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Altered trophoblast proliferation is insufficient to account for placental dysfunction in *Egfr* null embryos

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Abstract

Homozygosity for the *Egfr*^{tm1Mag} null allele in mice leads to genetic background dependent placental abnormalities and embryonic lethality. Molecular mechanisms or genetic modifiers that differentiate strains with surviving versus non-surviving *Egfr* nullizygous embryos have yet to be identified. *Egfr* transcripts in wildtype placenta was quantified by ribonuclease protection assay (RPA) and the lowest level of *Egfr* mRNA expression was found to coincide with *Egfr*^{tm1Mag} homozygous lethality. Immunohistochemical analysis of ERBB family receptors, ERBB2, ERBB3, and ERBB4, showed similar expression between *Egfr* wildtype and null placentas indicating that *Egfr* null trophoblast do not up-regulate these receptors to compensate for EGFR deficiency. Significantly fewer numbers of bromodeoxyuridine (BrdU) positive trophoblast were observed in *Egfr* nullizygous placentas and *Cdc25a* and *Myc*, genes associated with proliferation, were significantly down-regulated in null placentas. However, strains with both mild and severe placental phenotypes exhibit reduced proliferation suggesting that this defect alone does not account for strain-specific embryonic lethality. Consistent with this hypothesis, intercrosses generating mice null for cell cycle checkpoint genes (*Trp53*, *Rb1*, *Cdkn1a*, *Cdkn1b* or *Cdkn2c*) in combination with *Egfr* deficiency did not increase survival of *Egfr* nullizygous embryos. Since complete development of the spongiotrophoblast compartment is not required for survival of *Egfr* nullizygous embryos, reduction of this layer that is commonly observed in *Egfr* nullizygous placentas likely accounts for the decrease in proliferation.

1. Introduction

EGFR is the prototypical member of a family of related receptor tyrosine kinases (RTKs) that includes ERBB2, ERBB3, and ERBB4. The *Egfr* gives rise to multiple alternatively spliced and polyadenylated transcripts [1]. Phenotypic analysis of homozygous *Egfr* null mutants in mice exhibit peri-implantation to post-natal lethality, depending on the genetic background of the mouse [2,3]. *Egfr* homozygous null embryos on a 129/Sv background die around 11.5 days post-coitus (dpc) due to abnormal placental development, with a reduced spongiotrophoblast

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(ST) layer and severe disorganization of the labyrinth trophoblast (LT) layer [4]. On an outbred CD-1 stock the ST layer is similarly reduced, but there is rescue of the disorganized LT layer allowing *Egfr* null embryos to survive to birth. A more comprehensive characterization of *Egfr* null embryonic lethality on many genetic backgrounds revealed that the timing of lethality varies widely between strains [5]. Several Swiss-derived strains, on either a congenic FVB/NJ or ICR/HaROS.129F1 backgrounds exhibit lethality prior to 10.5 dpc. Similar to the 129/Sv strain LT defects are observed beginning at 11.5 dpc in many strains which do not support embryonic survival, including congenic BALB/cJ and BTBR-*T⁺ tf/J*. Some backgrounds, such as ALR.129F1 and to a lesser extent FVB.129F1 and BALB.129F1, support robust survival of *Egfr* null embryos past midgestation although null placentas are smaller than those from wildtype embryos. The existence of placental phenotypes that are strain specific suggests the effects of EGFR deficiency on normal growth and differentiation of placenta are mediated by background-specific modifiers.

Two separate mapping crosses have failed to yield significant quantitative trait loci associated with survival of *Egfr* nullizygous embryos suggesting the existence of many modifiers with complex relationships [5]. In addition, the molecular mechanism contributing to the *Egfr* null placental phenotype has yet to be elucidated. Placental defects have been reported in animals deficient for a number of signaling molecules downstream of EGFR. Mice deficient for the adaptor proteins GRB2 and GAB1, Ras-specific guanine nucleotide exchange factor, SOS1 and its target KRAS, components of MAPK cascades including RAF1, MAPK2K1, ERK2 and MAPK14 (p38), and downstream transcription factors JUNB, ETS2, and FOS all exhibit LT defects and embryonic lethality at midgestation [6–16]. Together these data are consistent with MAPK signaling being required for normal placental development and suggests that *Egfr* null strains surviving past midgestation probably use alternate pathways to activate MAPK signaling. Consistent with the importance of EGFR signaling for normal placental development, EGFR is expressed in human placenta and altered expression has been associated with intrauterine growth restriction (IUGR), preeclampsia, and placenta accreta [17–22].

In this study, we investigated potential mechanisms responsible for differential survival in the absence of EGFR signaling, including whether there is a compensatory upregulation of other ERBB receptors. We also measured proliferation and apoptosis in EGFR-deficient placentas. Lastly, mice double mutant for *Egfr* and either *Trp53*, *Rb1*, *Cdkn1a*, *Cdkn1b* or *Cdkn2c* were generated.

2. Materials and methods

2.1. Mice and genetic crosses

A null allele for *Egfr* (*Egfr^{tm1Mag}*) on outbred CD-1 stock or inbred 129/Sv, ALR/LtJ, FVB/NJ, C57BL/6J, and BALB/cJ strains have been previously described [5]. 129S1/SvImJ and 129S6/SvEvTAC strains were used interchangeably due to their highly similarity [23]. Males heterozygous for the *Egfr^{tm1Mag}* mutation, on either an ALR/LtJ, FVB/NJ, C57BL/6J, or BALB/cJ genetic background, were mated to 129/Sv-*Egfr^{tm1Mag}* females to generate F1-*Egfr^{tm1Mag}* homozygous embryos and *Egfr^{tm1Mag}* heterozygous adults. The ALR.129F1 heterozygous adults were backcrossed to 129/Sv-*Egfr^{tm1Mag/+}* to obtain N2 backcross embryos. Embryos and mice were genotyped by PCR for presence of the *Egfr^{tm1Mag}* allele as previously described [5].

Noon on the day that copulation plugs were observed was designated as 0.5 days post-coitus (dpc). Pregnant females were euthanized by CO₂ asphyxiation and embryos and placentas dissected from the uterine horns on the morning of 9.5 through 18.5 dpc into phosphate buffered saline (PBS). The placenta and extra-embryonic tissues were separated from the embryo by mechanical dissection and either whole embryos before 10.5 dpc or tail biopsies after 10.5 dpc

were collected for DNA extraction to determine the genotype of each individual embryo. Placentas were either flash frozen or preserved in RNAlater (Ambion) for extraction of RNA or fixed in 10% NBF (neutral buffered formalin) for histological analysis.

129/Sv-*Egfr^{tm1Mag}* heterozygotes were intercrossed with mice heterozygous for *Trp53^{tm1Tyj}*, *Rb1^{tm1Tyj}*, *Cdkn1a^{tm1Tyj}*, or *Cdkn1b^{tm1Mlf}* null alleles maintained on a similar 129/Sv background. *Egfr^{tm1Mag}* heterozygous mice from a BALB/cJ background were intercrossed with mice heterozygous for *Trp53^{tm1Tyj}*, *Rb1^{tm1Tyj}*, or *Cdkn2c^{tm1Yxi}* null alleles, also on a BALB/cJ background. Double heterozygous mice were intercrossed and embryos with placentas were collected and genotyped at 13.5 dpc (see Table 2). To increase numbers of double homozygous embryos, some *Trp53^{tm1Tyj}* and *Cdkn1a^{tm1Tyj}* crosses were set up by intercrossing animals homozygous for *Trp53^{tm1Tyj}* or *Cdkn1a^{tm1Tyj}*, and heterozygous for *Egfr^{tm1Mag}*, *Trp53^{tm1Tyj}*, *Rb1^{tm1Tyj}*, *Cdkn1a^{tm1Tyj}*, *Cdkn1b^{tm1Mlf}* and *Cdkn2c^{tm1Yxi}* null alleles were amplified by PCR and detected as previously described [24–27].

Mice were fed Purina Mills Lab Diet 5058 or 5010 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 an Institutional Animal Care and Use Committee.

2.2. Ribonuclease protection assay

Wildtype samples were collected daily from 9.5 to 16.5 dpc from CD-1 females mated to CD-1 males. Embryos were dissected from extraembryonic tissue in PBS and total RNA was isolated from the embryo or placenta by homogenizing tissue in 1–2 mls of Tri-reagent (Molecular Research Center). A *Pvu* II-*Sst* I restriction fragment generated from the region spanning exons 15–18 of the *Egfr* was subcloned into pBSK+ for use as a probe. The plasmid was linearized with *Xho* I and a 440 bp antisense probe was generated and radiolabeled with T3 polymerase and dUTP-³²P using the MAXIscript *in vitro* transcription kit (Ambion). The riboprobe was quantified by scintillation counting and 10 µg of total RNA from each embryonic or placental time point was incubated with 6 × 10⁵ CPM (250–300 ng) labeled riboprobe. Ribonuclease protection assays (RPAs) were carried out in triplicate using the RPA Kit II (Ambion), and analyzed using a phosphorimager system (Molecular Dynamics). The radioactivity of each fragment was quantified, normalized to a glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) probe, and expressed as normalized counts. Each experiment included a 20 µg sample of total liver RNA to ensure the probe was in excess of RNA. The mouse *Gapdh* probe was a 280-bp *Hin* dIII-*Pst* I fragment linearized with *Bam* HI and antisense probe was generated using SP6 polymerase as described above. Error is expressed as the standard error of the mean for three independent RPAs for the embryonic and placental timepoints.

2.3. Immunohistochemistry and Western blots

FVB.129F1 and BALB.129F1 placentas at 18.5 dpc were collected and fixed in 10% neutral buffered formalin (NBF) at 4°C overnight. Samples were then floated in 30% sucrose, embedded in Tissue Tek (Fisher), and cryosectioned. Slides were post fixed in 10% NBF for 10 minutes, endogenous peroxidases quenched using 3% hydrogen peroxide in MeOH, and subjected to heat-induced epitope retrieval in 10mM citrate buffer pH 6.0. Non-specific sites were blocked using blocking solution (0.1 mM Tris pH 8.0, 0.1% Tween-20, 1% BSA) incubated with sheep-anti-mouse-EGFR (1:100; Maine Biotech) or rabbit-anti-human-ERBB2 (sc284, 1:100), rabbit-anti-human-ERBB3 (sc285, 1:100), or rabbit-anti-human-ERBB4 (sc283, 1:100; Santa Cruz). Samples exposed to anti-EGFR were incubated in species-specific anti-sheep secondary antibody conjugated to HRP at 1:100, while the other anti-ERBB samples were incubated in goat anti-rabbit-HRP secondary antibody (Jackson Immunologics). Antigen was colorimetrically detected by reaction with 3-3'-diaminobenzidine (DAB) and

counterstained with hematoxylin. Slides were photographed at 200X and the levels of immunoreactivity were determined qualitatively.

For western analysis placentas were collected at 10.5 dpc from C57B6.129F1, 13.5 dpc from ALR.129F1, and 18.5 dpc from FVB.129F1 crosses. Tissues were mechanically homogenized on ice in protein lysis buffer (40mM HEPES pH 7.4, 10mM EDTA pH 8.0, 4mM EGTA pH7.5, 2% Triton X-100, 20 ng/mL aprotinin, 20 ng/mL leupeptin, 1mM sodium vanadate, 1mM PMSF) based on 10% weight/volume. Proteins were separated using 6% stacking and 10% resolving SDS-polyacrylamide gel electrophoresis in 1X Laemmli running buffer then transferred to nitrocellulose membrane. Membranes were washed in TBST (10mM Tris pH7.5, 150mM NaCl, 0.1% Tween 20), blocked with 5% powdered milk in TBST, and incubated with the following primary antibodies EGFR (1:500), ERBB3 (1:500), or mouse-anti-rat-beta tubulin (Sigma T5201, 1:500), washed and incubated with secondary antibody to either rabbit anti-sheep, goat anti-rabbit or rabbit-anti-mouse conjugated to HRP (Jackson Immuno) and antigen detected using ECL detection (Amersham).

2.4. Proliferation assays

Placentas, from N2 embryos generated by backcrossing ALR.129F1-*Egfr*^{tm1Mag} heterozygous mice to 129/Sv-*Egfr*^{tm1Mag} heterozygous mice, were collected two hours after maternal intraperitoneal injection with 10 μ l per gram of body weight of 25 mg/ml bromodeoxyuridine (BrdU) in PBS. Timed pregnancies were collected at 10.5, 13.5 and 18.5 dpc, the placenta washed in PBS, bisected, and fixed in 10% neutral buffered formalin at 4°C overnight. Tissue was then washed in PBS and 0.9% saline, dehydrated in ethanols and xylenes then embedded in paraffin before cutting seven-micron sections. A BrdU staining kit (Zymed) was used to determine proliferation rates in *Egfr*^{tm1Mag} homozygous null and wildtype placentas per manufacturer's protocol. Colorimetric reaction was detected using DAB-tetrahydrochloride horseradish peroxidase substrate, counterstained with hematoxylin, dehydrated in a series of ethanols, and mounted using Permount (Fisher Scientific). Samples were photographed at a magnification of 400X. BrdU positive nuclei were counted in one field of view for 5–10 independent placenta from 3–4 BrdU injected pregnant females per time point. Percentages are expressed as the number of BrdU positive nuclei over total number of nuclei in a field of view.

2.5. Real-time PCR

Total RNA was prepared from 129/Sv and BALB/cJ 10.5 dpc placentas using Trizol reagent and purified using an RNAeasy mini kit according to the manufacturer's protocol (Qiagen). One microgram of each RNA sample was reverse transcribed with the High Capacity cDNA Archive Kit (Applied Biosystems) and equivalent amounts of cDNA were used in a real-time PCR reaction to measure transcript levels of *Cdkn1a*, *Cdkn1b*, *Myc* and *Cdc25a*. Levels were normalized relative to expression of *Gusb* in each sample and fold change in gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method [28]. Primer and probe sets were Assays-On-Demand (Applied Biosystems) and used according to the manufacturer's protocol with a Mx3000P real-time PCR machine (Stratagene).

2.6. Statistical analysis

For BrdU experiments samples were collected from 2–5 independently injected females, with 2–10 placentas collected per gestational time. Error bars are expressed as the standard error of the mean (SEM). Significance was determined by the Mann-Whitney test with $p < 0.05$ being significant. For real-time PCR data p -values were determined by student's T-test with $p < 0.05$ being significant.

3. Results

3.1. *Egfr* transcripts are dynamically expressed in the developing mouse placenta

Three major transcripts of *Egfr* have been detected in rodent tissue; 10 and 6.5-Kb transcripts that encode the full-length 170 kD receptor and a 2.8-Kb transcript that encodes a secreted 95 kD protein corresponding to the extracellular ligand-binding domain of EGFR [29–31]. A quantitative RPA distinguishing the full-length and truncated receptor transcripts revealed a dynamic expression pattern during gestation. Full-length *Egfr* transcripts are expressed at very low levels in the embryo from 8.5 to 10.5 dpc with expression increasing steadily from 11.5 dpc to 14.5 dpc and then decreasing after 14.5 dpc (Fig. 1A). No truncated *Egfr* transcripts are detectable during embryogenesis.

In contrast to the embryonic expression pattern, both *Egfr* transcripts are detectable at all stages of placental development. Expression of the full-length transcripts decrease from 9.5 to 11.5 dpc and then increase from 12.5 to 16.5 dpc, where the highest levels of expression are observed (Fig. 1B). Interestingly, the lowest level of expression occurs around 11.5 dpc, coincident with abnormal development of the *Egfr* nullizygous placenta and embryonic lethality on many genetic backgrounds [2,3,5]. The highest level of truncated receptor transcript is observed at 9.5 dpc. When compared to levels of full-length transcript at the same time points, truncated transcripts in the placenta decrease from 81% at 9.5 dpc to 17% at 13.5 dpc of the total *Egfr* transcripts.

3.2. ERBB receptor expression is unaltered in EGFR deficient placenta

Since individual members of the ERBB family can activate similar signaling pathways, it is possible that other ERBB family members compensate for the loss of EGFR during placental development in strains with a less severe phenotype. To address this possibility, localization of EGFR, ERBB2, ERBB3, and ERBB4 in 18.5 dpc wildtype and *Egfr^{tm1Mag}* null placenta was determined by immunohistochemistry.

EGFR was detected primarily in the decidua, trophoblast giant and ST cells, with low levels detected in the LT layer (Fig. 2A). The EGFR was not detected in the *Egfr* null placenta at 18.5 dpc (Fig. 2B). The only ERBB family member not detected in the wildtype or *Egfr* null placenta was ERBB2 (Fig. 2C and D), suggesting that in the 18.5 dpc mouse placenta this receptor is expressed at undetectable levels or is not involved in normal placental formation. Up-regulation of ERBB2 was not detected in response to a lack of EGFR signaling. Both ERBB3 and ERBB4 were detected in the maternal decidua and in the trophoblast giant cells of wildtype and *Egfr* null placentas (Fig. 2E – H). Since dysregulation of ERBB2, ERBB3 or ERBB4 was not detected in the *Egfr* null placenta, it is unlikely that these ERBB receptors contribute to compensatory mechanisms supporting embryonic survival in the absence of EGFR.

Levels of the ERBB3 and EGFR were confirmed by western blot at 18.5 dpc, as well as at 10.5 and 13.5 dpc. No EGFR immunoreactivity was detected in homozygous *Egfr* null placenta, while a normal 170 kD EGFR protein was detected in corresponding wildtype samples (Fig. 3). Higher levels of ERBB3 were detected at 18.5 dpc compared to 10.5 and 13.5 dpc in wildtype placentas, with no significant differences noted between wildtype and *Egfr* null samples. Weak bands observed in 10.5 and 13.5 dpc samples likely reflect contribution from the deciduas, since EGFR, ERBB3 and ERBB4 are all highly expressed in the decidua.

3.3. Reduced trophoblast proliferation in EGFR deficient placentas

Differences in cellular proliferation in wildtype and *Egfr* null placenta were assessed by incorporation of the thymidine analog BrdU into cells that are in the S-phase of the cell cycle. Based on immunohistochemical analysis, the number of BrdU-positive nuclei appeared

reduced in 10.5 dpc *Egfr* null placentas from 129/Sv, a strain exhibiting abnormal LT and ST development and embryonic lethality by 11.5 dpc (Table 1). At 10.5 dpc a 60% decrease in proliferation in the *Egfr* null placenta was detected compared to wildtype placenta, suggesting that there is a significant reduction in proliferating trophoblast cells without EGFR. To determine whether the reduction in proliferation contributes to mid-gestation lethality of *Egfr* null embryos, BrdU incorporation was determined at 10.5 dpc in the ALR;129 mixed genetic background, which has a functional LT but reduced ST layer and supports embryo survival through late gestation. Similar to that observed in the 129/Sv background, EGFR deficiency on the ALR;129 background resulted in a 56% reduction in BrdU positive cells. These data suggest that proliferation does not contribute to differential survival of *Egfr* null embryos.

Interestingly at 13.5 dpc of gestation, there was no longer a statistically significant difference in BrdU incorporation between the ALR;129 wildtype and *Egfr* null placentas (Table 1). We also did not observe differences in proliferation rates in specific trophoblast layers when the amount of BrdU incorporation was analyzed specifically in the ST or LT layers at 13.5 dpc (data not shown). By 18.5 dpc few cells are actively replicating (Table 1). Additionally there was no significant difference in the number of trophoblast cells undergoing apoptosis when comparing ALR;129 wildtype and *Egfr* null placentas at 10.5, 13.5 or 18.5 dpc using a TUNEL assay (Table 1). We observed few TUNEL-positive trophoblasts across gestation, consistent with previous reports characterizing apoptosis in mouse placental tissue [7,32].

To confirm the BrdU data, markers of proliferation and cell cycle arrest were measured by real-time PCR in 10.5 dpc *Egfr* wildtype and null placentas. Expression of *Cdc25a* and *Myc*, two genes known to be transcriptionally up-regulated in proliferating cells [33,34], were significantly higher in wildtype (n = 11) versus *Egfr* null (n = 12) placentas (Fig. 4). *Myc* expression in *Egfr* null placentas was 74% of wildtype levels ($p = 0.01$), while *Cdc25a* expression was 61% of wildtype levels ($p < 0.02$) (Fig. 4). *Cdkn1a* and *Cdkn1b* are known to be transcriptionally up-regulated in cells arresting in the cell cycle at G1-S [33]. We found no significant changes in *Cdkn1a* expression, but *Cdkn1b* transcripts were significantly higher (117% compared to wildtype) in *Egfr* null placentas ($p < 0.05$) (Figure 4). These data are consistent with a greater number of *Egfr* nullizygous trophoblast cells undergoing cell cycle arrest.

3.4. Placental defects are not rescued by genetic reduction of cell cycle checkpoint regulators

Although genetic backgrounds showing disparate survival of *Egfr* null embryos have equivalent reductions in trophoblast proliferation at 10.5 dpc, it is possible that the ALR;129 background overcomes the 10.5 dpc proliferation defect and continues to develop a functioning placenta while the 129/Sv strain does not. To investigate this, we intercrossed *Egfr^{tm1Mag}* heterozygous mice with mice carrying null alleles of various cell cycle checkpoint genes that regulate the G1 to S transition [33,35], including three cyclin dependent kinase inhibitors (CKIs), *Cdkn1a* (*p21^{Cip1}*), *Cdkn1b* (*p27^{Kip1}*), and *Cdkn2c* (*p18^{INK4c}*), as well as two tumor suppressors, *Trp53* (*p53*), and *Rb1*. *Trp53* and *Cdkn1a* also regulate the G2 to M checkpoint [36]. Genetic ablation of these cell cycle checkpoint molecules would be expected to increase cellular proliferation and, if this contributed to the mid-gestation lethality observed on the 129/Sv background, increase survival of *Egfr* null embryos.

The 129/Sv background was used for *Trp53^{tm1Ty}*, *Rb1^{tm1Tyj}*, *Cdkn1a^{tm1Tyj}*, and *Cdkn1b^{tm1Mlf}* null allele crosses while the BALB/cJ background, which has a phenotype similar to 129/Sv, was used for *Trp53^{tm1Ty}*, *Cdkn2c^{tm1Yxi}* and *Rb1^{tm1Tyj}* null allele crosses. Surviving *Egfr^{tm1Mag}* homozygous embryos were not detected at 13.5 dpc irrespective of *Trp53^{tm1Tyj}* (n = 120), *Rb1^{tm1Tyj}* (n = 108), or *Cdkn1b^{tm1Mlf}* (n = 102) genotype (Table 2). At 13.5 dpc a low

number of viable embryos from the *Cdkn2c^{tm1Yxi}* cross were found to be homozygous for *Egfr^{tm1Mag}* (2 out of 119). However, one of these two embryos was wildtype for *Cdkn2c*. Similarly 12 out of 257 total embryos from the *Cdkn1a^{tm1Tyj}* cross were found to be *Egfr^{tm1Mag}* homozygous and viable at 13.5 dpc. Data from *Cdkn1a^{tm1Tyj}*, *Egfr^{tm1Mag}* heterozygous intercrosses revealed that 3 of the 5 surviving *Egfr* nullizygous embryos carried wildtype *Cdkn1a* alleles. Because genotypes of *Cdkn1a^{tm1Tyj}* or *Cdkn2c^{tm1Yxi}* did not correlate with embryonic survival it is likely that *Cdkn1a^{tm1Tyj}* and *Cdkn2c^{tm1Yxi}* congenic lines were not pure 129/Sv or BALB/cJ and were probably segregating unknown genetic background modifiers of the *Egfr^{tm1Mag}* phenotype. In composite, these data indicate that removing negative regulators of the cell cycle is not sufficient to support survival of *Egfr* null embryos.

4. Discussion

EGFR transcripts and protein are abundantly detected in human placenta and aberrant expression of the receptor has been associated with IUGR, preeclampsia, and placenta accreta [17–22]. Interestingly, alternative transcripts from the *Egfr* locus that encode a secreted 60 kD protein corresponding to the extracellular ligand binding domain of EGFR have been reported in both human and mouse placenta [1,21,37,38]. Using RPA we demonstrated dynamic expression of *Egfr* transcripts during mouse embryo and placental development. Truncated *Egfr* transcripts were not detected in the developing embryo, but low levels detected throughout placental development raise the possibility that secreted, ligand-binding EGFR may negatively regulate EGFR signaling in extra-embryonic tissue. In addition, we observed up-regulation of full-length *Egfr* transcripts after 11.5 dpc suggesting that EGFR is actively being turned-over around the time that the placental LT develops and begins to function. Since RPA provides no information on the localization of *Egfr* transcripts the dynamic expression patterns observed may reflect differences in the composition of the placenta related to gestational age rather than changes in *Egfr* expression; EGFR can be detected in all layers of the developing mouse placenta by immunohistochemistry.

One potential explanation for strain-specificity of the *Egfr* nullizygous placental phenotype is that surviving strains may developmentally compensate for a lack of *Egfr* signaling by up-regulating expression of other ERBB family members, similar to that demonstrated in the *Ptgs2* null mouse uterus where up-regulation of *Ptgs1* allows partial rescue of fertility defects in CD-1 females [39]. We compared expression of ERBB2, ERBB3, and ERBB4 in wildtype and *Egfr* null placentas from two hybrid strains that survive until late gestation. ERBB2 was not detected, and although ERBB3 and ERBB4 were detected in the decidua and trophoblast giant cells at late gestation, there was no difference in expression or localization between *Egfr* wildtype and null placentas. In fact, EGFR-deficient strains that survive past mid-gestation probably utilize a mechanism other than ERBB compensation since ERBB2, ERBB3, and ERBB4 are not expressed at detectable levels in the ST or LT, where defects in the *Egfr* nullizygous placenta are primarily observed.

EGFR plays an important role in regulating cellular proliferation and cell survival [40]. EGFR signaling has been shown to promote cell cycle progression through the G1-S, as well as G2-M checkpoints, and it is possible that the strain-specific placental phenotype observed in *Egfr* null mice could result from surviving strains using alternate pathways to enhance cell cycle progression in trophoblasts [41]. We found that *Egfr* null placentas on 129/Sv and ALR.129 genetic backgrounds have fewer BrdU-positive trophoblasts than wildtype at 10.5 dpc. However, proliferation defects in the *Egfr* nullizygous placentas do not correlate with embryonic lethality since *Egfr* null embryos on ALR.129 survive to late gestation despite having equivalently reduced trophoblast proliferation compared to embryos on a 129/Sv background at 10.5 dpc. This result does not exclude the possibility that the ALR.129, but not the 129/Sv genetic background, harbors modifiers allowing the strain to overcome proliferation

defects at 10.5 dpc and develop a functioning placenta. We tested this hypothesis by genetically reducing negative cell cycle regulators downstream of EGFR, thus potentially permitting cell cycle progression in the absence of EGFR on backgrounds that do not support survival of *Egfr* null embryos past mid-gestation. Hyper-proliferation of LT has been observed in mouse RB1-deficient placentas and in placentas deficient for both CDKN1B and CDKN1C [42–44]. We surmised that eliminating these and other cell cycle checkpoint components would rescue *Egfr* nullizygous placental development by increasing trophoblast proliferation. We intercrossed *Egfr^{tm1Mag}* heterozygotes with mice segregating *Trp53^{tm1Tyj}*, *Rb1^{tm1Tyj}*, *Cdkn1a^{tm1Tyj}*, *Cdkn1b^{tm1Mlf}* or *Cdkn2c^{tm1Yxi}* null alleles carried on 129/Sv and BALB/cJ backgrounds. Results from the intercrosses indicated that genetic reduction of negative cell cycle checkpoint components does not rescue the embryonic lethality of EGFR deficient embryos.

Together our data indicates that proliferation defects observed in 10.5 dpc *Egfr^{tm1Mag}* nullizygous placentas do not contribute to the midgestation embryonic lethality since the phenotype is observed in both surviving (ALR;129) and non-surviving (129/Sv) strains. At 10.5 dpc the ST is likely the primary lineage affected by the proliferation defect because both ALR;129 and 129/Sv show dramatic reduction of this trophoblast compartment. Consequently, a decrease in the ST population probably does not contribute to placental insufficiency in *Egfr* nullizygous embryos. Rather, survival of embryos beyond 11.5 dpc is prevented by an abnormal LT phenotype in strains such as 129/Sv. Additional experiments utilizing markers for trophoblast sub-type progenitors are necessary to evaluate differences in early LT cell populations that may distinguish surviving and non-surviving strains.

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Figure 1.

Expression profile of *Egfr* during development. Graphs of normalized counts from (A) embryonic and (B) placental RNase protection assays at gestational time points 9.5 to 16.5 dpc. Transcript levels were quantitated by phosphorimager analysis, normalized to *Gapdh* expression and expressed as normalized counts on the Y-axis. Blue lines represent full-length *Egfr* transcript levels and red lines represent truncated *Egfr* levels. Error is represented as standard error of the mean (SEM).

Figure 2.

Immunohistochemistry for ERBB family members on late gestation placentas.

Immunohistochemistry was done using FVB.129 or BALB.129N2 placentas at 18.5 dpc.

Panels (A, C, E, G) are wildtype samples, while (B, D, F, H) are *Egfr^{tm1Mag}* null samples. (A, B) Protein localization for an EGFR specific antibody. In wildtype sample EGFR is localized to both the spongiotrophoblast and labyrinthine layers. Note loss of localization of EGFR in the *Egfr^{tm1Mag}* null placenta, although there is some immunoreactivity in the maternally derived decidua. Also note the loss of the spongiotrophoblast cell layer. (C, D) ERBB2 is not detected in either wildtype or null samples. (E, F) Localization of ERBB3 protein to the TGC layer and the maternal decidua. Levels of protein appear equivalent in both the wildtype

and *Egfr^{tm1Mag}* null placenta. (G, H) Protein levels of ERBB4 appear unaltered between the wildtype and *Egfr^{tm1Mag}* null samples, with localization observed in the TGC and maternal decidua. SP, spongiotrophoblast layer; LT, labyrinthine trophoblast layer; De, decidua; TGC, trophoblast giant cells. Arrowheads indicate TGC. Scale bars are 50 μ m.

Figure 3. Western blot analysis of EGFR, ERBB3 and TUBB1 (beta-tubulin) during placental development. Protein lysates are from *Egfr* wildtype or null placentas isolated at the indicated time points. Beta tubulin represents loading control.

Figure 4.

Real-time PCR expression analysis of proliferation and cell cycle arrest markers. RNA from 10.5 dpc placenta are identified by open bars, *Egfr* wildtype and gray bars, *Egfr* null. Y-axis represents the percent change in gene expression compared to the average expression in wildtype samples adjusted to 100%. Expression of proliferation, *Myc* and *Cdc25a*, and cell cycle arrest, *Cdkn1a* and *Cdkn1b*, markers are presented.