5} heterozygous mice retain higher levels of EGFR activity than *Egfr*^{wa2} homozygous mice; however, the data are confounded by the fact that the crosses were performed using different mixed genetic backgrounds.

This study reports a comprehensive genetic analysis of reduced EGFR signaling in *Egfr*^{wa2} homozygotes and *Egfr*^{Wa5} heterozygotes in placental development and embryonic growth for three congenic backgrounds, C57BL/6J (B6), 129S1/SvImJ (129), and BTBR/J-*T*+, *tf/tf* (BTBR). Wild-type placenta weight, embryo weight, and mRNA levels of genes selected for their trophoblast-specific expression were found to be highly strain dependent. *Egfr*^{wa2} homozygous placentas are reduced in size in all three strains, and a proportion of 129-*Egfr*^{wa2} homozygotes die before 15.5 days post-coitus (dpc). *Egfr*^{wa2} homozygous embryos also display background-dependent intrauterine growth restriction (IUGR) in late gestation, which is most severe on 129 and BTBR backgrounds and models EGFR-associated IUGR in humans. *Egfr*^{Wa5} heterozygous placentas exhibit a minor reduction in size on all three backgrounds with no impact on embryonic growth. These results suggest that reduced levels of EGFR signaling can interfere with normal placental development and that embryo development is affected only after placental size is sufficiently reduced. In addition, our data show that the level of EGFR signaling in *Egfr*^{Wa5} heterozygous mice is higher than in *Egfr*^{wa2} homozygotes and suggests that different *Egfr* allele combinations can be generated to "genetically titer" total EGFR activity *in vivo*.

MATERIALS AND METHODS

Mice and genetic crosses: Congenic *Egfr*^{wa2} lines were generated by backcrossing mixed C57BL/6JEiC3H-a/A-*Egfr*$^{wa2/wa2}$ *Wnt3a*$^{vt/vt}$ mice obtained from The Jackson Laboratory to B6, 129, and BTBR wild-type inbred strains for ≥10

generations. Removal of the linked *Wnt3a*^{wt} allele, 20 cM distal to *Egfr* on chromosome 11, was verified by PCR-based genotyping. Congenic *Egfr*^{wa2} heterozygous mice were then intercrossed to produce litters from each background containing wild-type and *Egfr*^{wa2} heterozygous and homozygous congenic embryos and pups.

Congenic *Egfr*^{Wa5} mice were generated by backcrossing heterozygous *Egfr*^{Wa5} mice from a mixed genetic background to inbred B6, 129, and BTBR strains for ≥10 generations. Congenic heterozygous mice were then crossed to male or female wild-type animals of the same strain to produce litters containing wild-type and *Egfr*^{wa5} heterozygous congenic embryos and pups.

Mice were fed Purina Mills Lab Diet 5058 and water *ad libitum* under specific pathogen-free conditions in an American Association for the Accreditation of Lab Animal Care approved facility. All experiments were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Genotyping: DNA was extracted from adult ear punches or embryo tail biopsies for genotyping by incubating at 95° in 100 μl of 25 mm NaOH/0.2 mm EDTA for 20 min before neutralizing with 100 μl 40 mm Tris–HCl, pH 5.0. For the subsequent genotyping reactions, 1 μl of lysed tissue sample was used per reaction.

The *Egfr*^{wa2} allele was amplified by PCR with the primers Wa2F (5′-TACCCAGAAAGGGATATGCG-3′) and Wa2R (5′-GGAGCCAATGTTGTCCTTGT-3′) (Qiagen). PCR conditions were 30 cycles at 94° for 30 sec, 60° for 60 sec, and 72° for 60 sec. PCR products were digested for 3 hr at 37° with *Fok* I and restriction enzyme buffer 2 (NEB) and run on a 3% agarose gel to separate a 230-bp product corresponding to wild-type *Egfr* and a 130- and 100-bp set of products corresponding to the digested *Egfr*^{wa2} allele.

The *Egfr*^{Wa5} allele was detected by real-time PCR with the primers WA5F (5′-GTGAAGACACCACAGCATGTC-3′) and WA5R (5′-CTCTTCAGCACCAAGCAGTTTG-3′) along with the 5′ VIC-labeled probe WA5V1 (5′-AAGATCACAGATTTTGG-3′) to detect wild-type *Egfr* and the 5′ FAM-labeled probe WA5M1 (5′-AGATCACAGGTTTTGG-3′) to detect *Egfr*^{Wa5} (ABI). Genotyping was performed on an MXP-3000 real-time PCR instrument (Stratagene) with 2X Taqman Universal PCR Master Mix (Applied Biosystems). PCR conditions were 95° for 10 min followed by 40 cycles of 92° for 15 sec and 60° for 1 min. Amplification of the wild-type allele was detected by comparative quantification of VIC-labeled PCR products, and amplification of the *Egfr*^{Wa5} allele was detected by comparative quantification of FAM-labeled PCR products with positive and negative *Egfr*^{Wa5} adult tissue used as a reference sample.

Collection of placenta samples: Noon on the day that copulation plugs were observed was designated as 0.5 dpc. Pregnant females were euthanized by exposure to a lethal dose of isoflourane, and embryos with their corresponding placentas were dissected from the uterine horns on the morning of 15.5 or 18.5 dpc into phosphate buffered saline (PBS). The placenta and extra-embryonic tissues were separated from the embryo by mechanical dissection, and a tail biopsy was collected for DNA extraction to determine the genotype of each embryo. Wet weights of embryos and placentas were recorded at the time of dissection. Placentas were preserved in RNAlater (Ambion) for extraction of RNA or fixed in 10% neutral buffered formalin (NBF) for histological analysis.

Histology: After fixing placentas in 10% NBF overnight, tissues were washed in PBS, dehydrated in ethanols and xylenes, and embedded in paraffin. Seven-micrometer sections were cut using a Leica RM2165 microtome. Sections were deparaffinized, rehydrated in a graded series of ethanols, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Stained sections were dehydrated in a series of ethanols and mounted using Permount. Representative histological images were photographed on a Nikon FXA microscope at a magnification of ×1.25, ×10, or ×12 using a CCD digital camera.

Real-time PCR: Placentas were homogenized in 1.2 ml Trizol using a bead mill (Eppendorf), and RNA was isolated according to the manufacturer's protocol (Invitrogen). For each sample, 15 μg of RNA was DNAse treated, followed by a phenol–chloroform extraction. RNA was quantified (Nanodrop), and 1 μg of each sample was reverse transcribed using the cDNA archive kit (Applied Biosystems). The amount of cDNA corresponding to 20 ng of RNA was used for each 20 μl real-time PCR reaction on an MXP-3000 instrument (Stratagene). Assays-on-demand primer and probe sets for *Gusb*, *Eomes*, *Esrrb*, *Esx1*, *Dlx3*, *Gm52*, *Tcfeb*, *Ctsq*, *Timp2*, *Glut3*, *Cx31*, and *Pdch12* were run according to the manufacturer's protocol with 2X Taqman Universal Mastermix (ABI). Probes for *4311*, *Gcm1*, and *Pl1* were designed and synthesized in-house. *Gusb* was used as an endogenous control, and fold change of each gene was calculated using the ΔΔCt method (Livak and Schmittgen 2001). The average ΔCt of wild-type animals for each strain/allele combination was used as the control value to calculate ΔΔCt values for samples of the same strain and allele. Fold-change values were computed from the ΔΔCt for each sample and converted to a percentage increase over the wild-type average fold change for *Egfr*^{Wa5} heterozygous and *Egfr*^{wa2} heterozygous and homozygous samples.

For cluster analysis, ΔCt values for each sample and probe were uploaded into Cluster and median centered (Eisen *et al.* 1998). Data were visualized using TreeView (Saldanha 2004).

Statistical analysis: All placenta and embryo weights were analyzed using the Mann–Whitney test. A genotype by strain interaction test was performed using a standard least-squares regression analysis in JMP (SAS) for placenta and embryo weights. A χ^2 goodness-of-fit test was performed to determine if genotype distributions deviated from expected Mendelian ratios. Real-time PCR fold change values were analyzed using the Student's *t*-test.

RESULTS

***Egfr*^{wa2} homozygous placentas are reduced in size on all genetic backgrounds:** To determine the effect of the *Egfr*^{wa2} allele on placental development, placentas were collected at 15.5 and 18.5 dpc from each of the three congenic strains by intercrossing respective *Egfr*^{wa2} heterozygous mice. At 15.5 dpc, the placental weight of *Egfr*^{wa2} homozygotes was reduced 24% in B6 ($P < 0.001$), 19% in 129 ($P < 0.001$), and 39% in BTBR ($P < 0.01$) *vs.* wild-type and heterozygous littermates (Figure 2A). At 18.5 dpc, the placental weight of *Egfr*^{wa2} homozygotes was reduced 24% in B6 ($P < 0.05$), 37% in 129 ($P < 0.01$), and 28% in BTBR ($P < 0.01$) *vs.* wild-type and heterozygous littermates (Figure 2B). For all strains and time points examined, wild-type and *Egfr*^{wa2} heterozygous placental weights did not differ. The extent of placental growth restriction in *Egfr*^{wa2} homozygotes was significantly different between strains at 15.5 dpc ($P < 0.001$) but not at 18.5 dpc.

***Egfr*^{wa2} homozygous embryos display strain-dependent intrauterine growth restriction:** To assess the effect of changes in placental size on embryonic growth, wild-

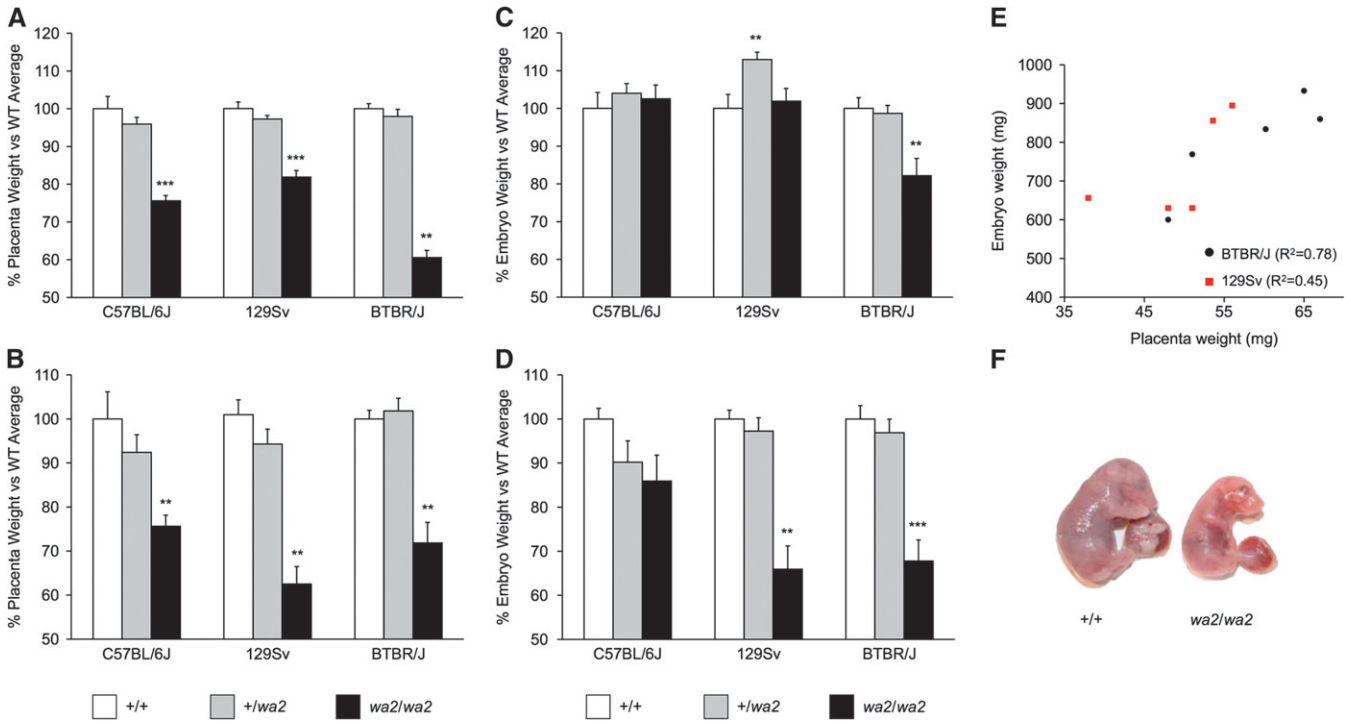

FIGURE 2.—Weights of placentas and embryos from wild-type, *Egfr*^{wa2} heterozygous, and *Egfr*^{wa2} homozygous littermates measured at 15.5 and 18.5 dpc on three genetic backgrounds. All strains included at least five embryos of each genotype. $^{**}P < 0.01$ compared to wild type; $^{***}P < 0.001$ compared to wild type. (A) At 15.5 dpc, *Egfr*^{wa2} homozygous placentas weighed 24% less than wild type on B6, 18% less than wild type on 129, and 39% less than wild type on BTBR. (B) At 18.5 dpc, *Egfr*^{wa2} homozygous placentas weighed 24% less than wild type on B6, 37% less than wild type on 129, and 28% less than wild type on BTBR. (C) At 15.5 dpc, *Egfr*^{wa2} homozygous embryo weights were not significantly different on B6 and 129 compared to wild type but homozygous embryos weighed 17% less than wild type on BTBR. *Egfr*^{wa2} heterozygous embryos on the 129 background weighed 13% more than wild-type embryos ($P < 0.01$). (D) At 18.5 dpc, *Egfr*^{wa2} homozygous embryo weights were not significantly different on B6 compared to wild type but homozygous embryos weighed 34% less than wild type on 129 and 32% less than wild type on BTBR. (E) Correlation between placenta weight and embryo weight in growth-restricted 18.5-dpc *Egfr*^{wa2} homozygous embryo on 129 and BTBR backgrounds. (F) Growth-restricted 129 *Egfr*^{wa2} homozygous embryo (right) at 18.5 dpc.

type and *Egfr*^{wa2} heterozygous and homozygous embryos were collected at 15.5 and 18.5 dpc for each strain. At 15.5 and 18.5 dpc, there were no significant differences in B6 embryo weight between the genotypes (Figure 2, C and D). At 18.5 dpc, there was a small, nonstatistically significant, *Egfr*^{wa2} dose-dependent effect on embryo weight for the B6 background (Figure 2D), indicating that the 24% reduction in placental weight in B6-*Egfr*^{wa2} homozygotes had little to no effect on embryonic growth.

At 15.5 dpc, 129-*Egfr*^{wa2} homozygous embryos did not weigh significantly differently from wild-type embryos but heterozygous embryos weighed 12% more than wild-type and *Egfr*^{wa2} homozygotes ($P < 0.01$ and $P < 0.05$, respectively; Figure 2C). In contrast, at 18.5 dpc, severe growth restriction was observed in 129-*Egfr*^{wa2} homozygotes with *Egfr*^{wa2} homozygous embryos weighing 34% less than wild-type and heterozygous littermates ($P < 0.01$ and $P < 0.001$, respectively; Figure 2, D and F), similar to the 37% reduction in placental weight. At 18.5 dpc, there were no differences in embryo weight between 129 wild-type and *Egfr*^{wa2} heterozygous embryos (Figure 2D).

At 15.5 dpc, BTBR was the only strain with significant embryonic growth restriction mirroring its more severe placental phenotype. *Egfr*^{wa2} homozygous embryos weighed 18% less than wild-type and heterozygous littermates ($P < 0.05$ and $P < 0.01$, respectively; Figure 2C). By 18.5 dpc, an even more severe embryonic growth restriction was observed in BTBR, with *Egfr*^{wa2} homozygous embryos weighing 32% less than wild-type and heterozygous littermates ($P < 0.01$ and $P < 0.001$, respectively; Figure 2D). There were no differences in embryo weight between BTBR wild-type and *Egfr*^{wa2} heterozygous embryos at either developmental stage. We found that embryo and placenta weights were highly correlated in 18.5-dpc BTBR-*Egfr*^{wa2} homozygous embryos ($R^2 = 0.78$) and to some extent in 129-*Egfr*^{wa2} homozygotes ($R^2 = 0.45$), suggesting that fetal growth restriction was caused by the placental phenotype (Figure 2E). Although embryonic growth restriction in *Egfr*^{wa2} homozygotes was not different among B6, 129, and BTBR strains at 15.5 dpc, the interaction between background and genotype approached significance at 18.5 dpc ($P = 0.0545$), consistent with an interaction between background and genotype relating to placental

TABLE 1

Survival of *Egfr^{wa2}* homozygotes on three congenic strains

Strain	Age	+/+	+/*Egfr^{wa2}*	*Egfr^{wa2}*/*Egfr^{wa2}*	Total viable	*P*
C57BL/6J	15.5 dpc	18 (24)	33 (44)	24 (32)	75	0.361
129/Sv	15.5 dpc	33 (38)	41 (48)	12 (14)	86	0.005
BTBR	15.5 dpc	7 (21)	19 (58)	7 (21)	33[a]	0.678
129/Sv	Weaning	38 (30)	70 (56)	17 (14)	125	0.01

dpc, days post-coitus. Numbers in parentheses are percentages.
[a] Three of three dead embryos genotyped were *Egfr^{wa2}*/*Egfr^{wa2}*.

weight at 15.5 dpc and resulting in altered embryo weight later in gestation.

129-*Egfr^{wa2}* homozygous embryo survival is reduced at 15.5 dpc: To determine the effect of *Egfr^{wa2}* on embryo survival, viable 15.5-dpc embryos were genotyped for each strain and evaluated for deviation from expected Mendelian ratios (Table 1). For B6, 32% of 75 viable embryos were *Egfr^{wa2}* homozygous, which was not significantly different than the expected 25%. However, a significant deviation from Mendelian ratios was observed on the 129 background as only 14% of 86 viable embryos were *Egfr^{wa2}* homozygous at 15.5 dpc ($P < 0.01$); a similar percentage of homozygotes was also observed at weaning (Table 1). This result suggests that a significant number of *Egfr^{wa2}* homozygous embryos die prior to 15.5 dpc. Although survival of BTBR embryos was similar to B6, three BTBR embryos were found dead at 15.5 dpc and all three were *Egfr^{wa2}* homozygous, suggesting that there may be some loss of *Egfr^{wa2}* homozygotes prior to 15.5 dpc in the BTBR background as well. There were also fewer than expected numbers of BTBR-*Egfr^{wa2}* homozygous weanlings observed in the breeding colony (data not shown).

***Egfr^{Wa5}* heterozygous embryos have a small reduction in placental size but no change in embryo weight:** To measure the effect of the *Egfr^{Wa5}* allele on growth of the placenta and embryo, litters were collected from crosses between *Egfr^{Wa5}* heterozygous and wild-type mice for the same three strains. Placenta weight of *Egfr^{Wa5}* heterozygotes at 15.5 dpc was reduced by 9% in B6 ($P < 0.001$) and 129 ($P < 0.001$) *vs.* wild-type littermates (Figure 3A), but embryo weight was not affected (Figure 3B). Unlike placenta from BTBR-*Egfr^{wa2}* homozygotes, *Egfr^{Wa5}* heterozygous placentas were more modestly affected at 15.5 dpc, showing only a 5% reduction in placenta weight with no difference in embryo weight compared to wild-type littermates ($P < 0.05$; Figure 3, A and B). Placenta and embryo weights were also measured at 18.5 dpc in the 129 strain but no significant differences were observed between 129-*Egfr^{Wa5}* and wild-type littermates at this later developmental stage (Figure 3C).

Viable 15.5-dpc embryos were genotyped for each strain to determine if the genotype distributions deviated from expected Mendelian ratios (Table 2). For B6 and 129, 53% and 51% of viable embryos, respectively, were *Egfr^{Wa5}* heterozygotes. Although the BTBR strain exhibited the smallest change in placental weight, only 40% of viable embryos were *Egfr^{Wa5}* heterozygotes ($P < 0.05$), suggesting that, although the reduction in placental size was more modest than in *Egfr^{wa2}* homozygotes, there was still an effect on placental function.

129 and BTBR *Egfr^{wa2}* homozygous placentas have few spongiotrophoblasts: Placentas from 18.5-dpc embryos were stained with H&E for general morphological characterization and with PAS to identify glycogen-containing cells of the spongiotrophoblast layer. Wild-type B6 placentas had a very thick layer of spongiotrophoblast with numerous protrusions into the labyrinth region (Figure 4A). The B6-*Egfr^{wa2}* homozygous placentas exhibited a reduction in spongiotrophoblasts compared to wild type (Figure 4B), but there were many glycogen-positive cells present (Figure 4C). Overall the B6 strain showed very intense PAS staining of the spongiotrophoblast, indicating an abundance of glycogen-storing cells in this layer. BTBR and 129 wild-type placentas exhibited a smaller layer of spongiotrophoblast compared to wild-type B6 (Figure 4, D and G), but the layer was well developed and stained strongly for PAS in all wild-type placentas examined. In contrast, there were only a few small clusters of spongiotrophoblasts in the BTBR and 129-*Egfr^{wa2}* homozygous placentas (Figure 4, E and H). Closer examination of these clusters revealed some PAS staining (Figure 4, F and I, arrowheads).

There were no detectable differences in the structure of the labyrinth region between 129 wild-type and *Egfr^{wa2}* homozygous 18.5-dpc placentas (Figure 4, J and K). Also, no obvious reduction was observed in the spongiotrophoblast layer of 129-*Egfr^{Wa5}* heterozygous placentas (Figure 4L) when compared to wild type (Figure 4G).

Expression of markers for specific trophoblast cell subtypes differed in *Egfr^{wa2}* homozygous *vs.* wild-type placentas: *Egfr^{wa2}* homozygous and *Egfr^{Wa5}* heterozygous placentas were molecularly characterized using a real-time PCR screen. The relative expression of trophoblast cell subtype markers *Gcm1*, *Dlx3*, *Tcfeb*, *Esx1*, *Esrrb*, *Eomes*, *Gm52*, *Ctsq*, *4311*, *Pdch12*, *Pl1*, *Timp2*, *Glut3*, and *Cx31* were measured by quantitative PCR (Table 3) and compared to an endogenous control, *Gusb*, in 15.5-dpc placentas. Five to 10 placentas were analyzed for each

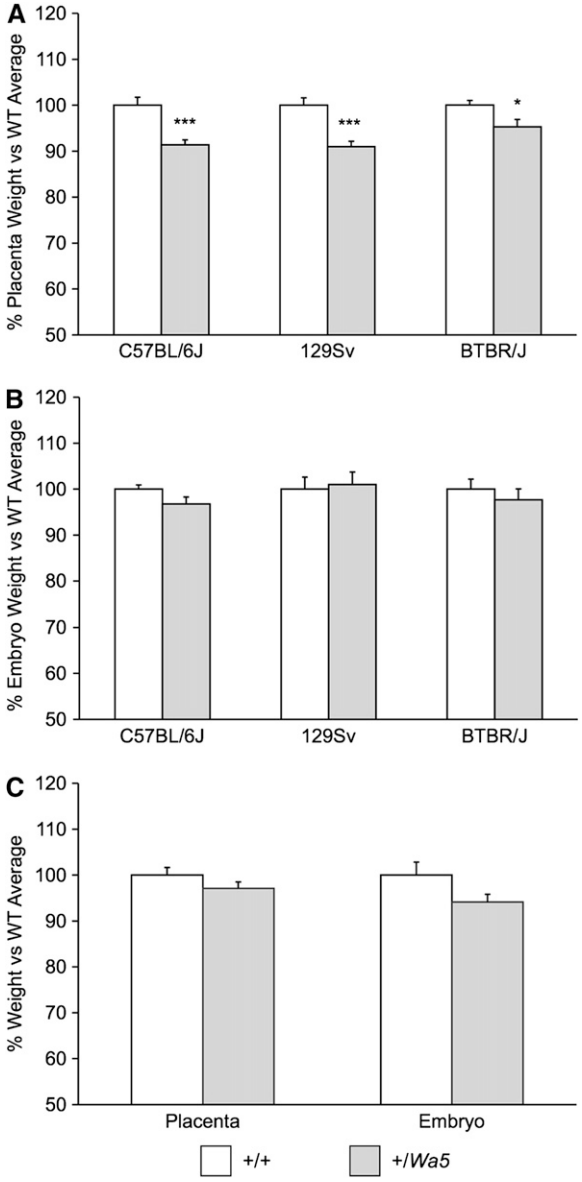

FIGURE 3.—Weights of placentas and embryos from wild-type and *Egfr*^{Wa5} heterozygous littermates on three genetic backgrounds. All strains included at least 26 embryos of each genotype for the 15.5-dpc time point and 18 embryos of each genotype for 18.5-dpc time point. *$P < 0.05$ compared to wild type; ***$P < 0.001$ compared to wild type. (A) At 15.5 dpc, *Egfr*^{Wa5} heterozygous placentas weighed 9% less than wild type on B6, 9% less than wild type on 129, and 5% less than wild type on BTBR. (B) At 15.5 dpc, none of the three genetic backgrounds showed significant differences between *Egfr*^{Wa5} heterozygous and wild-type embryo weights. (C) At 18.5 dpc, 129 *Egfr*^{Wa5} heterozygous placenta and embryo weights did not differ from wild type.

genotype and strain. Significant differences between *Egfr*^{wa2} homozygous and wild-type placentas were found in the expression of several placental genes (Table 4). In B6 and 129, several labyrinth-expressed genes, *Gcm1*, *Dlx3*, and *Tcfeb*, were expressed 30–40% higher in *Egfr*^{wa2} homozygous placentas compared to control littermates ($P < 0.001$–0.05). In 129 *Egfr*^{wa2} homozygous placentas,

TABLE 2

Survival of *Egfr*^{Wa5} heterozygotes on three congenic strains

Strain	Age	+/+	+/*Egfr*^{Wa5}	Total viable	*P*
C57BL/6J	15.5 dpc	42 (47)	48 (53)	90	0.526
129/Sv	15.5 dpc	32 (49)	33 (51)	65	0.901
BTBR	15.5 dpc	61 (60)	40 (40)	101	0.036

Numbers in parentheses are percentages.

Gm52 was 164% of wild-type levels ($P < 0.01$). *Pdch12*, a marker of glycogen cells, and *4311*, a marker of spongiotrophoblast, were significantly reduced in both B6 and 129 *Egfr*^{wa2} homozygotes with *4311* expressed at particularly low levels in the 129 homozygotes (17% of wild type, $P < 0.0001$). In B6 homozygotes, a gap junction protein expressed in the glycogen trophoblast *Cx31* was 64% of wild-type levels ($P < 0.001$), and a decidua marker, *Timp2*, was expressed at 74% of wild-type levels ($P < 0.01$). In 129 homozygotes, expression of a marker of sinusoidal labyrinth giant cells, *Ctsq*, was 78% of wild-type levels ($P < 0.01$). The only significant change in expression between *Egfr*^{wa2} and wild-type placentas was for the trophoblast giant cell marker *Pl1*, which was expressed at 175% of wild-type levels in 129-*Egfr*^{wa2} heterozygous placentas. This difference may be related to the higher weight of the 129-*Egfr*^{wa2} heterozygous placentas compared to placentas from wild-type littermates.

Changes in CT (ΔCT) values that were significantly different between *Egfr*^{wa2} homozygous and wild-type placentas were analyzed with Cluster and visualized with TreeView. Samples from the *Egfr*^{wa2} crosses clustered strongly by strain for all probes analyzed. Consistent with analysis of differences in fold change in gene expression, ΔCT values also clustered by genotype for the 129 background, with the *Egfr*^{wa2} homozygous samples showing high expression of labyrinth-specific genes and low expression of spongiotrophoblast-specific genes (Figure 5A). The B6 samples showed some genotype-specific clustering but not as strongly as 129 samples did.

Compared to placentas from *Egfr*^{wa2} homozygotes, there were fewer differences observed in expression between *Egfr*^{Wa5} heterozygous and wild-type placentas (Table 3). For the B6 background, *Egfr*^{Wa5} heterozygous expression of *Gcm1*, *Dlx3*, and *Esx1* was ~20% higher than in wild type ($P < 0.01$–0.05). BTBR-*Egfr*^{Wa5} heterozygotes showed higher expression of *Gm52* compared to wild type (133%, $P < 0.05$), and there were no significant expression differences between *Egfr*^{Wa5} heterozygous and wild-type placentas on the 129 background. The ΔCT values for genes from *Egfr*^{Wa5} samples clustered strongly by strain but not by genotype (Figure 5B).

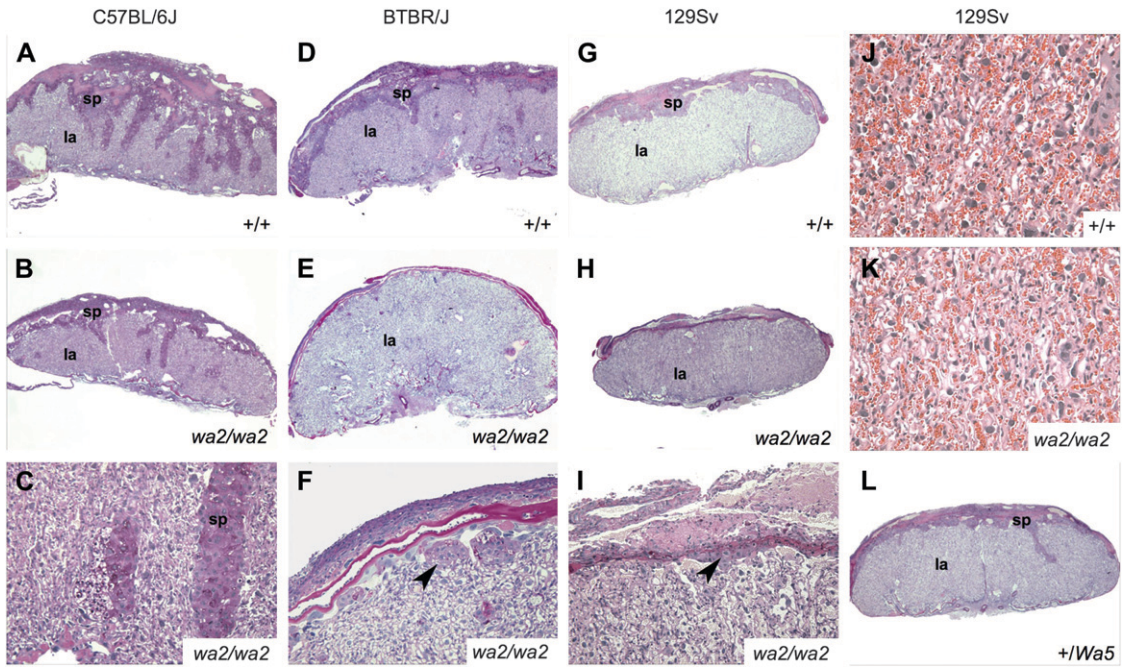

FIGURE 4.—Placentas from B6, BTBR, and 129 at 18.5 dpc. sp, spongiotrophoblast; la, labyrinth. (A) PAS-stained wild-type B6 placenta (×1.25 magnification). (B) PAS-stained *Egfr*^{wa2} homozygous B6 placenta (×1.25 magnification). (C) Higher magnification of PAS-stained spongiotrophoblasts in *Egfr*^{wa2} homozygous B6 placenta (×10 magnification). (D) PAS-stained wild-type BTBR placenta. (E) PAS-stained *Egfr*^{wa2} homozygous BTBR placenta. (F) Higher magnification of a very small cluster of PAS-stained spongiotrophoblasts (arrowhead) in *Egfr*^{wa2} homozygous BTBR placenta. (G) PAS-stained wild-type 129 placenta. (H) PAS-stained *Egfr*^{wa2} homozygous 129 placenta. (I) Higher magnification of a small cluster of PAS-stained spongiotrophoblasts (arrowhead) in *Egfr*^{wa2} homozygous 129 placenta. (J) Higher magnification of labyrinth region in wild-type 129 placenta (hematoxylin and eosin stained; ×12 magnification). (K) Higher magnification of labyrinth region in *Egfr*^{wa2} homozygous 129 placenta (hematoxylin and eosin stained stained). (L) PAS-stained *Egfr*^{Wa5} heterozygous 129 placenta.

Wild-type placenta weights, embryo weights, and expression of trophoblast markers are strain dependent: Wild-type placenta and embryo weights were compared at 15.5 dpc for the three strains (Figure 6). B6 placentas and embryos were the largest of the three strains at 15.5 dpc with an average weight of 98.3 and 385 mg for the placenta and embryo, respectively. Consistent with reduced survival of *Egfr* mutant embryos on 129 and BTBR backgrounds, wild-type 129 placentas and embryos were the smallest with an average of 73.9 mg for the placenta and 318.6 mg for the embryo, while BTBR placentas had an average weight of 82.3 mg and the embryos an average weight of 332.5 mg. Placenta and embryo weights were significantly different in all strain comparisons ($P < 0.001$).

Clustering the ΔCT values of the *Egfr*^{Wa5} data set by sample revealed interesting strain-specific differences in wild-type placenta gene expression. BTBR samples were not included in the cluster analysis because the endogenous control, *Gusb*, was expressed at a significantly higher level in BTBR placentas compared to 129 and B6 on the basis of total RNA levels ($P < 0.0005$). Placentas from the 129 background showed high expression of the labyrinth-specific genes *Tcfeb*, *Dlx3*, *Gm52*, *Gcm1*, *Esx1*, and *Ctsq* compared to B6 (Figure 5B), and B6 placentas showed higher expression of *Eomes*, *4311*, *Glut3*, *Pl1*, *Esrrb*, and *Pdch12* than 129.

DISCUSSION

EGFR and intrauterine growth restriction: Numerous studies have provided evidence that EGFR and its ligands are important for normal growth of the placenta and embryo. Overexpression of the EGFR ligand, EGF, has been found to reduce fetal growth in both humans and mice. In humans, a polymorphism in the 5′ untranslated region of *EGF* that results in increased EGF expression has been associated with lower birth weight and fetal growth restriction in pregnant women from Western Europe (DISSANAYAKE *et al.* 2007). In addition, transgenic mice that overexpress EGF are born at half the weight of their littermates and have lower levels of serum IGFBP3 (CHAN and WONG 2000). Interestingly, reduced EGF and EGFR phosphorylation have also been associated with low birth weight. Several groups have found associations between IUGR and diminished placental EGFR expression and/or activation in human pregnancies (FUJITA *et al.* 1991; FONDACCI *et al.* 1994; GABRIEL *et al.* 1994; FAXEN *et al.* 1998; CALVO *et al.* 2004). In pregnant mice, reduction of maternal EGF by sialoadenectomy results

TABLE 3

Expression pattern and placental function of genes used to quantify trophoblast cell subtypes

Gene	Function	Expression in placenta	Function in placental development	Reference
Gcm1	Transcription factor	Chorionic trophoblast, SynT layer II	Labyrinth branching morphogenesis, syncytiotrophoblast differentiation	Basyuk *et al.* (1999)
Dlx3	Transcription factor	Lz	Labyrinth morphogensis	Morasso *et al.* (1999)
Tcfeb	Transcription factor	Lz	Labyrinth branching morphogenesis	Steingrimsson *et al.* (1998)
Esx1	Transcription factor	TS cells, Lz	Labyrinth morphogenesis, syncytiotrophoblast differentiation	Li and Behringer (1998)
Esrrb1	Nuclear receptor	TS cells	Trophoblast pluripotency and proliferation	Luo *et al.* (1997)
Eomes	Transcription factor	TS cells, cuboidal Lz trophoblast	Trophoblast pluripotency and proliferation	Russ *et al.* (2000)
Gm52	Viral envelope protein	Lz	Syncytiotrophoblast fusion	Dupressoir *et al.* (2005)
Ctsq	Protease	Labyrinth sinusoidal TG	Unknown	Simmons *et al.* (2007)
4311	Cytokine	SpT, GT	Unknown	Lescisin *et al.* (1988)
Pdch12	Cell adhesion	GT	Unknown	Bouillot *et al.* (2006)
PL-1	Cytokine	TG	Unknown	Simmons *et al.* (2007)
Timp2	Metalloproteinase inhibitor	decidua	Decidual growth and remodeling	Teesalu *et al.* (1999)
Glut3	Glucose transporter	Lz	Nutrient transport	Ganguly *et al.* (2007)
Cx31	Gap junction	GT	Cell communication	Coan *et al.* (2006)
Gusb	Carbohydrate metabolism	Endogenous control		

SynT, syncytiotrophoblast; Lz, labyrinth; TS, trophoblast stem cells; TG, trophoblast giant cells; SpT, spongiotrophoblast; GT, glycogen trophoblast.

in growth restriction of embryos (Kamei *et al.* 1999). Also, EGFR-deficient mouse embryos exhibit placental defects that are dependent on strain and result in embryonic growth restriction and lethality (Sibilia and Wagner 1995; Threadgill *et al.* 1995). The effects of genetically reduced, but not abolished, EGFR signaling on placental development and embryo growth has not been previously reported. In this study, we examined the strain-specific effects of two reduced-function alleles of *Egfr* on placental and embryonic growth and the expression of trophoblast cell subtype markers in the placenta.

TABLE 4

Percentage of expression of trophoblast cell subtype markers in *Egfr*^{wa2} homozygous and *Egfr*^{Wa5} heterozygous placentas compared to wild-type littermates

	% *waved-2* homozygous		% *Waved-5* heterozygous		
Gene	129Sv	C57BL/6J	129Sv	C57BL/6J	BTBR/J
Gcm1	142**	129*	114	125*	130
Dlx3	143***	141***	109	120*	123
Tcfeb	134**	143*	95	110	135
Esx1	146	116	103	121*	114
Esrrb1	76	72	95	82	114
Eomes	114	94	104	105	119
Gm52	164**	135	115	104	133*
Ctsq	78**	82	88	113	99
4311	17***	58***	85	97	96
Pdch12	84*	71**	86	91	90
PL-1	105	116	97	80	130
Timp2	104	74**	106	94	109
Glut3	115	96	104	114	112
Cx31	95	64***	103	90	115

$n = 5$–10 placentas for each genotype and strain. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Egfr^{wa2} homozygous placentas were significantly smaller than wild type on all three genetic backgrounds examined, but the growth of *Egfr*^{wa2} homozygous placentas and embryos during late gestation differed by strain (Figure 7). Compared to B6 and BTBR, growth of the 129-*Egfr*^{wa2} homozygous placenta and embryo slowed the most during this time period and 129-*Egfr*^{wa2} homozygous embryos showed severe growth restriction at 18.5 dpc, a phenotype not observed at 15.5 dpc. The BTBR-*Egfr*^{wa2} homozygous placenta grew relatively more than *Egfr*^{wa2} homozygotes on other backgrounds between 15.5 and 18.5 dpc; however, BTBR-*Egfr*^{wa2} homozygous embryos were more growth restricted at 18.5 dpc compared to 15.5 dpc. In contrast, no significant changes were observed in the growth rate of B6-*Egfr*^{wa2} homozygous placentas and embryos across late gestation.

Trophoblasts in the placental labyrinth facilitate maternal-fetal exchange of nutrients required for normal embryonic growth and development. The *Egfr*^{wa2} homozygous labyrinth appeared well differentiated at the histological level, suggesting that defects in this placental layer do not significantly contribute to the growth restriction observed in *Egfr*^{wa2} embryos. Trophoblasts in the junctional zone, spongiotrophoblast,

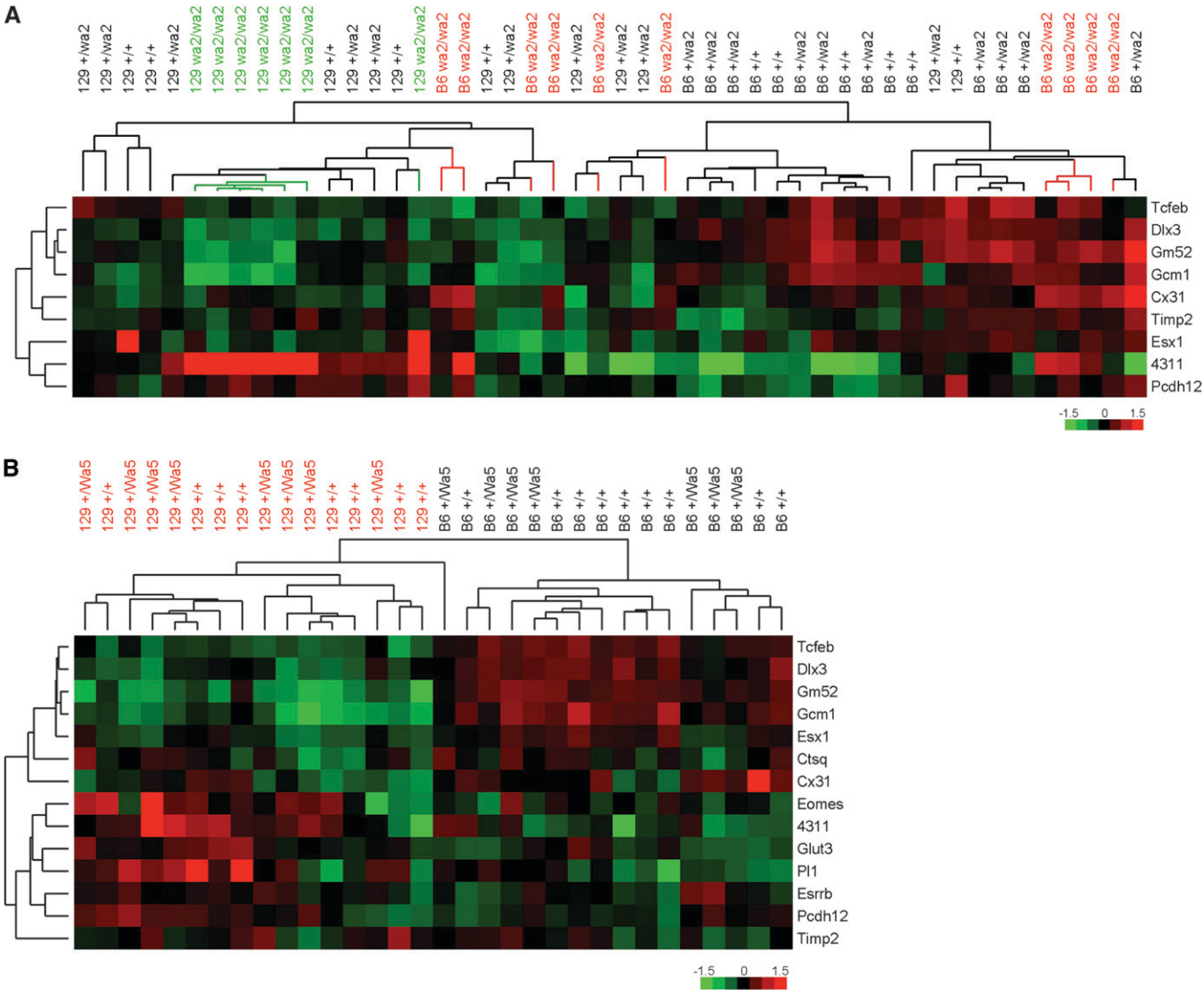

FIGURE 5.—ΔCT gene expression values for *Egfr* crosses clustered by gene and sample. Red blocks indicate low relative expression and green blocks indicate high relative expression. (A) Cluster analysis and dendrogram of ΔCT values for $Egfr^{wa2}$ homozygous, heterozygous, and wild-type samples. Cluster analysis included only genes that were significantly different between wild-type and $Egfr^{wa2}$ homozygous placenta. $Egfr^{wa2}$ homozygous samples are indicated by red (129) and green (B6) bars on dendrogram. (B) Cluster analysis and dendrogram of ΔCT values for $Egfr^{wa5}$ heterozygous and wild-type samples. Analysis included genes that were and were not significantly different between $Egfr^{wa5}$ heterozygous and wild-type samples. The 129 samples are indicated by red text; B6 samples are indicated by black text.

glycogen cells, and trophoblast giant cells have been shown to synthesize various hormones that regulate embryonic growth directly and/or indirectly through modulation of the maternal physiological response to pregnancy. Pronounced growth restriction was observed in 129 and BTBR-$Egfr^{wa2}$ homozygous embryos, the two strains that also exhibit severely reduced spongiotrophoblast and glycogen cells. Additionally, placental size was correlated with embryonic growth in these strains, further suggesting that the placental growth restriction causes the embryonic growth phenotype. In contrast, B6-$Egfr^{wa2}$ homozygous embryos were not growth restricted, and their placentas contained a more robust layer of spongiotrophoblast and glycogen cells.

Origins of genetic background-dependent placental phenotypes: Strain-dependent placental phenotypes have been previously reported in $Egfr^{tm1Mag}$ nullizygous embryos, but the specific role of modifying genes remains unknown. In this study, we demonstrated that strain-dependent differences also exist in wild-type placentas and embryos. Weights of placentas and embryos are significantly different among B6, 129, and BTBR strains with B6 exhibiting the largest and 129 the smallest placentas and embryos. Histological comparison of wild-type placentas from the three strains showed

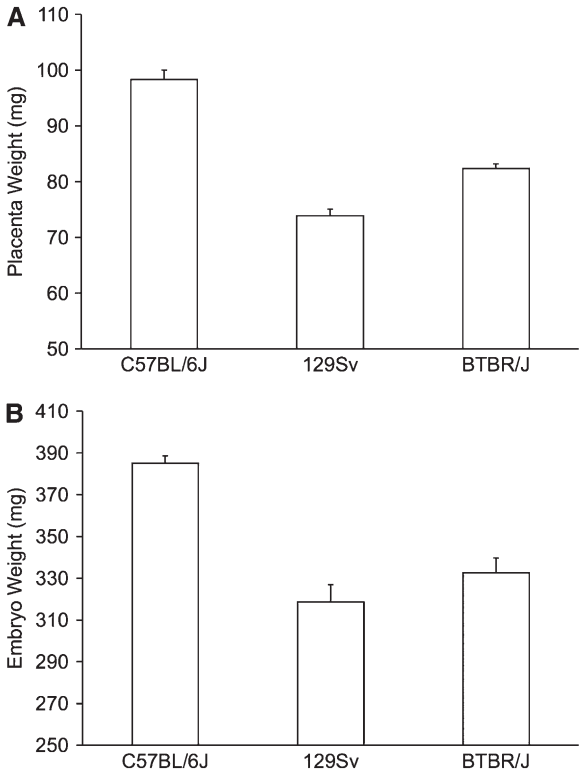

FIGURE 6.—Wild-type placenta and embryo weights of three inbred strains measured at 15.5 dpc. (A) Placenta weight is dependent on genetic background. The average weight of 15.5-dpc placentas was 98.3 mg for B6 ($n = 42$), 73.9 mg for 129 ($n = 27$), and 82.3 mg for BTBR ($n = 57$). Placental weights were significantly different in all three strains ($P < 0.001$). (B) Embryo weight is dependent on genetic background. The average weight of 15.5-dpc embryos was 385.0 mg for B6 ($n = 29$), 318.6 mg for 129 ($n = 26$), and 332.5 mg for BTBR ($n = 59$). Embryo weights were significantly different in all three strains ($P < 0.001$).

that the numbers of spongiotrophoblasts and the intensity of PAS staining also varied by strain.

Real-time PCR data comparing the expression of trophoblast cell subtype-specific genes in 129 and B6 suggest that, in addition to a difference in size, placentas from the strains may consist of different proportions of trophoblast layers, and/or the level of gene expression may vary. Clustering of ΔCT values revealed that even with *Egfr* alleles that affect placental composition, the data still clustered most strongly by strain rather than by genotype. Placentas from 129 embryos showed a relatively high expression of a set of labyrinth-specific genes while B6 exhibited the highest expression of a separate set of genes that included *Eomes*, *4311*, *Pl1*, and *Glut3*. The relatively high expression of *Glut3* in B6 is interesting, considering the role of GLUT3 in embryonic growth. Embryos heterozygous for a null allele of *Glut3* display late gestational IUGR, and placental *Glut3* expression is reduced in growth-restricted embryos from EGF-deficient sialoadenectomized dams (KAMEI *et al.* 1999; GANGULY *et al.* 2007). Elevated expression of *Glut3* in B6 placentas may allow $Egfr^{wa2}$ homozygous embryos to escape the severe growth restriction observed in the 129 and BTBR backgrounds.

These strain-specific differences in wild-type embryos are not surprising, given the fact that the placenta is an organ affected strongly by natural selection (COAN *et al.* 2005; ANGIOLINI *et al.* 2006). Many imprinted genes play a role in growth and development of the placenta, and during the derivation and maintenance of distinct mouse strains, polymorphic genes that influence placental growth may be fixed in different combinations. The unique placental composition and/or expression of genes known to play important roles in the trophoblast differentiation observed in standard wild-type laboratory mouse strains is interesting considering the large number of transgenic and mutant models with reported placental defects leading to embryonic lethality (ROSSANT and CROSS 2001; WATSON and CROSS 2005). For some of these models, such as the $Egfr^{tm1Mag}$ nullizygous mouse, the embryonic lethal phenotype is dependent on genetic background, suggesting that the causative placental defects probably vary by strain (STRUNK *et al.* 2004). The inherent strain-specific differences that we have observed in wild-type placenta indicate that the response of the placenta to genetic changes may be determined, in part, by strain-specific trophoblast characteristics.

$Egfr^{Wa5}$ heterozygotes retain more EGFR signaling than $Egfr^{wa2}$ homozygotes: In contrast to $Egfr^{wa2}$ homozygotes, we observed only small decreases (5–10%) in placental weight of $Egfr^{Wa5}$ heterozygotes for the three strains with no significant effect on embryonic weight. Histological changes were not obvious in $Egfr^{Wa5}$ heterozygous placenta and, molecularly, $Egfr^{Wa5}$ heterozygous placentas were more similar to wild type than were $Egfr^{wa2}$ homozygotes. Also unlike $Egfr^{wa2}$ homozygotes, there was no embryonic lethality of 129-$Egfr^{Wa5}$ heterozygotes prior to 15.5 dpc. However, we did observe significantly fewer $Egfr^{Wa5}$ heterozygotes than expected for the BTBR background, but the reason remains to be determined. Together, our placenta and embryo weight measurements, histology, and gene expression data show that the $Egfr^{Wa5}$ heterozygous phenotype is less severe than the $Egfr^{wa2}$ homozygous phenotype.

Recent reports provide evidence for an asymmetric dimer model of EGFR activation (ZHANG *et al.* 2006). Studies have shown that in an ERBB dimer one of the receptors, the activator, acts to hold the other, the activated receptor, in a conformation that promotes its activation and subsequent auto-phosphorylation. The N-lobe of the activated receptor makes critical contacts with the C-lobe of the activator, and mutations that disrupt this interaction generally result in reduced or abolished phosphorylation. A kinase-dead EGFR, such as from the $Egfr^{Wa5}$ allele, is capable of acting as the activator but not the activated receptor. According to this model, EGFR signaling in the $Egfr^{Wa5}$ heterozygote

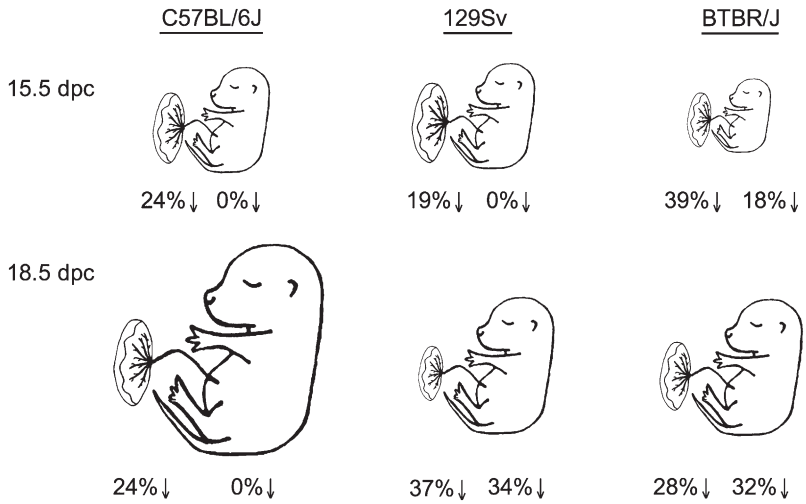

FIGURE 7.—Summary of strain-dependent late-gestation growth patterns in *Egfr*^{wa2} homozygous placentas and embryos. The percentage reduction in weight compared to wild-type littermates for the placenta and embryo is shown.

would occur normally through the wild-type EGFR dimer, to some extent through the EGFR^{Wa5}/EGFR dimer, and not at all through the EGFR^{Wa5}/EGFR^{Wa5} dimer. However, *in vitro* experiments have shown that EGFR^{Wa5} acts as a dominant negative and has a more severe effect on wild-type EGFR phosphorylation than does a kinase-dead receptor. EGFR phosphorylation is reduced by ~90% when cells express equal amounts of EGFR and EGFR^{Wa5} (LEE *et al.* 2004). Thus, the *Egfr*^{Wa5} mutation not only renders the receptor kinase-dead but also affects receptor activation through an additional mechanism, perhaps by modifying conformation of the receptor that it encodes and/or disrupting assembly of higher-order receptor oligomers.

Estimates of EGFR^{wa2} receptor-signaling capabilities have varied from 10% to almost wild-type levels of activity, depending on the cell type analyzed and the experimental approach (LUETTEKE *et al.* 1994). The *Egfr*^{wa2} mutation lies upstream of *Egfr*^{Wa5} in an α-helix portion of the receptor N-lobe (LUETTEKE *et al.* 1994; FOWLER *et al.* 1995). The effect of the *Egfr*^{wa2} mutation on EGFR phosphorylation is not well understood, but it is possible that the mutation compromises contact with the C-lobe portion of the activator directly or indirectly by altering conformation of the activated receptor. DU *et al.* (2004) proposed that *Egfr*^{wa2} homozygotes and *Egfr*^{Wa5} heterozygotes have approximately the same reduction in EGFR signaling. On the basis of the more severe phenotype observed in *Egfr*^{wa2} homozygous placentas, we propose that the following levels of EGFR signaling occur in the *Egfr* allelic series. The levels are arranged in order from wild-type levels of EGFR activity to complete absence of EGFR activity ("≈" indicates allele combinations predicted to produce similar levels of EGFR activity):

$$Egfr/Egfr \approx Egfr/Egfr^{tm1Mag} \approx Egfr/Egfr^{wa2} > Egfr/Egfr^{Wa5} > Egfr^{wa2}/Egfr^{wa2} \approx Egfr^{wa2}/Egfr^{tm1Mag} > Egfr^{wa2}/Egfr^{Wa5} > Egfr^{Wa5}/Egfr^{Wa5} \approx Egfr^{Wa5}/Egfr^{tm1Mag} \approx Egfr^{tm1Mag}/Egfr^{tm1Mag}$$

Our data also demonstrate that tissue-specific requirements for EGFR signaling can be determined using the allelic series. We have shown that normal development of the placenta requires less EGFR activity than morphogenesis of hair follicles since the *Egfr*^{wa2} and *Egfr*^{Wa5} mouse share the same wavy coat phenotype but not the same degree of placental defects.

In conclusion, our study highlights strain-dependent variation in placental development as well as the effect of diminished EGFR signaling on placental and embryonic growth. IUGR is a common condition with profound consequences for the fetus, including elevated risk for perinatal mortality and increased incidence of reduced cognitive function, diabetes, and heart disease later in life (BARKER *et al.* 2002). It is known that a large number of IUGR cases are caused by placental defects, but the precise developmental mechanisms are not well understood. *Egfr*^{wa2} homozygous embryos may serve as a model for investigating growth restriction arising from placental dysfunction. In addition, *Egfr*^{Wa5} heterozygotes can be used to study levels of EGFR signaling intermediate between the wild-type and the *Egfr*^{wa2} homozygote.

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