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Phenotypic Variation Resulting From a Deficiency of Epidermal Growth Factor Receptor in Mice Is Caused by Extensive Genetic Heterogeneity That Can Be Genetically and Molecularly Partitioned

Karen E. Strunk,*,†,[1] Vicky Amann* and David W. Threadgill*,†,[2]

*Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee 37221 and †Department of Genetics and the Carolina Center for Genome Sciences, University of North Carolina, Chapel Hill, North Carolina 27599

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ABSTRACT

The timing of lethality caused by homozygosity for a null allele of the epidermal growth factor receptor (*Egfr*^{tm1Mag}) in mice is strongly dependent on genetic background. Initial attempts to genetically map background modifiers using Swiss-derived, outbred CD-1 mice were unsuccessful. To investigate the genetic architecture contributing to survival of *Egfr*^{tm1Mag} homozygous embryos, the genetic variability segregating within the outbred population was partitioned by surveying viability of *Egfr*^{tm1Mag} mutants using intercrosses between 129S6/SvEvTAC-*Egfr*^{tm1Mag} and nine Swiss-derived, inbred strains: ALR/LtJ, ALS/LtJ, APN, APS, ICR/HaRos, NOD/LtJ, NON/LtJ, SJL/J, and SWR/J. The observations showed that these strains support varying levels of survival of *Egfr*^{tm1Mag} homozygous embryos, suggesting that genetic heterogeneity within the CD-1 stock contributed to the original lack of *Egfr*^{tm1Mag} modifier detection. Similar to the Swiss-derived intercrosses, nine congenic strains, derived from 129S6/SvEvTAC, AKR/J, APN, BALB/cJ, BTBR-*T*$^{+}$ *tf/tf*, C3H/HeJ, C57BL/6J, DBA/2J, and FVB/NJ inbred backgrounds, also supported varying levels of survival of *Egfr*^{tm1Mag} mutants. By intercrossing the congenic lines to create hybrid F_1 embryos, different genetic backgrounds were found to have complementary modifiers. Analysis of the congenic lines argues against heterosis of outbred backgrounds contributing to *Egfr*^{tm1Mag} phenotypic variability. A detailed analysis of the crosses suggests that modifiers function at three distinct stages of development. One class of modifiers supports survival of *Egfr*^{tm1Mag} homozygous embryos to mid-gestation, another class supports development through the mid-gestation transition from yolk-sac to placental-derived nutrient sources, and a third class supports survival through later stages of gestation. Data from microarray analysis using RNA from wild-type and *Egfr*^{tm1Mag} mutant placentas support the existence of extensive genetic heterogeneity and suggest that it can be molecularly partitioned. This method should be generally useful to partition heterogeneity contributing to other complex traits.

THE epidermal growth factor receptor (EGFR) is the prototypical member of a family of related receptor tyrosine kinases (RTKs) that includes ERBB2/NEU, ERBB3, and ERBB4 (Carpenter and Wahl 1990; Gullick 1998). Activation of the EGFR results in propagation of extracellular signals into the cytoplasm via activation of signaling cascades. These signals, resulting in the alteration of many cellular processes, including proliferation, migration, and differentiation, are initiated by seven known ligands: epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), heparin-binding Egf/diphtheria toxin receptor (DTR), and epigen (EPGN; Cohen 1962; Marquardt *et al.* 1984; Ciardiello *et al.* 1991; Elenius *et al.* 1991; Higashiyama *et al.* 1991; Shing *et al.* 1993; Toyoda *et al.* 1995; Riese *et al.* 1996; Strachan *et al.* 2001). The ligands encoded by *Egf*, *Tgf*α, *Epgn*, and *Areg* are specific for the EGFR, whereas those encoded by *Btc*, *Ereg*, and *Dtr* are reported to bind either EGFR or ERBB4 with varying affinities (Alroy and Yarden 1997; Riese *et al.* 1998). When misregulated, most RTKs can contribute to developmental disorders and to pathogenesis, such as cancer.

Homologous recombination in mouse embryonic stem (ES) cells was previously used to generate a null allele of the *Egfr* (Threadgill *et al.* 1995). Phenotypic analysis of mice generated from the targeted ES cells revealed that homozygous *Egfr*^{tm1Mag} null mutants exhibit peri-implantation to postnatal lethality, depending on the genetic background of the developing embryo. Consequently, the *Egfr*^{tm1Mag} null allele was the first targeted mutation to reveal profound variation in survival caused by differences in genetic background (Sibilia and Wagner 1995; Threadgill *et al.* 1995).

The most severely affected mutants were seen on non-Swiss-derived, outbred CF-1 stock; *Egfr*^{tm1Mag} homozygous

[1]*Present address:* Department of Genetics, CB#7264, University of North Carolina, Chapel Hill, NC 27599.

[2]*Corresponding author:* Department of Genetics, CB#7264, MBRB Room 4340, University of North Carolina, Chapel Hill, NC 27599. E-mail: dwt@med.unc.edu

mutant blastocysts form but then die shortly after implantation at 4.5 days post coitus (dpc). These embryos consist of a loosely arranged inner cell mass with no distinct endoderm. In contrast, on Swiss-derived, outbred CD-1 stock, *Egfr*^{tm1Mag} homozygous pups can survive longer than 3 weeks post-natally, but are severely runted and undergo progressive cerebral degeneration and wasting after birth. Newborn pups, homozygous for the *Egfr*^{tm1Mag} mutation, can be distinguished from littermates by waved whiskers and an absence of eyelids, a phenotype similar to mice homozygous for the hypomorphic *Egfr*^{wa2} allele (Luetteke *et al.* 1994; Fowler *et al.* 1995). On an inbred 129S6/SvEvTAC background, *Egfr*^{tm1Mag} homozygous embryos die at 11.5 dpc due to a decreased placental size, compared to littermate controls, which is caused by a reduced spongiotrophoblast layer and disorganization of the labyrinthine trophoblast layer. Surprisingly, on the outbred CD-1 stock, the *Egfr*^{tm1Mag} homozygous placentas still exhibit a reduced spongiotrophoblast layer; however, there is partial rescue of the disorganized labyrinthine trophoblast layer.

Several causes could contribute to the strain-dependent variation observed with survival of *Egfr*^{tm1Mag} homozygous mutants, including differences in background alleles, heterosis, and the maternal uterine environment. Previous experiments using reciprocal embryo transfers have eliminated the maternal uterine environment as a factor contributing to embryo survival (Threadgill *et al.* 1995). Likewise, tetraploid chimeras were used to definitively demonstrate that variation in embryonic survival is caused by abnormal placenta development from extraembryonic lineages and that embryonic lineages do not contribute significantly to differential survival of *Egfr*^{tm1Mag} homozygous embryos (Sibilia and Wagner 1995). The most probable factor contributing to differential survival is the existence of allelic differences between mice that function as modifying genes. However, heterosis cannot be discounted since most previous analyses used outbred mice. Conclusive evidence for the existence of modifying genes can be obtained only from an analysis of inbred or congenic mouse lines.

To address the question of how the same mutation can have different effects depending on genetic background, a backcross panel was set up with 129CD1 F_1-*Egfr*^{tm1Mag} heterozygous mice backcrossed to 129S6/SvEvTAC-*Egfr*^{tm1Mag} mice to identify genetic modifiers of *Egfr*^{tm1Mag}. Surviving 13.5 dpc N_2 generation *Egfr*^{tm1Mag} homozygous embryos were subjected to a whole-genome scan to identify CD-1 alleles contributing to survival past mid-gestation. Interestingly, no statistically significant loci were detected (D. W. Threadgill, N. J. Schork and T. Magnuson, unpublished results). However, since CD-1 is an outbred stock, the inability to localize specific modifiers of the *Egfr*^{tm1Mag} phenotype may have been due to the highly heterogeneous nature of the CD-1 stock.

To investigate the strong genetic-background-dependent phenotypic variation caused by the *Egfr*^{tm1Mag} allele and the apparent genetic heterogeneity of the modifiers, a detailed analysis of the underlying genetic architecture was performed. Genetic heterogeneity was investigated using inbred strains of mice, independently produced from Swiss-derived, outbred stocks that were assayed for their ability to modify survival of *Egfr*^{tm1Mag} homozygous offspring through intercrosses and backcrosses with 129S6/SvEvTAC-*Egfr*^{tm1Mag} heterozygous mice. A series of congenic strains containing the *Egfr*^{tm1Mag} mutation was also created and the resulting *Egfr*^{tm1Mag} homozygous mutants were characterized for their stage of lethality. Furthermore, the congenic strains were intercrossed to produce hybrid F_1 mutants to address the genetic complexity of the *Egfr* modifiers. These studies revealed complex patterns underlying the phenotypic diversity caused by homozygosity for the *Egfr*^{tm1Mag} mutation, suggesting that the numbers and interactions between modifiers are extensive and function at three distinct stages of placental development. To molecularly validate and to investigate a method for partitioning genetic heterogeneity, gene expression profiling was performed using microarrays on a subset of placentas. The results reveal that placentas from wild-type embryos are similar while those from different *Egfr*^{tm1Mag} homozygous embryos manifest highly diverse transcriptional differences.

MATERIALS AND METHODS

Mouse strains and crosses: To generate congenic lines carrying the *Egfr*^{tm1Mag} mutation, donor male mice heterozygous for the *Egfr*^{tm1Mag} allele, co-isogenic on the 129S6/SvEvTAC genetic background, were mated to wild-type female mice from eight recipient strains. The inbred strains of mice, selected for their genetic diversity, included AKR/J, APN, BALB/cJ, BTBR-*T*$^{+}$ *tf/tf*, C3H/HeJ, C57BL/6J, DBA/2J, and FVB/NJ. The resulting progeny were backcrossed to their respective recipient strain for at least 10 successive generations (N_{10}) to generate congenic lines. The backcross progeny were genotyped by PCR for presence of the *Egfr*^{tm1Mag} allele. To determine the effect of the *Egfr*^{tm1Mag} homozygous null mutation on the various genetic backgrounds, heterozygous female and male progeny from N_{10} or greater were intercrossed and embryos were collected and analyzed at 10.5, 13.5, or 18.5 dpc. Embryos were genotyped by PCR and those homozygous for the *Egfr*^{tm1Mag} allele were phenotypically classified for the ability to modify the mid-gestation placental lethality observed on the donor 129S6/SvEvTAC genetic background. The strains were classified as similar to the original 129S6/SvEvTAC background phenotype if the decidua contained either no viable embryo or residual extra-embryonic membranes at the 13.5 dpc time point. The strains were classified as rescuing (modifying) the mid-gestation lethality if they were phenotypically indistinguishable from wild-type or heterozygous littermates at 10.5 and 13.5 dpc. At 18.5 dpc *Egfr*^{tm1Mag} homozygous mutants were scored positive for rescue if embryos were alive and exhibited failure of eyelid closure, smaller size, and reduced placenta size characteristic of the *Egfr*^{tm1Mag} homozygous mutation previously described on outbred stocks (Miettinen *et al.* 1995; Sibilia and Wagner 1995; Threadgill *et al.* 1995); homozygosity was confirmed by PCR assay.

The congenic strains that did not support a robust survival to term of *Egfr*^{tm1Mag} homozygous embryos were intercrossed to

produce hybrid F_1 backgrounds to test for genome complementation. The backgrounds tested include 129S6/SvEvTAC, BALB/cJ, C57BL/6J, and FVB/NJ. Embryos were collected and genotyped at 13.5 or 18.5 dpc as described above to determine the ability of the combined genomes to support survival of *Egfr*^{tm1Mag} homozygous embryos.

Heterozygous 129S6/SvEvTAC-*Egfr*^{tm1Mag} mice were also mated to wild-type mice from nine Swiss-derived, inbred strains, ALR/LtJ, ALS/LtJ, APN, APS, ICR/HaRos, NOD/LtJ, NON/LtJ, SJL/J, and SWR/J, to generate F_1 animals heterozygous for the *Egfr*^{tm1Mag} allele. Heterozygous F_1 progeny from within each cross were intercrossed and, only with ALR/LtJ , also backcrossed to generate segregating F_2 and N_2 populations, respectively. At 10.5, 13.5, or 18.5 dpc, embryos were analyzed to determine the ability of each cross to rescue the mid-gestation lethality. The homozygous *Egfr*^{tm1Mag} F_2 embryos were phenotyped as described for the analysis of the congenic strains.

Successful female matings were determined by the presence of copulation plugs with the morning of the plugs designated as 0.5 dpc. Pregnant females were euthanized and embryos were dissected from the uterine horns on the morning of 10.5, 13.5, or 18.5 dpc into phosphate buffered saline (PBS). In all crosses the dam is listed first and the sire second unless otherwise noted. Mice were fed Purina Mills lab diet 5010 or 5058 and water *ad libitum* under specific pathogen-free conditions in an American Association for the Accreditation of Lab Animal Care approved facility. Mice were euthanized by CO_2 asphyxiation.

Genotyping: The placentas and extra-embryonic tissues were separated from the embryo by mechanical dissection and either whole embryos at 10.5 dpc or pieces of tail from 13.5- and 18.5-dpc embryos were collected for DNA extraction to determine the genotype of individual embryos. DNAs from these samples were isolated by lysing the tissues in 25 mM NaOH/0.2 mM EDTA at 95° for 20 min. The reaction was neutralized with an equal volume of 40 mM Tris pH 5.0 and centrifuged at 13,000 rpm for 5 min. A total of 200 ng of DNA from each lysis reaction was used to genotype embryonic samples by polymerase chain reaction (PCR) with the following primers: Egfr25, 5′-GCCCTGCCTTTCCCACCATA-3′; Egfr26A, 5′-AACGTCGTGACTGGGAAAAC-3′; and Egfr27, 5′-ATCAACTTTGGGAGCCACAC-3′. PCR conditions were as follows: denaturing at 94°, primer annealing at 63°, and extension at 72° for 40 cycles in a Perkin Elmer (Norwalk, CT) 9700. PCR products were separated on 1.5% agarose gels; the wild-type *Egfr* allele yields a 350-bp fragment and the targeted *Egfr*^{tm1Mag} allele a 450-bp fragment detected by ethidium bromide staining.

Statistical analysis: A one-group chi-square analysis using StatView (SAS Institute) was carried out for all genetic crosses.

Collection of placental samples: Timed pregnancies were collected at 18.5 dpc from *Egfr*^{tm1Mag} heterozygous ALR129 F_1 females mated to 129S6/SvEvTAC-*Egfr*^{tm1Mag} heterozygous males. From each pregnancy, the placenta was dissected from the uterus and separated from the embryo, yolk sac, and umbilical cord in PBS. Placentas were bisected and stored in RNA later (Ambion, Austin, TX) at −20° or flash frozen in SuperFriendly Freeze-it (Fisher Scientific) and stored at −80° until RNA was extracted. DNA was extracted from embryonic tissue and the genotype of each sample was determined by PCR. Total RNA was isolated from individual 18.5-dpc placentas using 1–2 ml of trizol (Invitrogen, San Diego) according to the manufacturer's protocol. Isolated RNA was quantified using a spectrophotometer and RNA quality analyzed on a Bioanalyzer 2100 (Agilent). Placental reference RNA was generated by combining 50 μg of total RNA from eight wild-type 18.5-dpc placentas, three *Egfr*^{tm1Mag} 18.5-dpc placentas, four wild-type 17.5-dpc placentas, two *Egfr*^{tm1Mag} 17.5-dpc placentas, and one post-natal day (P) 7 and P2 liver.

Microarray hybridization: RNA was reverse transcribed to cDNA using an anchored oligo(dT) primer, Cy3 or Cy5 dUTP, and Superscript II RNaseH$^-$ RT enzyme (Invitrogen) at 42° for 2 hr. For experimental samples, RNA from wild-type or *Egfr*^{tm1Mag} null placenta was labeled with Cy5 and the placental reference RNA was labeled with Cy3. Following reverse transcription, the mRNA was degraded by addition of NaOH and neutralized with HCl. Cy3- and Cy5-labeled cDNA samples were combined, free nucleotides were removed, and the samples were concentrated and washed with low TE (10 mM Tris pH 7.5, 0.1 mM EDTA pH 8.0) by filtration through a Microcon-30 filter (Amicon). Samples were collected into an Eppendorf tube and hybridization buffer was added [300 ng yeast tRNA, 600 ng poly(A) RNA, 3× SSC, 0.2% SDS]. Microarray slides were prehybridized for 45 min in 3× SSC, 0.1% SDS, 0.2% BSA at 65°, washed extensively in ddH_2O, dipped in isopropanol, and spun at 500 rpm for 5 min to dry. Heat-denatured probe samples were added to 7500-gene mouse microarrays prepared by the University of North Carolina-Chapel Hill Genomics Core and hybridized at 65° for 16–20 hr in humidified hybridization chambers (Telechem); microarrays were prepared from 60-mer oligo libraries (Compugen). The microarrays were washed once in 2× SSC/0.1% SDS for 2 min to remove the coverslips, once in 1× SSC for 5 min, and twice in 0.1× SSC for 5 min, all at room temperature with gentle agitation. After centrifugation at 500 rpm for 5 min at 20° to dry, the microarrays were scanned using a GenePix 4000B scanner (Axon Instruments, Foster City, CA). A laser detected the individual probes, with the Cy5 probe scanning at 635 nm and pseudocolored red and the Cy3 probe detected at 532 nm and pseudocolored green.

Microarray data analysis and clustering: GenePix Pro 4.0 (Axon Instruments) was used to locate and grid individual spots using the image generated by the scanner. After gridding, the raw fluorescent intensities were collected for each spot and for local background signal. Captured TIFF images corresponding to the two fluorescent labels and the raw intensity measurements were uploaded into a GeneTraffic v2.5 microarray database (Iobion). The data were normalized using a subgrid Lowess routine and the local background signals were subtracted (Yang *et al.* 2002). Hierarchical clusters were generated with Euclidean distance to the nearest neighbor to identify groups of genes with similar expression profiles. Expression levels across individual samples were represented relative to the median of all samples for each gene.

RESULTS

Variable survival of *Egfr*^{tm1Mag} homozygotes from intercrosses with Swiss-derived, inbred strains: Previous whole-genome scans using intercrosses with Swiss-derived, outbred CD-1 mice failed to localize *Egfr*^{tm1Mag} background modifiers (D. W. Threadgill, N. J. Schork and T. Magnuson, unpublished results). A likely reason that no modifier loci were genetically mapped in the initial cross is due to genetic heterogeneity of modifiers carried in the outbred CD-1 stocks. A prediction of this hypothesis is that independent Swiss-derived, inbred strains should have partitioned out the genetic heterogeneity and fixed independent and overlapping sets of modifiers during the inbreeding process. As a consequence, the Swiss-derived, inbred strains should show a variable ability to support embryonic survival of *Egfr*^{tm1Mag} homozygous mutants.

TABLE 1

Survival of *Egfr*^{tm1Mag} homozygous embryos from intercrosses with Swiss-derived, inbred strains

Intercross strain[a]	Age (dpc)	No. of embryos +/+:+/−:−/−[b]	*Egfr*^{tm1Mag} embryos (%)	Viable *Egfr*^{tm1Mag} embryos (%)	No. of litters
ICR/HaROS	10.5	30:43:15**	17.0	5.9	7
	13.5	25:34:10**	14.5	0.0	9
NON/LtJ	10.5	23:30:23	30.3	18.4	8
	13.5	25:28:12**	18.5	3.0	7
	18.5	8:11:1*	5.0	0.0	3
NOD/LtJ	10.5	9:31:13	24.5	17.0	5
	13.5	24:34:15**	20.5	6.8	7
	18.5	15:43:8**	12.1	1.5	8
SJL/J	10.5	10:33:20	32.0	12.7	6
	13.5	20:39:10**	14.5	4.3	7
	18.5	27:45:19**	20.9	6.6	11
SWR/J	13.5	14:45:21	26.3	21.3	10
	18.5	23:47:17	19.5	12.6	10
APS	13.5	17:47:23	26.4	18.4	8
	18.5	31:54:16**	15.8	12.9	10
APN	13.5	26:46:16*	18.2	13.6	8
	18.5	24:43:11**	14.1	11.5	7
ALS/LtJ	13.5	18:36:17	23.9	18.3	7
	18.5	29:71:34*	25.4	14.9	15
ALR/LtJ	13.5	24:46:9**	11.4	10.1	9
	18.5	15:44:17	22.4	19.7	6

[a] All strains were intercrossed with 129S6/SvEvTAC-*Egfr*^{tm1Mag}.

[b] *P*-values: *, <0.05 and **, <0.01 when using a χ^2 test for deviation from the expected 25% recovery of viable *Egfr*^{tm1Mag} homozygous embryos.

Nine strains, inbred for various phenotypic characteristics from Swiss-derived, outbred mouse stocks, were used to test these predictions; included were ALR/LtJ, ALS/LtJ, APN, APS, ICR/HaRos, NOD/LtJ, NON/LtJ, SJL/J, and SWR/J (Heston 1963; Crispens 1973; Evans *et al.* 1974; Casley *et al.* 1997; Leiter *et al.* 1998; Graser *et al.* 1999). Wild-type mice from each inbred strain were mated to heterozygous 129S6/SvEvTAC-*Egfr*^{tm1Mag} and the resulting F_1 progeny were intercrossed. The F_2 embryos were phenotyped at 10.5, 13.5, and/or 18.5 dpc (Table 1; Figure 1). Previous analysis showed that embryos homozygous for the *Egfr*^{tm1Mag} null mutation, co-isogenic on the 129S6/SvEvTAC genetic background, are viable and present at expected ratios through 10.5 dpc, but undergo rapid degeneration around 11.5 dpc due to severe placental abnormalities (Threadgill *et al.* 1995). Three groups of intercrosses were observed. The first group includes crosses with ICR/HaROS that display poor support for *Egfr*^{tm1Mag} survival at mid-gestation, worse than that on the co-isogenic 129S6/SvEvTAC genetic background. Interestingly, intercrossing with the ICR/HaRos strain produced only 5.3% viable *Egfr*^{tm1Mag} homozygous embryos at 10.5 dpc, with *Egfr*^{tm1Mag} homozygous embryos being smaller than either wild-type or heterozygous littermates. The lethality in ICR;129 intercrosses occurs significantly earlier than the *Egfr*^{tm1Mag} phenotype on the co-isogenic 129S6/SvEvTAC background, suggesting that ICR/HaRos is a sensitized background, potentially harboring modifiers that result in decreased viability. Although ICR;129 intercrosses support survival of *Egfr*^{tm1Mag} homozygous embryos better than non-Swiss-derived, outbred CF-1 stock does (Threadgill *et al.* 1995), the ICR/HaROS inbred strain may nonetheless have modifiers similar to those in the CF-1 stock.

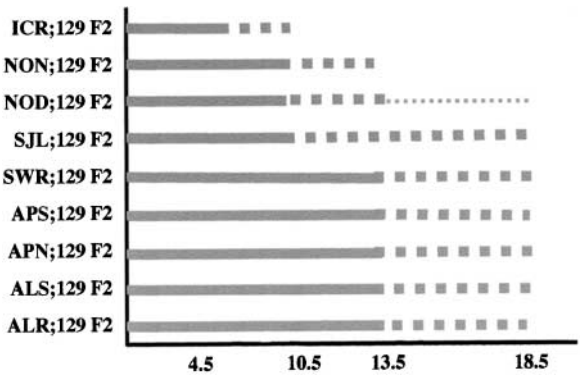

Figure 1.—Survival of *Egfr*^{tm1Mag} homozygous embryos from intercrosses. All crosses were between 129S6/SvEvTAC-*Egfr*^{tm1Mag} and a Swiss-derived, inbred strain. Solid bar, high frequency of survival; dotted bar, intermediate frequency of survival; dotted line, low frequency of survival.

Crosses with NOD/LtJ, NON/LtJ, and SJL/J represent the second group of intercrosses (Table 1; Figure 1); *Egfr*^{tm1Mag} homozygous embryos in these crosses showed a variable penetrance in survival past the mid-gestation lethality observed on the 129S6/SvEvTAC genetic back-

ground. Viable NOD;129, NON;129, and SJL;129 F_2-*Egfr*^{tm1Mag} homozygous embryos compose 1.5, 0, and 6.6% of the total embryos at 18.5 dpc, respectively. Although there is a statistically significant reduction in the number of viable *Egfr*^{tm1Mag} homozygous embryos at 13.5 dpc, a subset of these appear capable of surviving until late gestation; the *Egfr*^{tm1Mag} homozygous embryos surviving to late gestation may carry a complete set of modifying genes from the two parental strains. Interestingly, even though NON/LtJ and NOD/LtJ are sister strains that were derived for a difference in susceptibility to diabetes, with NON/LtJ being a nondiabetic and NOD/LtJ a diabetic strain (Leiter *et al.* 1998), the NON;129 mixed genetic background supports less survival past mid-gestation than NOD;129 does. Only 3% of the embryos collected from NON;129 intercrosses were viable *Egfr*^{tm1Mag} homozygous embryos at 13.5 dpc; no viable *Egfr*^{tm1Mag} homozygous embryos survived to 18.5 dpc in this cross. The observed difference between the two sister strains suggests that during the process of inbreeding, NOD/LtJ inherited *Egfr*^{tm1Mag} modifiers that better complement those on the 129S6/SvEvTAC background to support survival past mid-gestation and even to term at a low penetrance.

The remainder of the Swiss-derived, inbred strains compose the third group (Table 1; Figure 1), characterized by a robust ability to support survival of *Egfr*^{tm1Mag} homozygous mutants past the mid-gestation lethality observed on the 129S6/SvEvTAC background. Intercrosses with ALR/LtJ, ALS/LtJ, APN, APS, and SWR/J all supported viable *Egfr*^{tm1Mag} homozygous mutants to 18.5 dpc, previously observed only on the outbred CD-1 stock. The ALR;129 intercrosses supported the highest survival of *Egfr*^{tm1Mag} homozygous mutants, representing 19.7% of the total embryos at 18.5 dpc. Likewise, the sister strain to ALR/LtJ, ALS/LtJ, also contains modifiers capable of supporting survival past mid-gestation, with 14.9% of the total embryos being viable *Egfr*^{tm1Mag} homozygous mutants at 18.5 dpc; ALR/LtJ and ALS/LtJ were derived for their resistance or sensitivity, respectively, to alloxan-induced diabetes (Graser *et al.* 1999). However, in ALR;129 intercrosses, most of the surviving *Egfr*^{tm1Mag} homozygous mutants at 18.5 dpc were viable, whereas nonviable *Egfr*^{tm1Mag} homozygous progeny compose 10% of the total embryos from ALS;129 intercrosses. Interestingly, ALS/LtJ is more closely related to NON/LtJ than to NOD/LtJ on the basis of allelic similarity at microsatellite repeat polymorphisms (Graser *et al.* 1999), and both ALS/LtJ and NON/LtJ are less able than their sister strains to support survival of *Egfr*^{tm1Mag} homozygous mutants. This correlation yields additional support for the segregation of different modifiers from a larger heterogenous pool present in the founder Swiss-derived populations, with some inbred strains supporting functional rescue of the *Egfr*-dependent placental defect much better than other strains.

APS and APN represent a third pair of sister inbred strains derived for differential response to acetaminophen (Casley *et al.* 1997). Unlike the other sister strains, viable *Egfr*^{tm1Mag} homozygous progeny from APS;129 and APN;129 intercrosses are present in approximately equal frequencies at 18.5 dpc, composing 12.9 and 11.5% of total embryos, respectively. However, the APS;129 and APN;129 strain combinations, unlike ALR;129 and ALS;129, show a differential penetrance of surviving *Egfr*^{tm1Mag} homozygous mutants at mid-gestation, representing 18.4 and 13.6%, respectively. Nonetheless, viable *Egfr*^{tm1Mag} homozygous embryos make up 18.4 and 18.3% of the total embryos at 13.5 dpc in the nonsister strain combinations APS;129 and ALS;129, respectively. This observation suggests that different *Egfr* modifiers from those needed to support development during late gestation may be necessary to support survival of *Egfr*^{tm1Mag} homozygous mutants past mid-gestation.

The final strain analyzed through intercrosses, SWR/J, was initially derived for an increased susceptibility to tumor formation (Heston 1963). Viable *Egfr*^{tm1Mag} homozygous progeny from SWR;129 intercrosses composed 21.3 and 12.6% of all embryos at 13.5 and 18.5 dpc, respectively (Table 1; Figure 1). The SWR/J genetic background contains modifiers that support robust survival through mid-gestation, but with reduced survival during late gestation.

Variable survival of *Egfr*^{tm1Mag} homozygotes from congenic strains: Since extended survival of *Egfr*^{tm1Mag} homozygous mutants is dependent on genetic background and has been observed only on outbred or mixed backgrounds generated by intercrosses, the inability to genetically isolate modifiers in crosses using outbred stocks may also have been due to heterosis of the hybrid backgrounds. To test this possibility, the *Egfr*^{tm1Mag} mutation was bred onto eight inbred backgrounds, generating a panel of congenic lines and the original co-isogenic 129S6/SvEvTAC strain. The strains were chosen to maximize genetic diversity (Beck *et al.* 2000) and include 129S6/SvEvTAC, AKR/J, APN, BALB/cJ, BTBR-*T*$^{+}$ *tf/tf*, C3H/HeJ, C57BL/6J, DBA/2J, and FVB/J.

Although we cannot formally exclude some role for heterosis, analysis of the *Egfr*^{tm1Mag} congenic lines also revealed three phenotypic groups arguing against heterosis playing a significant role in the survival of *Egfr*^{tm1Mag} homozygous mutants. The three groups of strains, similar to the groups observed with the intercrosses, include strains that do not support survival past mid-gestation, those that show incomplete penetrance of survival past mid-gestation, and those that show high penetrance of mutant survival past mid-gestation (Table 2; Figure 2). The first group of congenic strains, BALB/cJ, BTBR-*T*$^{+}$ *tf/tf*, and FVB/NJ, resemble the phenotype previously described for 129S6/SvEvTAC (Threadgill *et al.* 1995), with no viable *Egfr*^{tm1Mag} homozygous embryos surviving to 13.5 dpc. Interestingly, homozygosity for *Egfr*^{tm1Mag} appears to be even more severe on an FVB/NJ background than on the 129S6/SvEvTAC background, with viable FVB/NJ-*Egfr*^{tm1Mag} mutant embryos composing only 6.7% of embryos at 10.5 dpc. The FVB/NJ

TABLE 2

Survival of *Egfr*^{tm1Mag} homozygous embryos from congenic strains

Congenic strain	Age (dpc)	No. of embryos +/+:+/−:−/−[b]	*Egfr*^{tm1Mag} embryos (%)	Viable *Egfr*^{tm1Mag} embryos (%)	No. of litters
FVB/NJ	10.5	30:46:13**	14.6	6.7	11
	13.5	26:56:7**	7.9	1.1	7
BALB/cJ	10.5	17:27:14*	24.1	17.2	6
	13.5	22:26:14**	22.6	0.0	6
BTBR-*T*$^{+}$ *tf/tf*	10.5	1:1:1	33.0	33.0	1
	13.5	11:19:8**	21.0	0.0	3
	18.5	4:18:2**	8.3	0.0	3
129S6/SvEvTAC	10.5	6:12:13	42.0	29.0	4
	13.5[a]	**		0.0	
C57BL/6J	10.5	29:20:18	26.9	17.9	10
	13.5	19:35:21**	28.0	5.3	10
	18.5	17:19:6**	14.3	0.0	7
DBA/2J	10.5	10:14:7	22.6	16.1	5
	13.5	17:25:5**	10.6	4.3	6
	18.5	22:33:3**	5.2	3.4	9
AKR/J	13.5	27:18:7	13.5	13.5	7
	18.5	22:18:6**	13.0	6.5	5
APN	13.5	6:21:5	15.6	12.5	2
	18.5	8:11:2	9.5	9.5	2
C3H/HeJ	13.5	4:5:4	31.0	15.4	2
	18.5	3:6:3	25.0	8.3	2

[a] Previously determined that no viable *Egfr*^{tm1Mag} homozygous embryos are present at 13.5 dpc (Threadgill *et al.* 1995).

[b] *P*-values: *, <0.05 and **, <0.01 when using a χ^2 test for deviation from the expected 25% recovery of viable *Egfr*^{tm1Mag} homozygous embryos.

results are reminiscent of *Egfr*^{tm1Mag} survival observed on the ICR/HaROS intercross or originally reported for the outbred CF-1 stock (Threadgill *et al.* 1995).

On C57BL/6J and DBA/2J genetic backgrounds, representing the second group, viable *Egfr*^{tm1Mag} homozygous embryos are recovered at expected ratios at 10.5 dpc, but are greatly reduced by 13.5 dpc composing only 5.3 and 4.3% of the viable embryos at mid-gestation, respectively. By 18.5 dpc, no viable *Egfr*^{tm1Mag} homozygous embryos are detected on the C57BL/6J genetic background, which may indicate that placentas from *Egfr*^{tm1Mag} homozygotes can support fetal development to mid-gestation with a low penetrance, but is further compromised at some point later during gestation. In contrast, on a DBA/2J background 3.4% of the embryos at 18.5 dpc are viable *Egfr*^{tm1Mag} homozygotes, similar to the 4.3% frequency observed at mid-gestation. This result suggests that although the DBA/2J background supports survival of *Egfr*^{tm1Mag} homozygous embryos only at a low penetrance through mid-gestation, those that do survive will do so to late gestation.

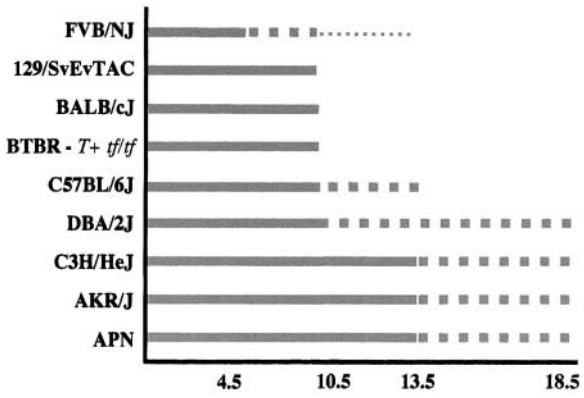

Figure 2.—Survival of *Egfr*^{tm1Mag} homozygous embryos on congenic backgrounds. Solid bar, high frequency of survival; dotted bar, intermediate frequency of survival; dotted line, low frequency of survival.

The third group, composed of AKR/J, APN, and C3H/HeJ backgrounds, supports relatively robust survival past mid-gestation, but is less able to support survival through late gestation. At 18.5 dpc, 6.5, 9.5, and 8.3% of the embryos are viable *Egfr*^{tm1Mag} homozygous mutants on AKR/J, APN, and C3H/HeJ backgrounds, respectively. However, the surviving *Egfr*^{tm1Mag} homozygous mutants are runted, exhibit failure of eyelid closure, and have smaller placentas when compared to wild-type littermates.

Despite these differences, occasional *Egfr*^{tm1Mag} homozygous escapers can be observed. For example, rare *Egfr*^{tm1Mag} homozygous mutants are born on 129S6/SvEvTAC and BTBR-*T*$^{+}$ *tf/tf* backgrounds while a single viable mutant was observed on the FVB/NJ background

TABLE 3

Survival of *Egfr*^{tm1Mag} homozygous F_1 hybrid embryos

Hybrids	Age (dpc)	No. of embryos +/+:+/−:−/−[a]	*Egfr*^{tm1Mag} embryos (%)	Viable *Egfr*^{tm1Mag} embryos (%)	No. of litters
FVB129 F_1	13.5	49:71:28**	18.9	8.1	18
	18.5	39:69:15**	12.2	8.1	15
BALB129 F_1	13.5	17:43:29	32.6	14.6	13
	18.5	20:45:14*	17.7	13.9	10
C57129 F_1	13.5	22:23:9	16.7	16.7	7
	18.5	22:22:11*	20.0	10.9	9
FVBBALB F_1	13.5	15:31:20	30.3	12.1	6
	18.5	19:29:5**	9.4	3.8	6
C57FVB F_1	13.5	8:17:12	32.4	27.0	5
	18.5	14:34:4**	7.7	1.9	5

[a] *P*-values: *, < 0.05 and **, < 0.01 when using a χ^2 test for deviation from the expected 25% recovery of viable *Egfr*^{tm1Mag} homozygous embryos.

at mid-gestation, suggesting that developmental stochastism also contributes to intrastrain variability.

Genomic complementation between *Egfr*^{tm1Mag} congenic lines: To further support the existence of heterogeneous groups of modifiers, pairwise F_1 hybrid *Egfr*^{tm1Mag} homozygous embryos were produced by outcrossing the congenic lines incapable of supporting development past mid-gestation (Table 3; Figure 3). While combining the genomes of inbred lines in F_1 hybrids appears to confer the ability to support survival past mid-gestation, albeit with variable penetrance, not all combinations of genetic backgrounds result in equivalent survival and none of the hybrid backgrounds support survival past mid-gestation to the level observed in the outbred CD-1 stocks, suggesting that the alleles carried by these strains either are not robust modifiers or are insufficient in number to provide complete support for embryonic survival in the absence of *Egfr*.

Although neither the FVB/NJ nor BALB/cJ backgrounds alone support survival of *Egfr*^{tm1Mag} homozygous embryos past 13.5 dpc (Table 2), when either background is crossed to the 129S6/SvEvTAC background, embryonic survival is supported at moderate levels past mid-gestation, with all of these embryos surviving to term. FVB129 and BALB129 F_1-*Egfr*^{tm1Mag} homozygous embryos represent 8.1 and 13.9% of the total viable embryos at 13.5 dpc, respectively, similar to the numbers surviving at 18.5 dpc (Table 3). Interestingly, all C57129 F_1 embryos were viable at 13.5 dpc; however, by 18.5 dpc viable *Egfr*^{tm1Mag} homozygous embryos account for only 10.9% of the total embryos (Table 3), a reduction of ~50% over the total number of *Egfr*^{tm1Mag} homozygous embryos observed.

Viable *Egfr*^{tm1Mag} homozygous embryos of FVBBALB F_1 hybrids account for 12.1% of the total embryos at 13.5 dpc, similar to that observed when either strain is crossed to 129S6/SvEvTAC, but unlike the prior crosses, survival drops to 3.8% at 18.5 dpc. While half of the FVBBALB F_1-*Egfr*^{tm1Mag} homozygous embryos and all of those from C57FVB F_1 are viable at 13.5 dpc, both genome combinations show a dramatic reduction in survival of *Egfr*^{tm1Mag} homozygous embryos by 18.5 dpc. Thus the combination of either BALB/cJ or C57BL/6J with the FVB/NJ genome results in robust survival to mid-gestation, but a significant reduction in the number of viable *Egfr*^{tm1Mag} homozygous embryos occurs by 18.5 dpc, with only 3.8 and 1.9% viable FVBBALB and C57BALB F_1-*Egfr*^{tm1Mag} homozygous embryos, respectively (Table 3). This indicates that the FVB/NJ genome potentially lacks or has a reduction in the number or strength of modifiers for late gestation. Potentially, the FVB/NJ genetic background may harbor modifiers that exhibit a negative effect on other modifiers of the *Egfr*^{tm1Mag} homozygous phenotype since FVB/NJ, when congenic for the *Egfr*^{tm1Mag} mutation, only partially supports survival to 10.5 dpc (Table 2).

Robust, cross-independent recovery of *Egfr*^{tm1Mag} homozygous mutants with ALR/LtJ: As an initial step to identify modifiers influencing survival of *Egfr*^{tm1Mag} ho-

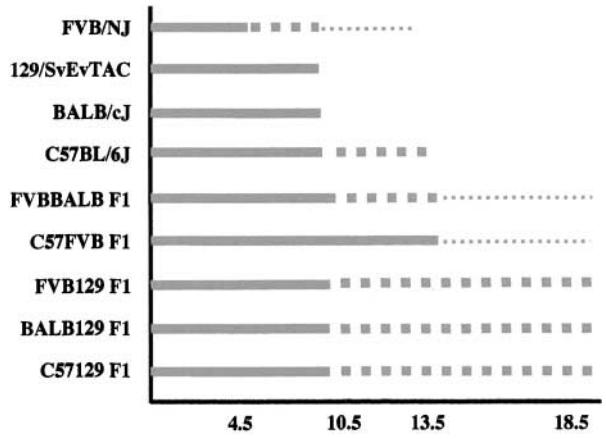

FIGURE 3.—Survival of F_1 hybrid *Egfr*^{tm1Mag} homozygous embryos from outcrosses. Solid bar, high frequency of survival; dotted bar, intermediate frequency of survival; dotted line, low frequency of survival.

TABLE 4

Survival of *Egfr*^{tm1Mag} homozygous embryos from ALR/LtJ crosses

Cross	Age (dpc)	Wild-type embryos[a] (+/+ and +/−)	*Egfr*^{tm1Mag} homozygous embryos[a]			No. of litters	*P*-value
			Viable	Dead	Resorbed		
(ALR129) × 129	18.5	186 (76)	26 (10.6)	7 (2.8)	26 (10.6)	19	0.74
129 × (ALR129)	18.5	58 (75)	9 (12)	5 (6.5)	5 (6.5)	8	0.95
ALR;129 F_2	18.5	59 (77.7)	15 (19.7)	2 (2.6)	0 (0)	6	0.37

[a] Total number of embryos of each genotype and class. Percentage of total embryos is in parentheses.

mozygous embryos using inbred strains and bypassing the genetic heterogeneity present in outbred stocks, ALR129 F_1-*Egfr*^{tm1Mag} mice were intercrossed or backcrossed to 129S6/SvEvTAC-*Egfr*^{tm1Mag} mice to generate ALR;129 F_2 and N_2 progeny, respectively, ostensibly segregating ALR/LtJ-derived loci contributing to the robust survival observed with this genetic background (Tables 1 and 4). Although only 11% were viable *Egfr*^{tm1Mag} homozygous embryos in the backcrosses, roughly half that observed in the intercrosses, no statistically significant reduction in the total number of *Egfr*^{tm1Mag} homozygous progeny was observed at 18.5 dpc, with 22–25% of the recovered embryos being homozygous for *Egfr*^{tm1Mag} in all crosses. The nonviable *Egfr*^{tm1Mag} homozygous mutants at 18.5 dpc had died within a day or two prior to collection. These results suggest that the ALR/LtJ genome has captured multiple survival modifiers for *Egfr*^{tm1Mag} mutants from the genetic heterogeneity originally detected in the outbred CD-1 stocks. These alleles need to be partitioned further via subsequent backcrossing or alternative approaches to efficiently identify individual or groups of modifiers capable of altering survival of *Egfr*^{tm1Mag} homozygous mutants.

Molecular heterogeneity of *Egfr*^{tm1Mag} modifiers revealed by microarray analysis: Microarray technology, widely used to distinguish tumor classes on the basis of global similarities to steady-state differences in cellular transcript levels (DeRisi *et al.* 1996; Perou *et al.* 1999; Rickman *et al.* 2001), was used to investigate the genetic heterogeneity associated with the number and intensity of modifying alleles segregating in the ALR;129 crosses. For the microarrays, total placental RNA was extracted from viable 18.5 dpc wild-type and *Egfr*^{tm1Mag} homozygous embryos and compared to a common reference RNA. The placental samples were obtained from N_2 embryos, produced by backcrossing ALR129 F_1-*Egfr*^{tm1Mag} females to 129S6/SvEvTAC-*Egfr*^{tm1Mag} males such that individual samples would be segregating heterogeneous ALR/LtJ-derived alleles capable of modifying survival of *Egfr*^{tm1Mag} homozygous embryos.

Gene expression profiles using placental RNA from five individual *Egfr*^{tm1Mag} homozygous and three wild-type matched littermate controls were analyzed to reveal numerous differentially expressed genes between wild-type and *Egfr*^{tm1Mag} homozygous placentas (Figure 4). Furthermore, the level of individual variation across the two genotypic groups molecularly confirmed the existence of extensive genetic heterogeneity across individual placentas and indicates that microarrays will be a generally useful tool for partitioning genetic heterogeneity contributing to complex traits.

After normalization, 4462 valid probes were retained across all eight hybridizations, revealing interesting trends after hierarchical clustering of the data from each placenta (Figure 4A). While variation across wild-type placentas was observed, the extent and levels of variation are significantly less than that observed across the *Egfr*^{tm1Mag} homozygous placentas. The significant gene expression variation observed across the individual placenta from *Egfr*^{tm1Mag} homozygous embryos suggests that not all *Egfr*^{tm1Mag} homozygous placentas are molecularly similar. This supports the genetic data indicating that the *Egfr*^{tm1Mag} modifiers are genetically heterogeneous and that survival of *Egfr*^{tm1Mag} homozygous embryos is due to differential segregation of a heterogeneous group of modifying genes. Even with these limited data, it appears that there are at least two major classes of *Egfr*^{tm1Mag} homozygous placentas at 18.5 dpc: those that are more similar to the wild-type placental gene profile (Figure 4A, samples 4–5) and those that show more divergence in gene expression (Figure 4A, samples 6–8).

The eight hybridizations were further analyzed by sampling only those genes with a twofold or greater change in gene expression across all valid spots, resulting in 141 genes meeting the more stringent criteria (Figure 4B). Similar to the entire data set, the 141 genes in the three wild-type samples were generally more similar in expression than those in the five *Egfr*^{tm1Mag} homozygous placentas with greater heterogeneity noted across the *Egfr*^{tm1Mag} homozygous samples. Gender of the embryos did not influence the clustering results (data not shown). Likewise, littermates did not cluster together, suggesting that the observed molecular profiles are dependent on the alleles carried by the individual placental samples.

DISCUSSION

The placenta is a highly specialized yet transitory organ necessary for nutrient and waste exchange between the mother and the developing fetus in mammals and

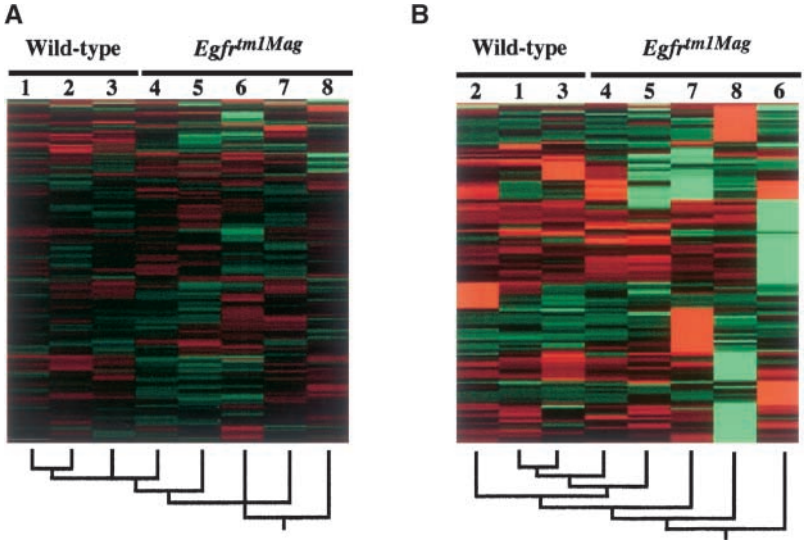

FIGURE 4.—Clustering of placental samples using transcriptional data obtained from microarrays. All samples were from 18.5 dpc placentas. (A) One-dimensional clustering of placental samples using 4462 genes differentially expressed among samples. (B) One-dimensional clustering of placental samples using the 141 genes with a twofold or greater difference in gene expression among samples. Brackets represent relative similarity in overall transcriptional profiles with more related samples appearing closer together and with shorter brackets. Samples 1–3 and 4–8 represent placentas collected from *Egfr* wild-type and *Egfr*^{tm1Mag} homozygous mutant embryos, respectively.

is composed of multiple cell types, including mesenchymal cells, endothelial cells of the labyrinthine vasculature, hormone-secreting and glycogen cells of the spongiotrophoblast, and trophoblast giant cells. Given the diverse roles of the various cell types in the placenta, variability in lethality of *Egfr*^{tm1Mag} homozygous mutants may be caused by functional differences in hormone production, cytokine signaling, transcription factors, cell cycle regulation, and growth factor signaling in one or more cell types.

One possible mechanism to explain the variable phenotypes observed with *Egfr*^{tm1Mag} homozygous embryos is heterosis. However, the current results do not support this mechanism as heterosis cannot explain the reduced survival of *Egfr*^{tm1Mag} homozygous embryos from NON;129 and ICR;129 intercrosses. If heterosis were to account for the ability to rescue the mid-gestation lethality and the inability to genetically map specific modifiers, one would expect a uniform rescue across various hybrid backgrounds, which was not observed in this study. Furthermore, since *Egfr*^{tm1Mag} homozygous embryos can survive, albeit at a low penetrance, to birth on the inbred AKR/J, APN, C3H/HeJ, and DBA/2J genetic backgrounds (Table 2), homozygosity is sufficient to support survival providing the optimal combination of alleles is present. Likewise, the *Egfr*^{tm1Mag} null mutation on a non-Swiss-derived, outbred CF-1 background is peri-implantation lethal at e4.5, which is earlier than would be expected if heterosis was responsible for the genetic variation observed.

A much more likely explanation for *Egfr*^{tm1Mag} phenotypic variability is through the action of modifier genes. Two groups of strains were used in this study to investigate the genetic architecture of *Egfr*^{tm1Mag} modifiers: the Swiss-derived strains, ALR/LtJ, ALS/LtJ, APN, APS, ICR/HaRos, NON/LtJ, NOD/LtJ, SWR/J, and SJL/J, in intercrosses and a panel of genetically diverse strains, 129S6/SvEvTAC, AKR/J, APN, BALB/cJ, BTBR-*T*$^{+}$ *tf/tf*, C3H/HeJ, C57BL/6J, DBA/2J, and FVB/NJ, developed into congenics. Most of the Swiss-derived strains have the ability, at least partially, to support survival of *Egfr*^{tm1Mag} homozygous mutants through mid-gestation, while more distantly related strains, like AKR/J, APN, and C3H/HeJ, and to a lesser extent DBA/2J, also have this ability. Although many Swiss-derived strains modify the mid-gestation lethality similarly, the data in their entirety suggest that there are differential effects of modifying genes segregating among the various genetic backgrounds (Table 5). Our data also suggest that EGFR is required at three distinct stages of development: prior to mid-gestation, at mid-gestation, and during late gestation (Figure 5). Various combinations of overlapping or independent modifiers likely act at critical stages throughout gestation and contribute to the genetic heterogeneity observed in previous attempts to map modifiers of the *Egfr*^{tm1Mag} homozygous phenotype.

The ICR/HaRos and FVB/NJ backgrounds resemble the outbred CF-1 background in that the embryo is already compromised prior to the switch from yolk sac to the placental-mediated nutrient exchange that occurs at mid-gestation. The FVB/NJ strain, a Swiss-derived strain, appears to exhibit the same inability to support *Egfr*^{tm1Mag} homozygous embryo survival that is observed with ICR;129 intercrosses, but not as severely as the non-Swiss-derived, outbred CF-1. This indicates that the first critical window for the EGFR activity during development occurs prior to 10.5 dpc. Since, the *Egfr*^{tm1Mag} mutation on FVB/NJ and ICR/HaRos backgrounds cannot support survival to mid-gestation, they either lack specific modifying alleles or have strong suppressor alleles. Furthermore, of the parental strains not supporting survival past mid-gestation, the BALB.129 and FVB.129 F_1 hybrids most effectively rescue the placental mid-gestation lethality. However, combinations of FVB/NJ and BALB/cJ show a reduced ability to rescue the mid-gestation lethality. These observations suggest that the FVB/NJ

TABLE 5

Predicted classes of modifiers present in different inbred strains

Strain	Group[a]	Presence of modifiers[b]		
		Prior to mid-gestation (4.5–9.5 dpc)	Mid-gestation (10.5–13.5 dpc)	After mid-gestation (14.5–18.5 dpc)
FVB/NJ	1	N	N	ND[c]
ICR/HaROS	1	N	N	ND
129S6/SvEvTAC	2	Y	N	ND
BALB/cJ	2	Y	N	ND
BTBR-T^{+} *tf/tf*	2	Y	N	ND
NON/LtJ	2	Y	N	N
NOD/LtJ	2	Y	N	N
C57BL/6J	2	Y	Y/N	N
SJL/J	2	Y	Y/N	Y
DBA/2J	2	Y	Y/N	Y
AKR/J	3	Y	Y	Y/N
C3H/HeJ	3	Y	Y	Y/N
SWR/J	3	Y	Y	Y/N
ALR/LtJ	3	Y	Y	Y
ALS/LtJ	3	Y	Y	Y
APN	3	Y	Y	Y
APS	3	Y	Y	Y

[a] Category of strains based upon level of support for survival of *Egfr*^{tm1Mag} homozygous mutants.
[b] Predicted class modifiers that are present (Y), absent (N), or weakly present (Y/N) from each strain.
[c] ND, cannot determine since embryos did not survive long enough.

and BALB/cJ genetic backgrounds contain similar overlapping and noncomplementing alleles modifying mid-gestation survival.

The second critical window for EGFR activity occurs at mid-gestation when the placenta replaces the yolk sac as the primary mediator of nutrient and waste exchange. Most of the strains tested are able to survive, at least with reduced penetrance, past mid-gestation. Combinations among ALR/LtJ, ALS/LtJ, APN, and APS and 129S6/SvEvTAC as well as AKR/J, APN, and C3H/HeJ and, to a lesser extent, C57BL/6J, DBA/2J, NOD/LtJ, SJL/J, and SWR/J, all support viable *Egfr*^{tm1Mag} homozygous embryos at 13.5 dpc. Given the robust and highly heterogeneous composition of *Egfr*^{tm1Mag} homozygous modifiers in Swiss-derived, outbred mice, it is not surprising that during the process of inbreeding, most strains captured alleles that support survival of *Egfr*^{tm1Mag} homozygous embryos at some level through mid-gestation. The alleles involved in modifying the *Egfr*^{tm1Mag} homozygous phenotype appear to be numerous and since they also confer an increase in fecundity via increases in viability, they may have been inadvertently selected for during the derivation of the Swiss-derived, inbred strains.

The third window for activity of *Egfr*^{tm1Mag} modifiers

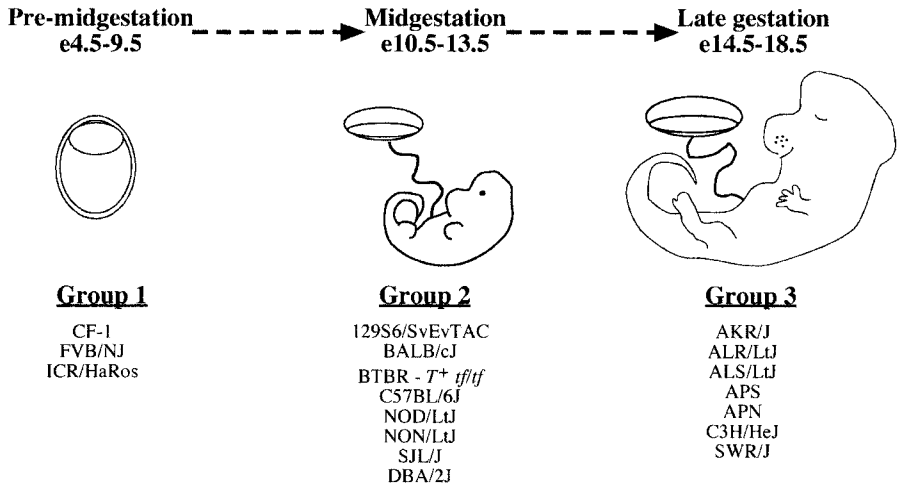

FIGURE 5.—Schematic of embryonic lethality of *Egfr*^{tm1Mag} homozygous mutants. The time line depicts when the majority of *Egfr*^{tm1Mag} homozygous embryos on specific strains are affected by modifying alleles. Group 1 strains primarily die prior to mid-gestation, group 2 at mid-gestation, and group 3 during late gestation.

occurs between 13.5 and 18.5 dpc of gestation. For ALR;129, ALS;129, APS;129, APN;129, and SJL;129 intercross progeny homozygous for *Egfr*^{tm1Mag} as well as the congenic APN and DBA/2J strains, it appears that if the embryos can live past 13.5 dpc, then they will survive to term. The comparable numbers of viable embryos observed at 13.5 and 18.5 dpc for each of these strains compared to strains with similar survival at 13.5 dpc but with dramatically reduced survival at 18.5 dpc, like AKR/J, C3H/HeJ, and SWR;129 intercrosses, further support the hypothesis for a third critical window during development when the EGFR is needed.

Currently the dissection of complex traits has moved to the forefront of genetics with many disease processes being controlled or influenced by complex genetic interactions. The experiments with *Egfr*^{tm1Mag} homozygous phenotypes are complicated by the fact that the quantitative variable is the time of embryonic survival. Consequently, this trait requires the use of alternative methods for identifying the modifier genes. Microarray technology is one method that has been used frequently to partition genetic heterogeneity using the relative change in gene expression between samples (Jansen and Nap 2001; Wayne and McIntyre 2002). The advantage of microarrays is that the technique enables large-scale analysis of many genes concurrently, thus giving a global view of the relationship between samples or individuals at the molecular level. In the case of the *Egfr* locus, the use of microarrays may allow for the identification of subclasses of mutants that possess similar or identical sets of modifiers that segregate in an otherwise heterogeneous background. Analysis of many samples may permit the partitioning of the *Egfr*^{tm1Mag} homozygous survivors into distinct molecular classes; these individual classes can then be potentially linked through classical quantitative trait analysis to specific genomic regions modifying the placental phenotype. Preliminary analysis of five *Egfr*^{tm1Mag} homozygous placentas shows variation in gene expression across individual placentas, consistent with a heterogeneous modification at the molecular level.

There are limits to the information that can be mined from gene expression profiling. DNA microarrays estimate only the relative levels of transcription between two samples, but within the cell there are many other levels of regulation that are being carried out, such as enzyme activity, protein-protein interactions, translational regulation, and phosphorylation. Using microarrays, the effects of signal transduction pathways, such as the EGFR pathway, are observed only at the end of the pathways when transcription is either up- or down-regulated. It is possible that the homozygosity for the *Egfr*^{tm1Mag} allele has an effect on the intermediate steps of the signaling pathway and thus will not be detected directly by the microarray analyses. However, the preliminary data presented here suggest that there is variation across individual *Egfr*^{tm1Mag} homozygous placentas and that it is likely that the variation observed is due to, or influenced by, the segregation of genes that modify the *Egfr*^{tm1Mag} placental phenotype.

The role of EGFR during placental development is proving to be complex, and the EGFR-deficient phenotype has the potential for being modified by many genes. Since the phenotypic consequences of *Egfr*^{tm1Mag} phenotype modification can be attributed to many genes segregating within individual animals, their detection has remained elusive. Furthermore, the variation in the *Egfr*^{tm1Mag} homozygous phenotypes may be attributed to the presence of many interchangeable modifier variants with strong effects or to larger groups of genes, with each gene having only a small effect on the *Egfr*^{tm1Mag} phenotype. When all of these genes are combined in the appropriate context, the resulting genetic complement has a synergistic effect on the *Egfr*^{tm1Mag} homozygous phenotype. The genetic studies presented here support the existence of multiple genes of varying strength that can modify the *Egfr*^{tm1Mag} homozygous phenotype.

The current results also have significant implications for various disease states. For example, aberrant EGFR signaling has been implicated in intrauterine growth retardation (IUGR) and in cancer (Dassonville *et al.* 1993; Rusch *et al.* 1993; Fondacci *et al.* 1994; Grandis *et al.* 1998; Tewari *et al.* 2000; Umekita *et al.* 2000). EGFR inhibitors are currently being widely tested to treat many forms of cancer and the early results have been mixed (Arteaga and Baselga 2003; Grunwald and Hidalgo 2003; Mendelsohn and Baselga 2003). Modifying genes that compensate for EGFR loss in the *Egfr*^{tm1Mag} homozygous mutants may also contribute to resistance to anti-EGFR therapy. Consequently, it is probable that EGFR inhibitors currently being evaluated in human clinical trials will have highly variable rates of success that are determined by an individual patient's genetic background and the *EGFR* modifying alleles that they harbor. Similarly, the *Egfr*^{tm1Mag}-modifying genes present in mouse strains may provide useful diagnostic markers and therapeutic targets to treat IUGR and to identify patients that will respond to anti-EGFR therapy.

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