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ARTICLE

Progressive Loss of the Spongiotrophoblast Layer of *Birc6/Bruce* Mutants Results in Embryonic Lethality

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Summary: We have generated a mouse line with a mutant allele of the mouse *Bruce/Birc6* gene induced by gene trap mutagenesis. Based on its structural features, *Bruce* is a member of the family of apoptosis inhibitor proteins (IAPs). This mutation leads to a truncated transcript and protein and results in a complete loss of the wildtype *Bruce* protein. *Bruce* mutant mice die from a progressive loss of their placental spongiotrophoblast layer between day 11.5 and 14.5 of embryonic development. The cause of the *Bruce* homozygous mutant phenotype is a lack of proliferation of spongiotrophoblast cells in the developing placenta. In contrast to in vitro data, which indicate a function for Bruce in apoptosis inhibition, the in vivo results presented here suggest instead a role for Bruce in cell division. genesis 42:91–103, 2005. **© 2005 Wiley-Liss, Inc.**

Key words: IAP; apoptosis; spongiotrophoblast; gene trap

During evolution the eutherian organisms invented the placenta as a direct connection between an embryo and its mother, which allows development independent from most environmental influences. In contrast to the closely related sauropsida, the mammalian egg suffers from a secondary lack of vitelline fluid and the embryo relies mostly on a maternal supply of oxygen and nutrients. To establish the close contact between the embryo and its mother, extraembryonic trophoblast cells are actively invading the uterine wall during implantation. In contrast to the human, the mouse placenta consists of three cell layers. Two of them, the giant cell layer and the spongiotrophoblast, are of trophectodermal origin, whereas the labyrinthine layer is constituted of extraembryonic mesoderm and trophoblast cells (for a review, see Rossant and Cross, 2001).

Several signals from the embryo, the mother, and the environment control the complex processes of placental development. Oxygen content is one of the key triggering signals, capable of inducing immediate responses by trophectodermal cells in vivo and in vitro. For example, trophoblast cells are sensitive to hypoxia and produce *HIF1/ARNT* and *VEGF* in response, which subsequently stimulates the proliferation of endothelial cells by transcriptional activation of a number of genes (Adelman *et al.*, 2000; Damert *et al.*, 1997). In addition, low O_2 levels promote differentiation into spongiotrophoblast and thus regulate the fate of placental cells (Adelman *et al.*, 2000; Schaffer *et al.*, 2003; Levy *et al.*, 2000). Growth factor signals from the embryo are also required for differentiation of trophoblast cells. Since the placenta needs to constantly keep up with the growing embryo, it undergoes permanent tissue remodeling, which is facilitated by the release of matrix metalloproteinases (Teesalu *et al.*, 1999). As a consequence, cells of the placenta are not only undergoing proliferation, but also extensive apoptosis (for a review, see Levy *et al.*, 2000). Apoptosis in the placenta can be triggered by extrinsic signals, namely *FasL/Fas* or the *TNFalpha/TNFR* systems (Uckan *et al.*, 1997; Crocker *et al.*, 2001; Straszewski-Chavez *et al.*, 2004). Molecularly best understood are intrinsic apoptotic cascades which are initiated by mitochondrial release of *cytochrome c* and *diablo/smac* in response to cellular triggers such as DNA damage or hypoxia. Both apoptotic pathways involve the activation of initiator and effector caspases. Once activated, the proteolytic caspase pathway ultimately leads to the

The Supplementary Material in this article can be accessed at http://www.interscience.wiley.com/jpages/1526-954X/suppmat.

Abbreviations: VEGF, vascular endothelial growth factor; FGF4, fibroblast growth factor 4; IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; UBC, ubiquitin conjugating; *BRUCE*, baculovirus repeat UBC containing enzyme.

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cleavage of a wide spectrum of cellular targets and causes cell death (Thornberry and Lazebnik, 1998; Shi, 2001). For the human it has recently been shown that caspase 8-mediated cell fusion is a prerequisite for the maintenance of the syncytiotrophoblast, which is homologous to the murine labyrinth (Black *et al.*, 2004). The delicate balance between proliferation, differentiation, and apoptosis needs to be tightly regulated in the placenta and any imbalance can lead to malformation or death of the developing embryo. Molecules with a potentially negative impact on apoptosis in vivo are the inhibitor of apoptosis proteins (IAPs), most of which have been shown to bind and inhibit caspases in vitro (Wilson *et al.*, 2002; Shiozaki *et al.*, 2003). In addition, several IAPs are able to bind to the mammalian RHG motif-containing proteins *Smac/diablo* and *HtrA2/Omi*, which are potent apoptosis inducers (Du *et al.*, 2000; Suzuki *et al.*, 2001).

The huge *Bruce* protein of 528 kDa contains one N-terminal baculovirus inhibitor of apoptosis repeat (Bir) domain as well as a C-terminal ubiquitin-conjugating (UBC) domain (Hauser *et al.*, 1998). More recently, IAP family members have been divided into two subgroups, based on their Bir repeat type. One group contains between one and three N-terminal Bir repeats and either a C-terminal ring finger or an additional caspase recruitment domain (CARD); the other contains a "*survivin*-like" Bir domain and comprises *Survivin* and *Bruce* (Verhagen *et al.*, 2001). Proteins with the slightly larger *survivin*-like Bir repeat sequence can be found in yeast (*Spbir1P*), nematode (*BIR-1, BIR-2*), and fly (*d-Bruce, Deterin*) and are therefore older in evolutionary terms compared to other IAPs. In *Caenorhabditis elegans* and the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* the Bir proteins do not inhibit apoptosis but instead have been shown to have a role in cell division (Uren *et al.*, 1999; Silke and Vaux, 2001; Fraser *et al.*, 1999). This observation is further supported by the fact that no caspase or Bcl-2 gene has been found in single-celled organisms, although examples of cell suicide have been described (Vaux and Korsmeyer, 1999). Bir-1 mutant zygotes of *C. elegans* do not finish cytokinesis, *bir1* deficient *S. pombe* exhibit defects in mitotic spindle elongation, and the *Bir1p* protein of *S. cerevisiae* has been shown to be associated with kinetochore proteins. The exact molecular function of nematode and yeast IAPs is not known. It has been proposed for Bir-1 that it may serve as an adaptor molecule in larger complexes (Yoon and Carbon, 1999; Rajagopalan and Balasubramaian, 1999; Speliotes *et al.*, 2000). Mutations in the *Drosophila* homolog of *Bruce* (*d-Bruce*) leads to enhanced apoptosis during spermatogenesis induced by *reaper* and *grim* and causes male sterility. However, in *Drosophila* the apical cell death caspase, *Dronc*, is not blocked by *d-Bruce* (Vernooy *et al.*, 2002; Arama *et al.*, 2003). Mammalian *Bruce* is probably also not directly interacting with caspases but antisense oligonucleotides directed against *Bruce* accelerate the sensitivity of SNB-78 glioma cells to apoptosis induced by DNA-damaging agents (Chen *et al.*, 1999). More recently, it has been demonstrated, that a knockdown of *Bruce* by RNAi in HeLa and MCF-7 cells was sufficient to induce apoptosis. Moreover, it was shown that the ubiquitin ligase Nrdp1 is capable of triggering apoptosis through ubiquitination of *Bruce* (Qiu *et al.*, 2004). In contrast to most other usually ring finger containing IAP family members, which are therefore ubiquitin ligases (E3; Joazeiro and Weissman, 2000), and in contrast to the closely related *Survivin*, which does not contain any other functional domain, the second domain of *Bruce* provides E2 ubiquitin-conjugating enzyme activity (E2). The functionality of the UBC domain has been demonstrated in vitro (Hauser *et al.*, 1998). Based on its unique protein structure, it has been suggested that *Bruce* may ubiquitinate caspases directly and could serve as a cellular link between the 26S proteasome and the apoptosis cascade. However, depending on the number of ubiquitin residues, which are transferred to a cellular target, other cellular processes such as endocytosis, DNA repair, and transcriptional control may be triggered and only multi-ubiquitination of at least four or more ubiquitin residues leads to degradation by the proteasome (Jesenberger and Jentsch, 2002). *Bruce* is expressed ubiquitously both in embryonic and extraembryonic tissue as well as in embryonic stem cells.

In a large-scale gene trap screen, we produced an allelic series of gene trap vector integrations in the *Birc6/Bruce* gene on mouse chromosome 17, the homolog of the human IAP protein APOLLON. For reasons yet unknown, *Bruce* is a hot spot for gene trap mutagenesis, with currently 30 integrations generated by the International Gene Trap Consortium (IGTC; in ensembl.org/mus musculus: ENSMUSG00000024073; check "gene trap" in DAS sources). In a recent bioinformatic analysis of gene trap data, we excluded at least its impressive gene size of 175 kb and 73 exons as the most likely reason for being a gene trap hot spot (Hansen *et al.*, 2003).

From an ES cell clone carrying an integration in intron 45 (also referred to as "W036C08" according to the GGTC database clone identifier), we established a mutant mouse line (Birc6$^{Gt(pT1Betageo)45Flo}$) by blastocyst injection. The mutation in the murine *Bruce* leads to embryonic lethality at variable stages of embryonic development. Since the mutant embryos did not always show obvious alterations, we performed a morphological analysis of extraembryonic structures in the mutants. We can show that homozygous embryos carrying a gene trap vector integration in the *Bruce* gene die from a relative regression of their spongiotrophoblast layer between embryonic day (E)11.5 and E14.5.

RESULTS

The integration of gene trap vectors into the genome is a random event and with splicing acceptor containing gene trap vectors usually integrations in introns are selected. According to the initial 5′ RACE sequence of the clone W036C08, the pT1betageo gene trap vector

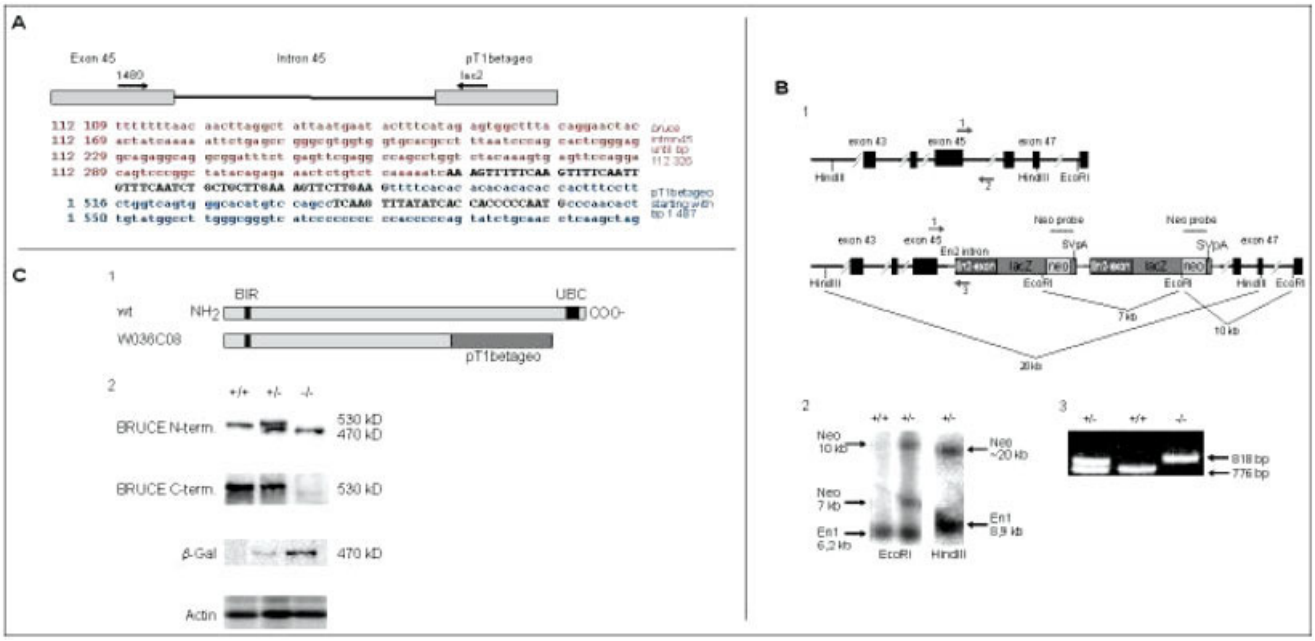


FIG. 1. **A:** The integration of pT1betageo in intron 45 of the Bruce gene (W036C08). This allele was designated (Birc6$^{Gt(pT1Betageo)45Flo}$). Note that 202 bp of the engrailed 2 intron were lost during the integration. Red: Bruce genomic sequence, blue: en-2 intron sequence, black: sequence of unknown origin. **B1:** The integration of pT1betageo in intron 45 (W036C08) occurred in tandem as shown in a Southern blot (**B2**) using an internal neomycin probe after EcoRI and HindIII digest of genomic DNA from wildtype (wt) and heterozygous animals. **B3:** Genotyping assay using a multiplex PCR with primers 1–3 (**B1**). **C1:** Schematic overview of the wildtype Bruce protein with the N-terminal Bir domain and the C-terminal UBC domain and the predicted fusion proteins after integrations in intron 45 (W036C08). **C2:** Western analysis using protein of Bruce mutant embryos at E10.5: W036C08: Upper lane: the antibody against the N-terminus of Bruce recognizes both the wt and the fusion protein. No wt protein was found in homozygous embryos. Second lane: The antibody against the Bruce C-terminus recognizes only the larger wt protein. Third lane: The antibody against betagal recognizes only the smaller fusion protein. Lower lane: An antibody against beta actin was used as a loading control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

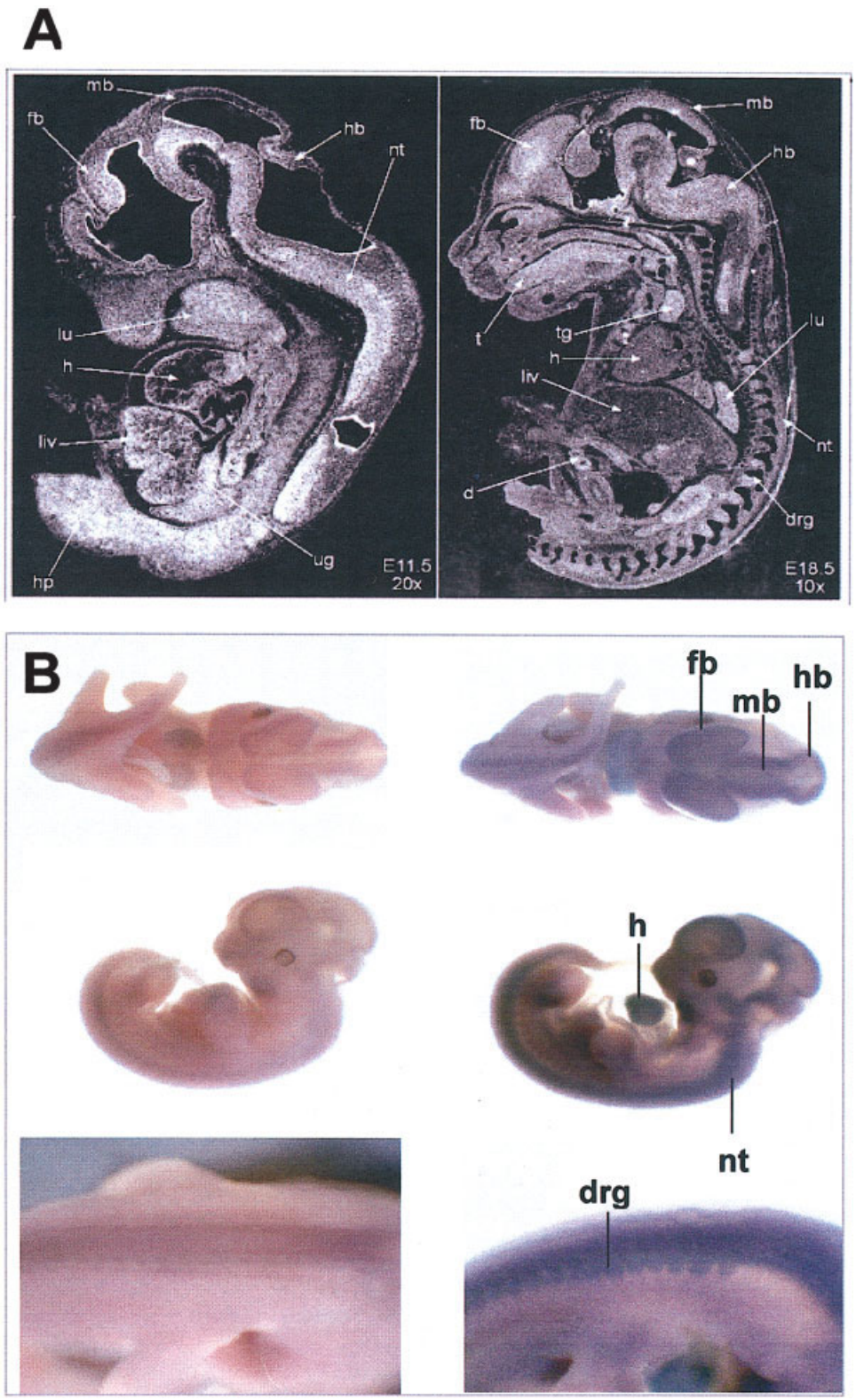
had integrated in intron 45 of the *Bruce* gene. The identity of the clone and the exact intronic integration site was confirmed by repetition of 5′ RACE-PCR by Southern blot analysis and by genomic PCR (Fig. 1). The integration site at base 112.326 of the *Bruce* gene (ensembl.org; MUS MUSCULUS | CHROMOSOME: NCBIM32: 17:75105982:75280750:1) was determined by PCR amplification of genomic DNA obtained from the respective ES cell clone with primer pair "1489" and "lac2" and sequencing of the cloned PCR product.

We determined that 1486 bases of the initial 1688 bases of the intron of the mouse *Engrailed-2* splicing acceptor were lost during the integration event (Fig. 1A). Southern analysis was done using an internal neomycin probe after digestion with either HindIII or EcoRI. After genomic integration of pT1betageo, no HindIII restriction sites are left (Wiles *et al.*, 2000). EcoRI has one restriction site in pT1betageo (Fig. 1B). Since we obtained a single 20 kb band after HindIII digestion but a 7 kb and a 10 kb band after EcoRI digestion, we concluded that in the ES clone W036C08 two pT1betageo plasmid vectors had integrated in tandem into the *Bruce* locus (Fig. 1B). As a hybridization control, we used an unrelated *Engrailed-1* genomic probe (ESN) simultaneously.

We established five chimeric males of the clone W036C08, two of which transmitted the mutation through the germline and were further bred to homozygosity (Birc6$^{Gt(pT1Betageo)45Flo}$). A genotyping strategy using a triplex PCR assay was developed based on the integration sequences (Fig. 1B).

We determined the expression of *Bruce* in wildtype embryos at E11.5 and E18.5 by in situ hybridization using a *Bruce* antisense probe. *Bruce* was determined to be widely expressed with strongest expression in brain and neural tube, dorsal root ganglia, trigeminal ganglia, lung, liver, urogenital fold, and heart (Fig. 2A). The wildtype expression pattern was reflected in the mutant by the lacZ reporter in a gene dosage-dependent manner, as expected. The strongest lacZ staining was found in brain, neural tube, heart, and dorsal root ganglia (Fig. 2B).

In order to determine whether the integration of the gene trap vector had resulted in a truncated protein as predicted, we performed a Western analysis on protein from F2 littermates at E10.5 (Fig. 1C). By using an N-terminal antibody for the *Bruce* protein, which recognizes amino acids N-terminal of the Bir repeat of the wildtype protein (a gift of K. Lotz), we determined for W036C08 that heterozygous embryos express two different proteins in a 1:1 ratio, the 528 kDa wildtype *Bruce* protein and a 470 kDa fusion protein of *Bruce* and betageo, the result of the utilization of the *En-2* splicing acceptor of the gene trap vector pT1betageo, as indicated also by a lacZ antibody, which detects only the mutant protein.



COLOR

FIG. 2. Bruce expression at E11.5 and E18.5 by in situ hybridization and lacZ staining at E11.5. **A:** left: E11.5, right: E18.5: The Bruce wild-type (wt) in situ hybridization reveals widespread expression mainly in brain, neural tube, dorsal root ganglia, trigeminal ganglion, tongue, lung, and heart. **B:** lacZ staining reflects the wt expression pattern in the mutant. LacZ is expressed in a gene dosage-dependent manner: left heterozygous mutant at E11.5, right side: homozygous littermate. fb: forebrain, mb: midbrain, hb: hindbrain, nt: neural tube, h: heart, drg: dorsal root ganglia, ug: urogenital fold, hp: handplate, lu: lung, liv: liver, t: tongue, tg: trigeminal ganglion.

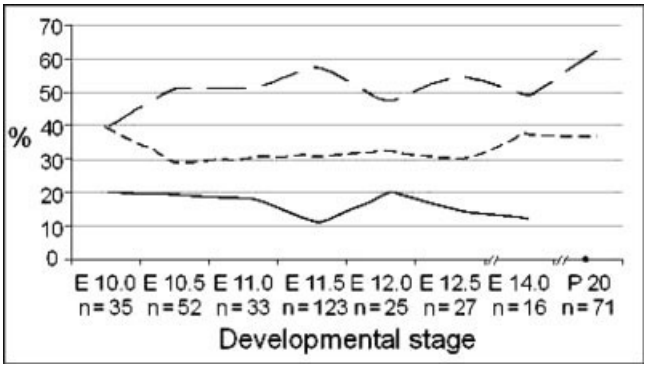


FIG. 3. Genotyping of Bruce embryos over time: Embryos from het/het matings were prepared at different stages of development. No more than 10–20% mutant embryos were found for W036C08 starting at E10 with a dramatic decrease of mutants in the litters after E12.5. All homozygous embryos found at E14.5 displayed severe malformations and no homozygotes were found after this stage. Dashed line: heterozygote embryos; dotted line: wildtype embryos; continuous line and dot at P20: homozygous embryos. n = total number of genotyped embryos.

The wildtype *Bruce* protein is entirely replaced by the smaller fusion protein in homozygous mutants. The C-terminal antibody, which is directed against the UBC domain of *Bruce* (K. Lotz, pers. commun.), does not yield any signal in homozygous mutants, indicating utilization of the *En-2* splicing acceptor of the gene trap vector and truncation of *Bruce* after exon 46. As a loading control, an antibody against beta actin was used (Fig. 1C). After determining that no wildtype protein is made from the mutant allele of W036C08, we systematically investigated the embryonic development of the mutants.

After genotyping a total of 80 F2 offspring of the strain W036C08, we determined that no homozygous mutants had survived until weaning. We therefore genotyped F2 embryos at different stages of development (Fig. 3). Starting at E10.0, a total of seven homozygous W036C08 embryos were found among 35 pups from five litters, thus at a lower rate than expected. An average below 20% homozygotes could be found throughout embryonic development until E14.5. No homozygous mutants were found among embryos older than E14.5 or newborns, indicating embryonic or perinatal lethality of the mutation. Morphologically, the majority of homozygous W036C08 mutant embryos appeared normal (Fig. 2B); however, 10% of those were being resorbed and presently underwent extensive necrosis starting as early as E11.5 (Fig. 4).

The LacZ expression pattern of embryos and their corresponding placentas revealed that the morphology of the mutant placentas differed from heterozygous littermates at E11.5 (Fig. 4C). Compared to heterozygous littermates, there was no spongiotrophoblast layer visible;

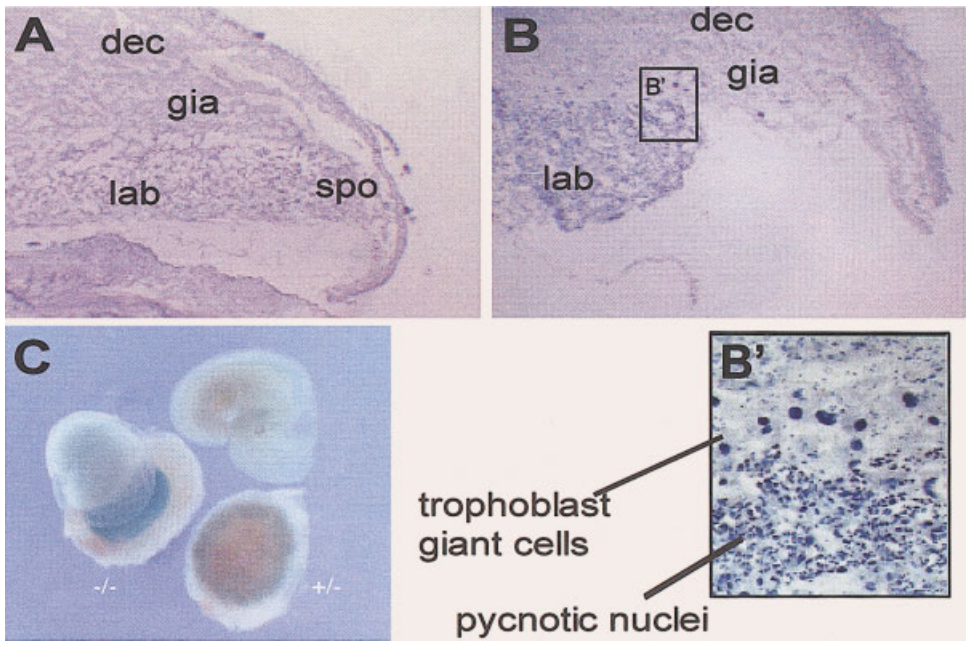


FIG. 4. Placental phenotype of W036C08. **A:** Sections through heterozygous placenta from **C** stained with hematoxylin at E11.5. **B:** Placenta of a homozygous littermate from **C**. Note that the spongiotrophoblast of a severely affected embryo (**C** left side) is strongly regressed. **B':** Higher magnification from **B** with trophoblast giant cells and adjacent cell layers. A large number of pycnotic nuclei indicates extensive necrosis. Both placentas of **A** and **B** are shown in **C** with the respective embryos after lacZ staining: Left side: homozygous embryo attached to its placenta. Right side: heterozygous placenta with its corresponding embryo above. Note that strongest lacZ expression was detected in the spongiotrophoblast layer. Note that the embryo is currently being resorbed and that the lacZ staining pattern of the placenta indicates the absence of the spongiotrophoblast layer. All lacZ staining appears to be located in the labyrinth. Dec: decidua, gia: trophoblast giant cells, lab: labyrinth, spo: spongiotrophoblast.

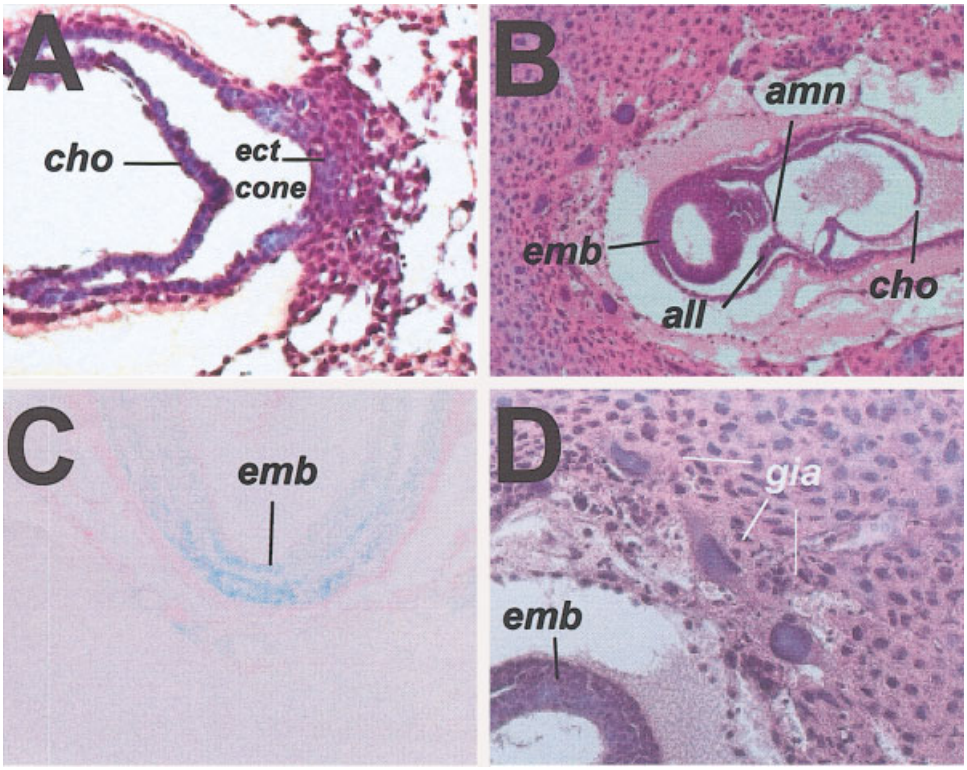


FIG. 5. LacZ expression of W036C08 in extraembryonic tissue at E7.5. **A:** LacZ is found in the chorion and the ectoplacental cone, which is the precursor of the spongiotrophoblast layer. **B:** LacZ is not expressed in the amnion or allantois prior to chorio-allantoic fusion. **C:** LacZ expression in the embryonic ecto- and endoderm. **D:** LacZ expression in trophoblast giant cells. Cho: chorion, etc., cone: ectoplacental cone, emb: embryo, amn: amnion, all: allantois, gia: trophoblast giant cells.

instead, all residual lacZ staining appeared to be restricted to the labyrinthine layer in these mutants. Embryos connected to these severely malformed placentas presently underwent resorption. After sectioning and hematoxylin stainings we found that the spongiotrophoblast layer of the affected embryos was either largely reduced or missing (compare Fig. 4A,B). At this stage most of the labyrinthine cells underwent necrosis, as indicated by large numbers of pycnotic nuclei. In order to determine whether *Bruce* is already expressed in extraembryonic tissue before the layers of the placenta are established after chorio-allantoic fusion, we took advantage of the lacZ reporter cassette. Heterozygous embryos were sacrificed at E7.5 and stained for lacZ expression. We determined that *Bruce* is expressed in the chorion and ectoplacental cone, which is the spongiotrophoblast precursor (Fig. 5A). Expression was also found in trophoblast giant cells and in the late gastrula stage embryo (Fig. 5B,D). No lacZ expression was found in the amnion and in the allantois (Fig. 5B).

In order to determine whether early spongiotrophoblast development occurs normally in nonaffected embryos, we generated in situ hybridization probes specific for the labyrinth and the spongiotrophoblast layer. Both layers have been shown to express different VEGF receptors: flt-1 is exclusively expressed in the spongiotrophoblast and flk-1 is restricted to the labyrinth (Dumont *et al.*, 1995).

Placentas were chosen from embryos between stage E10.5 and E12.5. All homozygous placentas of morphologically unaffected embryos were isolated, fixed in 4% >paraformaldehyde (PFA), embedded in paraffin, and subsequently sectioned. As indicated in Figure 6, the gross appearance of mutant placentas was normal at E10.5. The *Bruce* mRNA is highly abundant in both the labyrinth and the spongiotrophoblast. In the mutant placentas no *Bruce* mRNA was detectable by in situ hybridization (Fig. 6B4,D4). The spongiotrophoblast layer is initially established in the mutants, as shown by the presence of flt-1 positive cells. However, with some variability, there is no further extension of flt-1-positive tissue after E10.5 (Fig. 6D2,F2) in the fast-growing placenta. The labyrinthine layer, as shown by flk-1-positive signals, is apparently developing normally between E10.5 and E12.5. However, the labyrinthine layer appears to be more loosely packed in the mutant as compared to the wildtype starting at E11.5 (not shown). From the significant reduction of flt-1-positive cells in the mutant, we concluded that the primary impact of this *Bruce* mutation is on the spongiotrophoblast layer of the mutant placentas. This finding is further supported by a strong reduction of mash2 and Hif1 alpha expression and EGFR protein in mutant spongiotrophoblast (Fig. 7). In order to determine whether spongiotrophoblast cells of morphologically yet unaffected embryos undergo enhanced apoptosis, we performed Tunel analyses and determined the presence of cleaved caspase 3 (Fig. 8). Between 0 and 12 cleaved caspase 3-positive cells were detected in both the wildtype and in the mutant spongiotrophoblast and labyrinth. (Fig. 8B,D and Supplementary data). We confirmed that no significant differences in the number of apoptotic events by Tunel

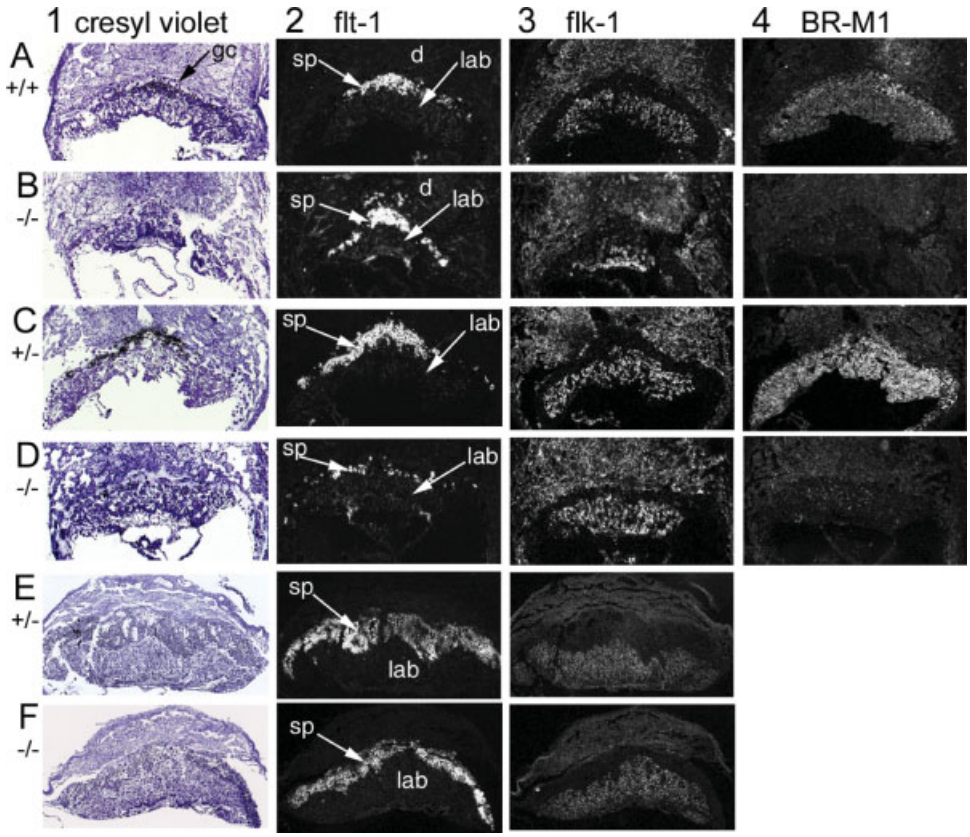


FIG. 6. In situ hybridization of placentas at different developmental stages reveals progressive loss of the spongiotrophoblast layer. **A,C,E:** Wildtype. **B,D,F:** Mutant. **A,B:** E10.5. **C,D:** E11.5. **E,F:** E12.5. 1: Cresyl violet staining; 2: flt-1 probe; 3: flk-1 probe; 4: Bruce probe. d: decidua; gc: giant cells; sp: spongiotrophoblast; lab: labyrinth. As indicated by the VEGF receptor flt-1, which is a spongiotrophoblast specific marker, Bruce mutant placentas show a relative regression of the spongiotrophoblast (compare C2 and D2 as well as E2 and F2).

and cleaved caspase 3 staining were evident between mutants and wildtype (Fig. 8E,F). These results suggest that apoptosis is not involved in the early growth retardation of the mutant spongiotrophoblast layer. In order to further determine whether *Bruce* mutants undergo enhanced apoptosis in the embryo proper, we used an antibody for cleaved caspase 3 on dorsal root ganglia which express high levels of lacZ in the mutant (Fig. 2). As shown in Figure 8G,H, there was no difference in the number of apoptotic events in the wildtype compared to the mutants with respect to cleaved caspase 3. Therefore, we concluded that the primary effect of this *Bruce* allele is not an elevation of apoptosis. In order to determine whether spongiotrophoblast cells proliferate normally after E10.5, we utilized an antibody specific for phosphorylated histone H3, which is exclusively detectable in metaphase cells. Interestingly, as shown in Figure 9, no dividing cells were detected in the spongiotrophoblast layer of three mutants. In contrast, between 18 and 53 proliferating cells in metaphase were found in the spongiotrophoblast of three heterozygous littermates (Supplemental data). From these experiments we conclude that *Bruce* is involved in the regulation of cell division of spongiotrophoblast cells. The strong reduction of Hif1 expression in mutant spongiotrophoblast layers (Fig. 7) suggests that Bruce mutant spongiotrophoblast cells do not respond to their hypoxic environment. Taken together, our data suggest that the establishment and patterning of the placenta is normal in the mutants. Even in the absence of wildtype *Bruce* protein the spongiotrophoblast layer is established. Also, chorioallantoic fusion and branching takes place with no detectable differences between mutant and wildtype. After E10.5, however, the cells of the spongiotrophoblast do not appropriately respond to signals from their microenvironment with further growth and expansion, which is finally followed by death and resorption of the embryo.

DISCUSSION

As shown in this study, a mutation of the mouse *Bruce/Birc6*, a gene which is widely expressed during mouse development, has a very specific impact on the proliferation of placental spongiotrophoblast cells. Although initially established as determined by flt-1 expression, few spongiotrophoblast cells enter metaphase after E10.5. However, in contrast to what has been predicted from the structure of the *Bruce* protein, apoptosis is not the primary phenotype of the mouse mutants. Instead, it appears that spongiotrophoblast cells of *Bruce* mutants are unable to respond to triggering signals from their microenvironment with proliferation. Since many cellular events that lead to a remodeling of the growing placenta are triggered by oxygen, we hypothesize that *Bruce* is acting downstream of hypoxia/VEGF signaling where the *Bruce* protein is directly involved in cell cycle regulation. The placental phenotype of *Bruce* mutants that lead to embryonic lethality occurred with some vari-

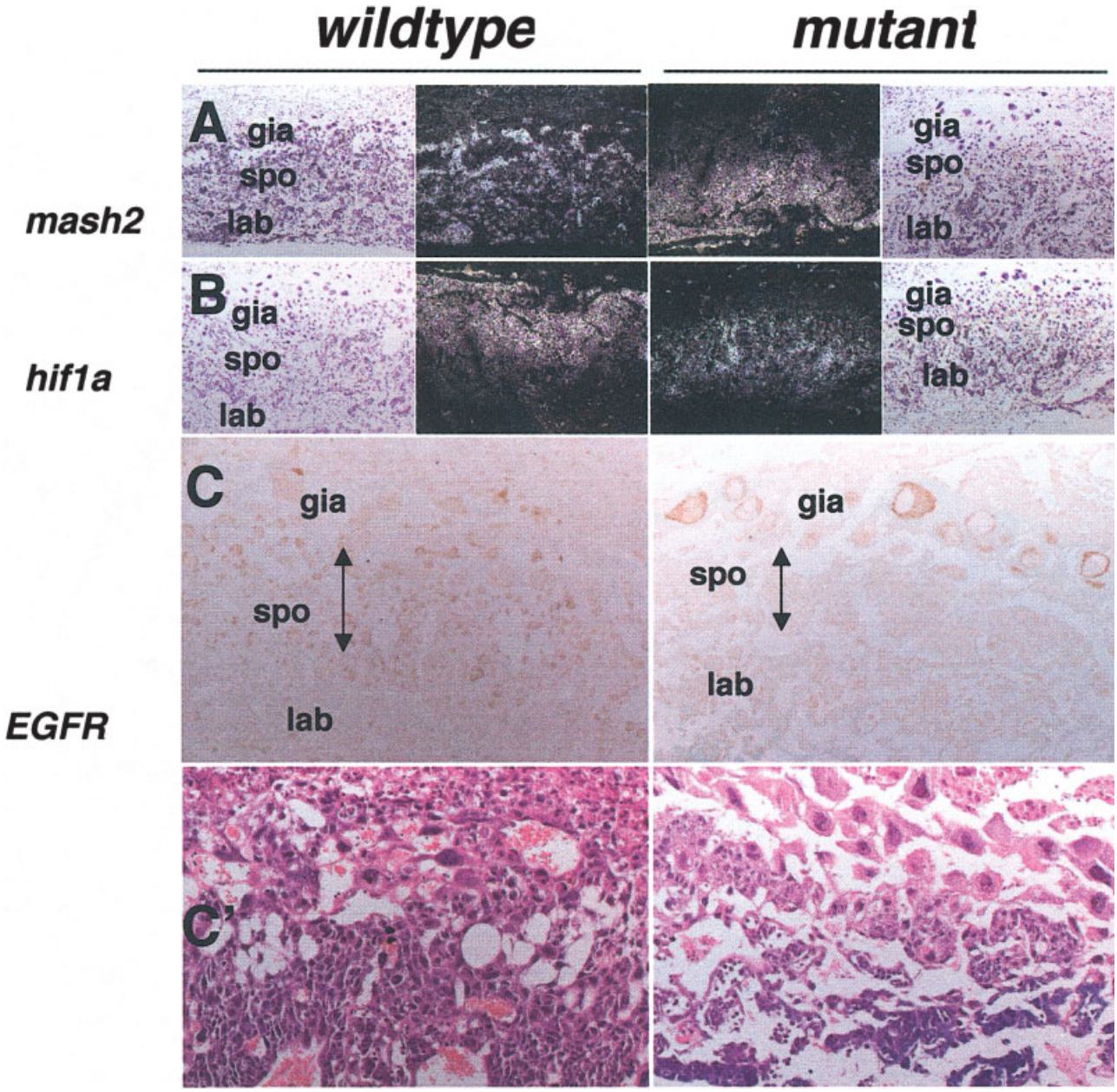


FIG. 7. The expression of the spongiotrophoblast markers *mash2* and *EGFR* as well as the hypoxia-inducible factor *hif1alpha* are strongly reduced in the spongiotrophoblast of the Birc6 mutant. **A:** In situ hybridization with a *mash2* antisense probe. **B:** In situ hybridization with a *hif1alpha* antisense probe. **C:** Immunohistochemistry using an *EGFR* antibody. The spongiotrophoblast layer of the mutants is almost negative for all three markers. A strong signal for *EGFR* was found around mutant giant cells but was absent from mutant spongiotrophoblast, in contrast to the wildtype. **C′:** H&E staining of sections in **C**.

ability between E11.5 and E14.5 of embryonic development.

The variability of the phenotype could be due to influences of the genetic background with variable contribution of 129S2 and C57BL/6 in F2 intercrosses. This hypothesis was further supported by more recent observations of homozygous mutants detected at E16.5 after six backcrosses to C57BL/6. From a deletion mutant of Bruce, obtained by homologous recombination, a phenotype similar to the gene trap mutant was obtained. Genetic background influences have also been observed in the knockout mutant, indicating no dominant negative effects of the truncated Bruce protein described in this study (Lotz *et al.*, 2004). More recently, another gene trap mutant with an integration in intron 54 of Bruce has been reported. Similar to our own integration, this mutation is lethal between E11.5 and E16.5. However, in contrast to our findings and the Bruce knockout mutant, the intron 54 integration leads to an upregulation of apoptosis in both the placenta and the embryo

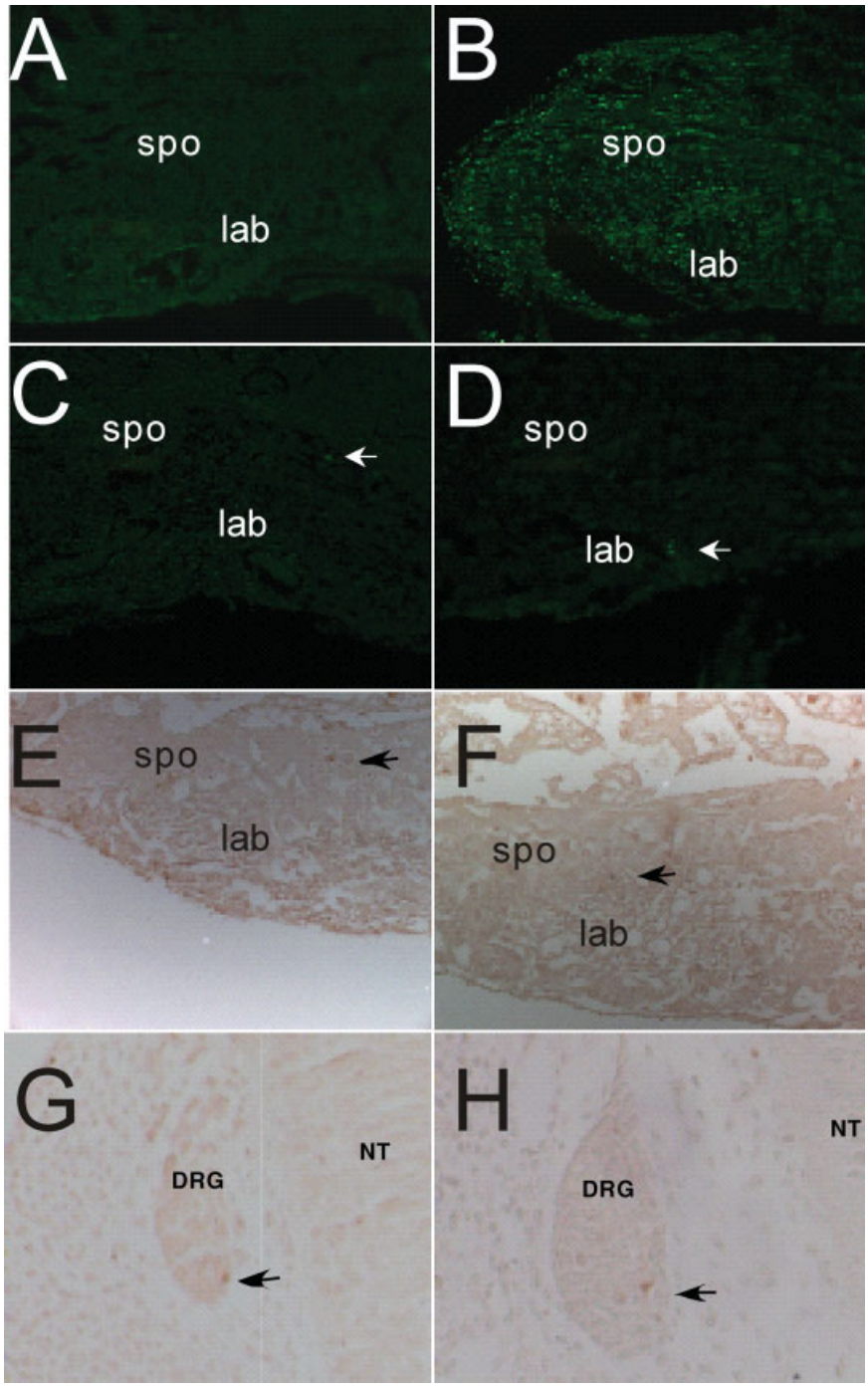


FIG. 8. Apoptosis is not the cause of the spongiotrophoblast regression of Bruce ($Birc6^{Gt(pT1Betageo)45Flo}$) mutants. Tunel assay. **A:** No terminal transferase added (negative control). **B:** Partially DNAseI digested (positive control). **C:** Mutant placenta E11.5. **D:** Heterozygous placenta E11.5: most apoptotic cells can be found in the labyrinth, both in the mutant and in the wildtype littermate. No differences between mutants and wildtype were observed. **E:** Cleaved caspase 3 in mutant placenta E11.5. **F:** Cleaved caspase 3 in heterozygous placenta of littermate E11.5. At E11.5 most cleaved caspase 3-positive cells are located in the labyrinth, both in the mutant and in the wildtype placenta. No differences between mutant and wildtype placenta were observed for cleaved caspase 3. **G:** Cleaved caspase 3 immunohistochemistry on dorsal root ganglia of mutant embryos at E11.5 as a control tissue, in which Bruce is strongly expressed and where apoptosis is frequent, indicates no enhanced apoptosis phenotype as compared to wildtype littermates (**H**).

(Ren *et al.*, 2005). Further experiments using compound mutants of the three different Bruce alleles may help to dissect direct and indirect influences on the Bruce phenotypes.

Other IAP family members have been deleted in the mouse by homologous recombination but none exhibited an apoptosis phenotype in vivo (Lens *et al.*, 2003; Harlin *et al.*, 2001; Holcik *et al.*, 2000). Similarly, knockout mice of the IAP inhibitor protein *smac/diablo* did not exhibit any obvious phenotype, indicating high redundancy among IAP proteins and their inhibitors (Holcik *et al.*, 2000). As determined for the human placenta, all IAP proteins tested were expressed in this tissue (Ka and Hunt, 2003) and could therefore compensate for the absence of *Bruce* in terms of caspase inhibition. Theoretically, it is possible that the *Bruce*-betageo fusion protein of W036C08 is still partially functional with its residual Bir repeat. Considering the similar knockout phenotype of Bruce, however, our data suggest that the nonredundant function of Bruce is a role in

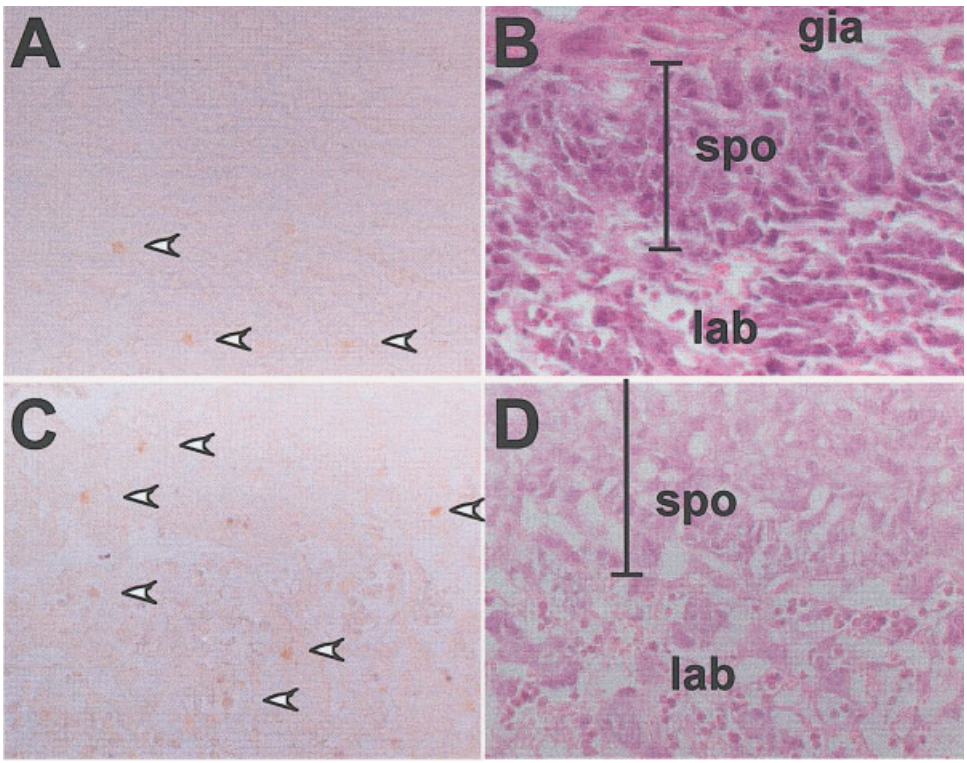


FIG. 9. No detectable proliferation of −/− spongiotrophoblast cells at E11.5. By utilizing an antibody against phosphohistone H3, which detects only cells in metaphase, we detected proliferating cells (arrows) in mutant labyrinth (**A**) but not in the spongiotrophoblasts at E11.5. However, metaphase cells were found in the wildtype spongiotrophoblast and in wildtype labyrinth (**C**). Note also that the mutant spongiotrophoblast is significantly reduced in size as compared to the wildtype. **A,B:** Mutant. **C,D:** Wildtype. **B,D:** H&E staining. **A,C:** Phosphohistone H3 immunohistochemistry. gia: giant cells, spo: spongiotrophoblast, la: labyrinth.

cell cycle regulation. *Bruce* is the only IAP protein known with E2 enzyme capabilities (Hauser *et al.*, 1998). Thus, none of the other IAP members could compensate for the ubiquitin-conjugating activity of *Bruce*. In the mutant protein, the UBC domain of *Bruce* is replaced by betageo (Fig. 1). Ubiquitination is an important tool of the cell to rapidly and selectively eliminate proteins that need to be kept under tight regulation. A well-known example is the inactivation of p53 via ubiquitination through mdm2 (for a review, see Vogelstein *et al.*, 2000). The amount of p53 protein as well as its state of activity is crucial to life or death decisions within a cell. A pathway directed against mdm2 activity on p53 is $p14^{ARF}$, which is capable of sequestering mdm2 in the nucleoli and drives the cell irreversibly into senescence (Tao and Levine, 1999). The role of p53, mdm2, and $p14^{ARF}$ during placentation is yet unknown; however, administration of the DNA-damaging agent 1-β-D-Arabinofuranosylcysteine (Ara-C) to pregnant rats has led to p53-mediated defects in the placental labyrinth. Therefore, p53 can play a role during the differentiation of placental cells under certain conditions (Yamauchi *et al.*, 2004). Under normal conditions proliferation, differentiation, or apoptotic events of placental cells are triggered by hypoxia, as shown in vivo and in vitro (Adelman *et al.*, 2000; Levy *et al.*, 2000). More recently, it has been demonstrated that hypoxia can lead to an accumulation of unfolded proteins in the endoplasmic reticulum (ER stress). Under normal conditions, ER stress leads to phosphorylation and subsequent transportation of p53 into the cytoplasm, where it can be degraded by ubiquitination (Stavridi and Halazzonetis, 2004; Qu *et al.*, 2004). According to the results of Ren *et al.* (2005), p53 is a direct target of Bruce and it is tempting to speculate that p53 protein in spongiotrophoblast cells of the $Birc6^{Gt(pT1Betageo)45Flo}$ mutant leads to irreversible cellular senescence rather than proliferation or apoptosis. The answer to this question and an explanation for the different gene trap mutant phenotypes may be addressed by breeding the different *Bruce* alleles on a p53-negative genetic background.

Based on our data, we conclude that *Bruce*, similar to its evolutionary closest IAP relative *Survivin*, does not have a function in cell death in vivo, at least none that cannot be compensated for by one of the other IAP proteins. However, a unique function of *Bruce* during mouse development lies in the regulation of spongiotrophoblast cell proliferation. The cellular function of *Bruce* in proliferation control needs to be further investigated by biochemical and cell biological means.

Interestingly, in lower organisms like nematode and yeast, mutations in IAP proteins had a primary impact on cell cycle and not apoptosis. In vertebrate cells, however, all IAP proteins are capable of inhibiting apoptosis when overexpressed in vitro, but only *Survivin* (Lens *et al.*, 2003) has been demonstrated to have retained its direct impact on the cell cycle. In this study, we present evidence that *Bruce/Birc6* also plays a role in cell cycle regulation and does not cause an increase of apoptotic events in vivo when mutated. The molecular events that lead to unresponsiveness of spongiotrophoblast cells to triggering signals on a *Bruce*-negative background

remains to be revealed. Most interestingly, the situation of the developing placenta under low oxygen and high IAP protein expression level is similar to tumor development. The expression of Apollon, the human homolog of *Bruce*, was found to be upregulated in four of six brain cancer (glioma) as well as in one of five ovarian cancer cell lines (Chen *et al.*, 1999). The mutant allele of *Bruce* presented in this study will provide a valuable resource to understand its role during placental development and cancer.

MATERIALS AND METHODS

Generation of ES Cells

The generation of the ES cell clone W036C08 has been described (Hansen *et al.*, 2003; Wiles *et al.*, 2000). In brief, the plasmid vector pT1betageo was HindIII linearized and electroporated into tbv-2 ES cells (129S2). G418 selection was applied at 200 ng/ml for 8 days. Mutant ES cell clones were identified by 5′ RACE and direct sequencing of PCR products (Wiles MV *et al.*, 2000).

Production and Breeding of Mouse Line

ES cells were injected as described (Hansen *et al.*, 2003) and male chimeras were bred with C57BL/6 females. The resulting mouse line W036C08 was bred on a C57BL/6 background for six generations. The initial analysis was done using F2 animals.

Genomic DNA Isolation and Southern Blotting

Genomic DNA was isolated from mouse tails or ES cells using the Wizard genomic DNA system from Promega (Madison, WI). DNA was digested overnight, heated to 60°C in loading buffer and run on 0.8% agarose gels at 30V for 12 h. Gels were blotted on Hybond N plus membranes (Amersham, Arlington Heights, IL) according to standard procedures (Ausubel *et al.*, 1989). An 800-bp PstI fragment was isolated from a PGK-neo plasmid (Adra *et al.*, 1987; gift of T. Braun) and used as an internal probe. In order to distinguish between single and multiple vector integration, genomic DNA from the W036C08 ES clone was digested with HindIII, which does not cut in pT1betageo, and with EcoRI, which cuts once in pT1betageo (Wiles *et al.*, 2000; Fig. 1). As a loading control we used an 850-bp SalI/EcoRI genomic probe for engrailed-1 (ESN; a gift of J. Guimera), which will yield a 6.2-kb fragment after EcoRI digestion and a 8.9 kb fragment after HindIII digestion.

PCR Primers and Conditions

PCR was performed with 1.25U Taq polymerase from Fermentas and 20–50 ng DNA or cDNA in 50 μl total according to standard conditions after initial melting at 94°C for 60 s and with final extension at 72°C for 7 min on an Eppendorf Master Cycler Thermocycler Gradient.

Mouse flk1 cDNA probe: "flk1–3199": 5′-TCTTTCGGTGTGTTGCTCTG-3′, "flk1–3574": 5′-CATAATGGAATTTGGGGTCG-3′; 95°C for 20 s, 51°C for 20 s, 72°C for 70 s, repeat 35 cycles, product size: 376 bp.

Mouse flt1 cDNA probe: "flt1-424": 5′-CTTTCTCAAGTGCAGAGGGG-3′, "flt1–725": 5′-TCATGTGCACAAGTTTGGGT-3; 95°C for 20 s;, 51°C for 20 s, 72°C for 70 s, repeat 35 cycles, product size: 302 bp.

Determination of integration site of clone W036C08: "Intrev_1489": 5′-GCAAGAGTGTCCGTGACCAC-3′, "lac2": 5′-CAAGGCGATTAAGTTGGGTAACG-3′; 95°C for 60 s, 51°C for 40 s, 72°C for 60 s, repeat 35 cycles, product size: 830bp.

Routine genotyping of W036C08: "722F": 5′-TGCAGGAATGGGGTACATTT-3′, "5′5 Race": 5′-GCCGCTTGTCCTCTTTGTTAGG-3′, "1489": 5′-GCAAGAGTGTCCGTGACCAC-3′; 94°C for 60 s, 56.5°C for 40 s, 72°C for 60 s, repeat 35 cycles, product sizes: 767 bp (wt), 820 bp (mut).

All PCR products were subcloned initially in TOPO TA (Invitrogen, La Jolla, CA) and sequence-verified. 5′RACE was performed as described previously (Hansen *et al.*, 2003).

Western Analysis

Western blotting was performed using the following antibodies: Anti-β-galactosidase, mouse monoclonal (Roche, Nutley, NJ); 1:4,000. Anti-β-actin, goat polyclonal (Santa Cruz Biologicals, Santa Cruz, CA); 1:1,000. Anti-Bruce N-terminal, mouse monoclonal; (Transduction Laboratories, Lexington, KY); 1:1,000. Anti-Bruce C-terminal, rabbit polyclonal (Hauser, AG Jentsch) 1:100. Antimouse, polyclonal, goat peroxidase coupled (Dianova), 1:10,000. Antirabbit, polyclonal, goat peroxidase coupled (Dianova), 1:10,000. Antigoat, polyclonal, donkey peroxidase coupled (Sigma, St. Louis, MO), 1:10,000.

E10.5 embryos or tissues were homogenized in 150 μl of PBS (phosphate-buffered saline) containing protease inhibitor (complete, Roche) using a micropipette, subsequently, 45 μl of 5× Laemmli buffer (Laemmli, 1970) were added, mixed, and incubated at 94°C for 10 min. Then 12.5 μl of the lysate per lane were either loaded directly on SDS gels or stored at −80°C until use.

Gradient acrylamide gels of 4–20% were run in Mighty Small Gelsystems (Hoeffer Scientific, San Francisco, CA) in 1× SDS buffer at 20–40 mA for 2–3 h. Bench Mark Prestained Protein Ladder (Invitrogen) was used as a molecular standard.

Proteins were transferred to a PVDF membrane (Millipore, Bedford, MA) using a tankblot system at 70V at 4°C overnight (Burnette, 1981). Immunochemistry was performed using specific antibodies in blocking solution (4% slim milk) for 1 h at room temperature (RT), after 5× washing in TBST and incubation with a peroxidase-coupled secondary antibody for 45 min at RT and subsequent 5× washing in TBST, immunoreaction via chemiluminescence was carried out using ECL Western Blotting Detection reagents (Amersham) according to the manufacturer's recommendations.

In Situ Hybridization

In order to generate a *Bruce* antisense probe, the plasmid pBR-M1 (a gift of S. Jentsch, Munich) was XhoI digested and transcribed with T7 RNA polymerase. For the sense control, the same plasmid was digested with SacI and transcribed with T3 RNA polymerase. The RT-PCR products from wildtype placenta for flt-1 and flk-1 were cloned in TOPO TA (Invitrogen) and recloned as EcoRI fragments in pBluescript II KS+ (Stratagene, La Jolla, CA). The plasmid flt1 was digested BamHI and transcribed T3 for antisense or HindIII/T7 for sense transcript. As for the plasmid flk1: HindIII/T7 (antisense) or PstI/T3 (sense). A mash 2 probe was amplified from placental cDNA using the primers CTCTGTCCTGCGCCTCTACGTC and AGTCACCCAGGGATGCAGCTTA. The probe was cloned in pCRII-TOPO and sequenced. The plasmid mash2 was digested with HindIII and transcribed T7 for antisense or BamHI/T3 for sense transcript. A Hif1alpha probe was amplified from placental cDNA using the primers CGACACCATCATCTCTCTGG and AGTGGCAGTGATGGTAGG. The probe was cloned in pCRII-TOPO and sequenced. The plasmid Hif1alphaA1 was digested XhoI and transcribed T3 for antisense or digested BamHI/T7 for sense transcript. In situ hybridizations were performed as described previously (Dagerlind *et al.*, 1992).

β-Gal Staining of Embryos

β-Gal staining of embryos was performed between E8.5 and E12.5 as described previously (Floss and Wurst, 2002).

Paraffin Sections

For immunohistochemistry and in situ hybridization, tissues were fixed in 4% PFA solution at 4°C for 60 min and incubated in 70% EtOH at 4°C for 60 min; 96% EtOH at RT for 45 min; 96% EtOH at RT for 30 min; 100% EtOH at RT for 45 min; 100% EtOH at RT for 30 min; Xylol at RT for 30 min; Xylol at RT for 30 min; paraffin at 65°C for 120 min. The tissue was sectioned at 4–8 μm on a microtome, air-dried at 37°C overnight, and stored at 4°C or used directly.

Immunhistochemistry

Immunhistochemistry was performed on paraffin sections using antibodies against phospho-Histone H3 (Lot 26414; Upstate Biotechnology, Lake Placid, NY), EGFR (sc-03; Santa Cruz) and Cleaved Caspase 3 (Asp175; Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocols.

Tunel Assays

Tunel assays were performed on 8 μm paraffin sections after deparaffination with xylene and decreasing alcohol solutions (100%, 95%, and 70%, water) and permeabilization for 3 min in 0.3M sodium citrate at RT and 4 min heating in a microwave at 600W. After cooling to RT, sections were washed 2× with PBS.

Assays were performed using a Tunel kit (Lot D16560; UniTect) according to the manufacturer's protocols. Histochemistry was done with cresyl violet or hematoxylin-eosin by standard protocols (Bancroft and Stevens, 1990).

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