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The fgl2 prothrombinase/fibroleukin gene is required for lipopolysaccharide-triggered abortions and for normal mouse reproduction

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Increased fgl2 prothrombinase activity in maternal decidua and fetal trophoblasts may trigger abortions by proinflammatory cytokines induced by bacterial lipopolysaccharide (LPS) in mice and is implicated in human recurrent miscarriages and pre-eclampsia. Defining the physiological and pathological role of the fgl2/fibroleukin gene required an fgl2-knockout mouse and data on normal pattern of fgl2 expression during pregnancy. Expression of fgl2 protein was determined by immunostaining with specific antibody. Fgl2 knockout mice were generated and typed by PCR for presence of the altered gene. Immunostaining of timed CBA×DBA/2 mouse matings in a low-abortion-rate colony showed a distinct pattern of development of fgl2 protein expression in maternal decidua, and in embryonic tissues in early pregnancy. Outbred (mixed background) heterozygous fgl2 +/–×+/– matings with a similar low abortion rate showed selective occult loss of both +/– and, to a greater extent, –/– embryos prior to gestation day 11.5, in association with haemorrhage at the anti-mesometrial pole of fgl2-deficient embryo. LPS injected on day 6.5 caused classical abortions at mid-pregnancy in fgl2 +/+×+/+ matings, but not –/–×–/– matings. Physiological expression of fgl2 in fetal trophoblast may prevent occult loss in early pregnancy, along with other coagulation factors, but fgl2 expression is required for LPS to induce abortion pathology.

Key words: bacterial lipopolysaccharide/fgl2 prothrombinase/fibroleukin/haemostasis in pregnancy/spontaneous abortion

Introduction

Fgl2 prothrombinase is a fibrinogen-like protein associated with lethality in hepatitis, spontaneous abortion (fetal loss syndrome), and graft rejection (Ding *et al*., 1997; Clark *et al*., 1999a,b, 2001a; Levy *et al*., 2000; Marsden *et al*., 2003). Prothrombinase activity is only demonstrable when the protein is associated with phospholipids, typified by cell membranes, coagulation factor V is required for maximal activity, and this activity is independent of factor Xa (Chan *et al*., 2002). Cells expressing fgl2 prothrombinase include macrophages, vascular endothelial cells, fetal trophoblast, and a subset of decidual stromal cells (Ding *et al*., 1997; Levy *et al*., 2000; Clark *et al*., 2001a; Chan *et al*., 2002; Marsden *et al*., 2003). Fgl2 can also occur as a soluble T cell-secreted protein, named fibroleukin, which lacks prothrombinase activity (Marazzi *et al*., 1998). Recent data indicate that soluble fgl2 has immunosuppressive activity via an effect on T cells and dendritic cells (Chan *et al*., 2003). Although the membrane-associated prothrombinase and 'soluble fgl2'/fibroleukin are functionally distinguishable, they are coded for by the same gene.

Increased expression of fgl2 prothrombinase has been linked to increased rates of T helper-1 (Th1)-cytokine-dependent spontaneous abortion/resorption in CBA/J×DBA/2 mouse matings, and in a subset of women with recurrent unexplained miscarriages of chromosomally normal embryos (Clark *et al*., 1998, 2001a; Knackstedt *et al*., 2002). Expression of fgl2 protein is also increased in trophoblast in pre-eclampsia (Knackstedt *et al*., 2002), and recently, increased expression of fgl2 has been associated with stimulation of labour (Pan *et al*., 2003). Understanding the regulation of the fgl2/fibroleukin gene in animal models is therefore relevant to normal and abnormal human pregnancy. Fgl2 expression is up-regulated by the pro-inflammatory 'abortogenic' cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α in the uterus of pregnant CBA/J mice, which, via activation of thrombin, trigger inflammation and fibrin deposition that terminates the blood flow to the developing placenta (Clark *et al*., 2001a). These cytokines are also stimulated by the potent abortogen, bacterial lipopolysaccharide (LPS), which acts via the toll-like receptor tlr4; co-presence of a tlr signal, possibly LPS, appears necessary for IFN-γ and TNF-α to act (Clark *et al*., 2003a), probably due to the need to simultaneously down-regulate expression of the CD200 (OX-2) protein that

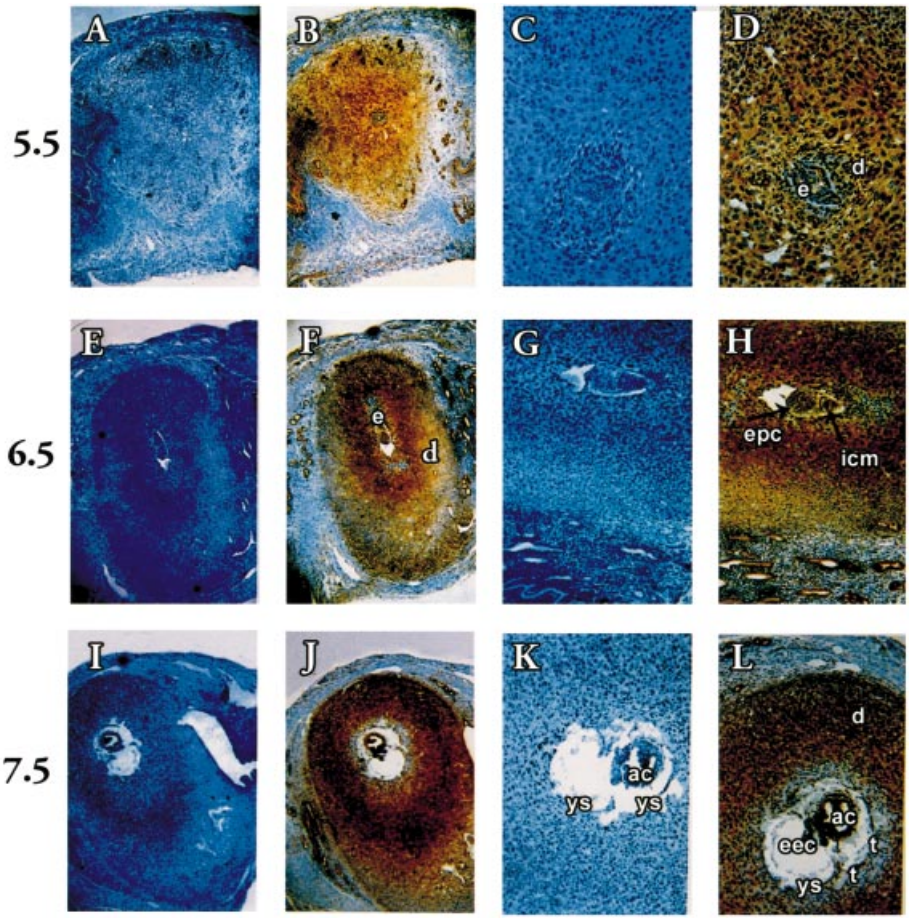

Figure 1. Fgl2 immunostaining of uteri from CBA×DBA/2 matings. (**A**–**D**) Gestation day 5.5; (**A** and **C**) negative control staining. (**E**–**H**) Gestation day 6.5; (**E** and **G**) negative control. (**I**–**L**) Gestation day 7.5; (**I** and **K**) negative control. The panels to the left (**A**, **B**, **E**, **F**, **I**, **J**) photographed at ×40; to the right, ×100. e = embryo; d = decidua; t = trophoblast; ac = amniotic cavity; eec = extraembryonic coelomic cavity.

counteracts fgl2 at the feto-maternal interface (Clark *et al*., 2001a). Since sensitivity to cytokine-triggered abortions can vary, depending on the nature of the endogenous microbial flora (a source of LPS) in the mouse colony at the time experiments are done, it is more reliable to inject LPS to trigger abortions (Clark *et al*., 2003a). Blocking fgl2 with specific neutralizing antibody given after day 6.5 of gestation prevents IFN-γ + TNF-α injected on gestation day (gd) 7.5 from triggering abortions in the CBA×DBA/2 mating model (Clark *et al*., 1998, 2001a). Pregnant mice, of a variety of different strains, abort in response to an injection of IFN-γ + TNF-α, and show the same classical resorptions if given LPS derived from *Salmonella enteritidis* (Clark *et al*., 1998, 2003a). LPS given later in pregnancy can also induce premature parturition in mice, similar to triggering of human labour by infections, but here, the role of fgl2 has not been tested (Chaouat, 1994; Kaga *et al*., 1996; Amory *et al*., 2001).

LPS is a potent inducer of proinflammatory cytokines of the type that triggers abortions in mice, and we predicted that blocking fgl2 with a sufficient amount of anti-fgl2 antibody would prevent abortions. To provide a more definitive test of the idea that fgl2 is obligatory for LPS-triggered abortions, we used a previously developed fgl2 knockout mouse on a heterozygous 129SvJ/C57Bl6 background. Fgl2 mRNA is endogenously expressed in decidua and trophoblast at levels lower than seen with abortions (Clark *et al*., 2001a), and the development of this knockout mouse (Marsden *et al*., 2003) allowed us to test a second hypothesis that physiological fgl2 expression plays an important role during normal pregnancy.

Materials and methods

Mice

C57Bl/6 (B6) mice of both sexes were obtained from the Jackson Laboratories, Bar Harbour, ME, and maintained in a barrier facility with food and water *ad libitum* and a 12 h:12 h light:dark cycle. Female CBA/J and male DBA/2 mice were similarly obtained and housed. All of the studies were done with approval of the institutional Animal Care Committee.

Generation and breeding of fgl2-knockout mice

Homologous recombination was used to inactivate the mouse fgl2/fibroleukin gene in 129Sv/J embryonic stem cells, and colonies were screened for homologous recombination using multiplex PCR analysis of *Bam*HI digested DNA (Marsden *et al*., 2003). Multiplex PCR$^+$ clones were injected into B6×B6 blastocysts and implanted in pseudopregnant CD1 females. Chimeric (129/B6) males were mated to identify those with an fgl2 mutation in the germ line, and the progeny were intercrossed for three generations (N1–N3), and N3 fgl2 +/– heterozygous offspring were then backcrossed to B6 for 10 generations (N10). At each generation, the pups were typed by multiplex PCR analysis to define +/+, +/– and –/– genotypes; the heterozygotes were then used for the next backcross. Healthy fgl2 –/– male and female mice from the N3 breeding generation were mated for the study of LPS-induced abortion susceptibility, and the day of sighting a vaginal plug was denoted as day 0.5 of pregnancy.

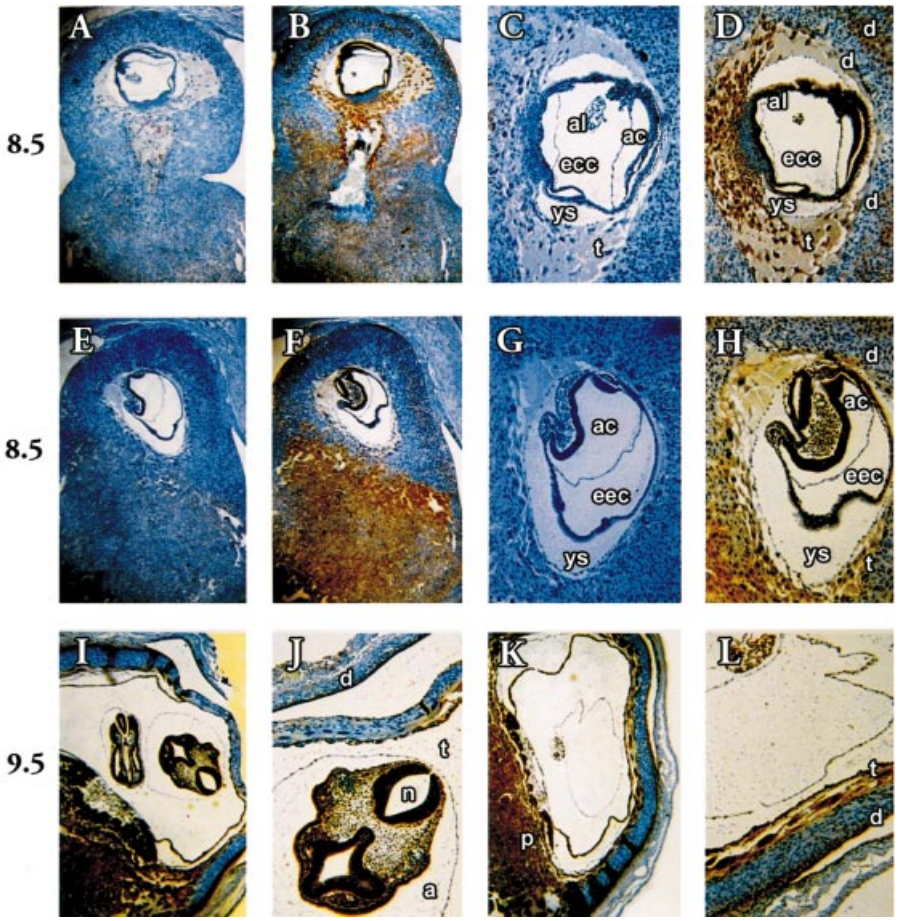

Figure 2. Fgl2 staining on day 8.5–9.5 of CBA×DBA/2 pregnancy. (**A**–**H**) Gestation day 8.5; (**A**, **C**, **E** and **G**) negative controls; ys = yolk sac; al = allantois; other labels as in Figure 1. (**I**–**L**) From gestation day 9.5; n = neural tube of embryo; p = placenta. **A**, **B**, **E**, **F**, **I**, **K**: magnification ×40; **C**, **D**, **G**, **H**, **J**, **L**: magnification ×100. Negative controls (not shown) gave same absent staining as in Figure 1.

Pregnancy loss studies

Heterozygous +/–×+/– matings from the N3 generation were killed at different times after mating to determine if there was pregnancy loss during normal reproduction. Uteri from mice at gestation day 8.5 were fixed in 4% paraformaldehyde for 18 h, transferred to 70% ethanol, and were processed for paraffin embedding and sectioning as described elsewhere (Clark *et al*., 2001a). Some tissue sections were stained with haematoxylin and eosin or Masson's trichrome to characterize histological changes. Other sections of normal or abnormal embryos were stained with polyclonal rabbit anti-mouse fgl2 (rabbit 6) at a 1/10 000 dilution or control (rabbit IgG 1.25 µg/ml or normal rabbit serum at 1/10 000) followed by horseradish peroxidase (HRP)-tagged anti-rabbit IgG and DAB (USA-HRP50 Test System, Rabbit Polyclonal DAC 2232; Signet/Cedarlane Labs, Canada), essentially as described by Knackstedt *et al*. (2002) for human tissues, but for mouse tissue, heating was not required to reveal fgl2 antigen (Marsden *et al*., 2003). Homozygous –/–×–/– matings of N3 generation mice were injected on day 6.5 of gestation with 5 µg *S. enteritidis* LPS as described elsewhere (Clark *et al*., 2003a). These mice were killed on day 13.5 of gestation and the numbers of viable and resorbing (aborting) sites were counted. CBA/J females mated to DBA/2, and N3 +/+×+/+ control matings, were similarly studied to verify activity of the LPS.

Statistical analysis

The significance of differences was determined by χ^2 or by Fisher's exact test, where appropriate. For analysis of deficient production of fgl2 +/– and –/– pups in breeding of heterozygous +/–×+/– parents, expected numbers were calculated from the expected Mendelian proportions of 1:2:1 (for +/+:+/–:–/–). A 'goodness of fit' χ^2 statistic could be calculated by dividing the total number of genotyped pups according to the 1:2:1 prediction (Snedecor and Cochrane, 1971), or by assuming that the number of +/+ pups observed was correct, in which case the number of –/– pups should be the same and the number of +/– twice. Both methods gave a similar result, and as there was no *a priori* reason to expect that the presence of a defective fgl2 gene could increase the number of fgl2 + gametes at the ovarian or testicular level, we chose to use the latter method. This facilitated appreciation of the magnitude of deficient production of pups of the +/– and –/– genotypes. Confidence intervals of resorption rates (100%×number of resorptions (abortions)/total number of implantations) were determined from the binomial.

Results

Expression of fgl2 protein in normal CBA×DBA/2 mated mice was carried out using timed matings on day 4.5–13.5 of gestation. Serial sections stained with haematoxylin and eosin were used to identify individual implantations. Adjacent sections were immunostained for fgl2. Implanted early embryos were first noted on day 5.5, and there was intense staining for fgl2 in decidualizing maternal tissue surrounding the embryo (Figure 1B and D compared with the negative control in A and C). There was staining of uterine epithelium including glandular epithelia (see Figure 1B), and some light staining of stromal cells remote from implantation sites. By day 6.5, the embryo itself had acquired staining for fgl2 (Figure 1F and H compared with E and G), and by day 7.5, there was more focal staining of specific tissue layers within the developing embryo, in fetal trophoblasts, and a zone of non-staining decidua had appeared at the maternal–fetal trophoblast interface (Figure 1J and L compared with I

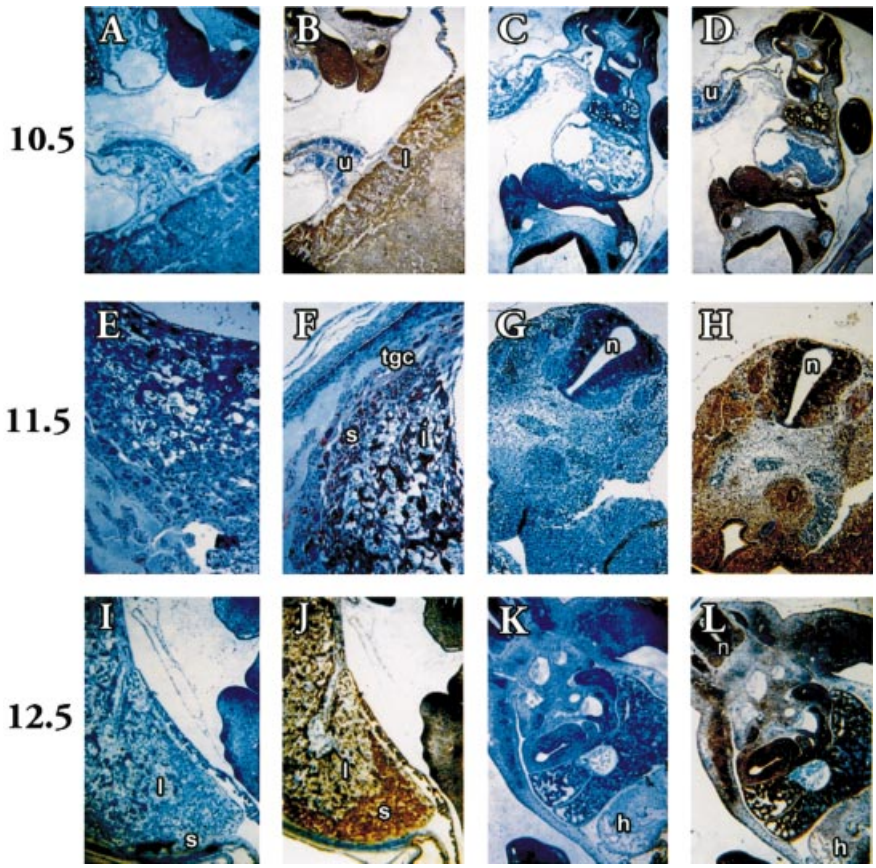

Figure 3. Fgl2 staining on days 10.5–12.5 of CBA×DBA/2 pregnancy. (**A**–**D**) Day 10.5; (**A** and **C**) negative controls; u = umbilical cord; l = labyrinthine trophoblast of placenta. (**E**–**H**) Day 11.5; (**E** and **G**) corresponding negative controls; tgc = trophoblast giant cell layer; l = labyrinthine zone; s = spongiotrophoblast zone of placenta. (**I**–**L**) Day 12.5; (**I** and **K**) negative control staining; h = heart. All magnifications ×40.

and K). On day 8.5, staining of decidua was more clearly separated from that of embryonic trophoblasts, and within the embryo fgl2$^-$ areas were noted (Figure 2A–H). A similar expression pattern continued to day 9.5 (Figure 2I–L). By day 10.5 (Figure 3A–D), there was staining of placental labyrinthine and spongiotrophoblast, as well as focal staining of certain developing organs within the embryo. Similar observations were made for day 11.5 (Figure 3E–H) and day 12.5 (Figure 3I–L). On gestation day 13.5 (Figure 4A–H), fgl2 staining in placental labyrinthine and spongiotrophoblast was again noted (4B and 4 D). Within the embryo, certain areas of developing nervous system tissue, lungs, limb buds, and somites (future vertebra) were positive (Figure 4F–H). We concluded that fgl2 protein expression occurred in a variety of developing embryonic tissues, including trophoblasts. Moreover we found that there was striking up-regulation of fgl2 in maternal decidua in contact with the embryo in early implantation, and that separation of fgl2$^+$ decidua from fgl2$^+$ trophoblasts began by day 7.5 in the CBA×DBA/2 mating system.

Expression of fgl2 mRNA in trophoblast and maternal decidua on gestation day 8.5 is required for high rates of spontaneous abortion in the CBA×DBA/2 model, and direct contact between fgl2$^+$ trophoblasts and decidua has been implicated in triggering resorptions (Clark *et al*., 2001a). The abortion rate in the lot of mice studied in Figures 1–4 was only 6%; such a low rate has been attributed to reduced exposure to 'danger' signals, such as bacterial flora LPS, which can vary considerably between shipments of mice (Clark *et al*., 2003a). Exposure to LPS stimulates local release of the abortogenic cytokines INF-γ and TNF-α, which, together with the LPS signal, down-regulates expression of the CD200 (OX-2) tolerance molecule at sites of fgl2 expression in the uterus; this down-regulation is obligatory for fgl2 to trigger loss. It was of interest to know if LPS could trigger abortions in the absence of fgl2. To further evaluate the need for fgl2 gene expression for LPS-induced/cytokine-dependent abortion, fgl2 knockout mice were generated as described in Materials and methods. The genotyping result for the N2 + N3 generations at weaning is pooled and summarized in Table I. Surprisingly, there appeared to be a selective loss of fgl2 –/– homozygotes of ~40–45%, and also a significant loss of +/– heterozygotes of ~25–30%; a small proportion of the neonates was lost before they could be genotyped, but even if these had all been from the +/– and –/– populations, most of the loss had to occur during pregnancy. For example, at the N2 + N3 (mixed-background) generations, –/– pups from 95 litters of 660 pups were 62.1% of expected, and in 39 litters of 292 pups with losses, –/– were 45.5% of expected. There was very little effect of post-natal loss on the frequency of +/– heterozygotes. Results after nine or ten backcrosses to C57Bl/6 (denoted as N9 and N10, which are pooled in the Table I) were consistent with selective loss of both +/– and –/– type embryos during pregnancy, and post-natal losses were more evident. The cause of the post-natal deaths did not appear to be haemorrhage, and further studies are in progress to elucidate the cause. Nevertheless, a significant proportion of fgl2 –/– pups survived to sexual maturity, enabling further studies of pregnancy in the absence of maternal and fetal fgl2 to be done. For these experiments, mixed-background N3 generation pups were used.

Figure 5 shows the number of embryos present in N3 generation heterozygous fgl2 +/–×+/– matings at different days of gestation. It can be seen that the average number of implantations began to drop

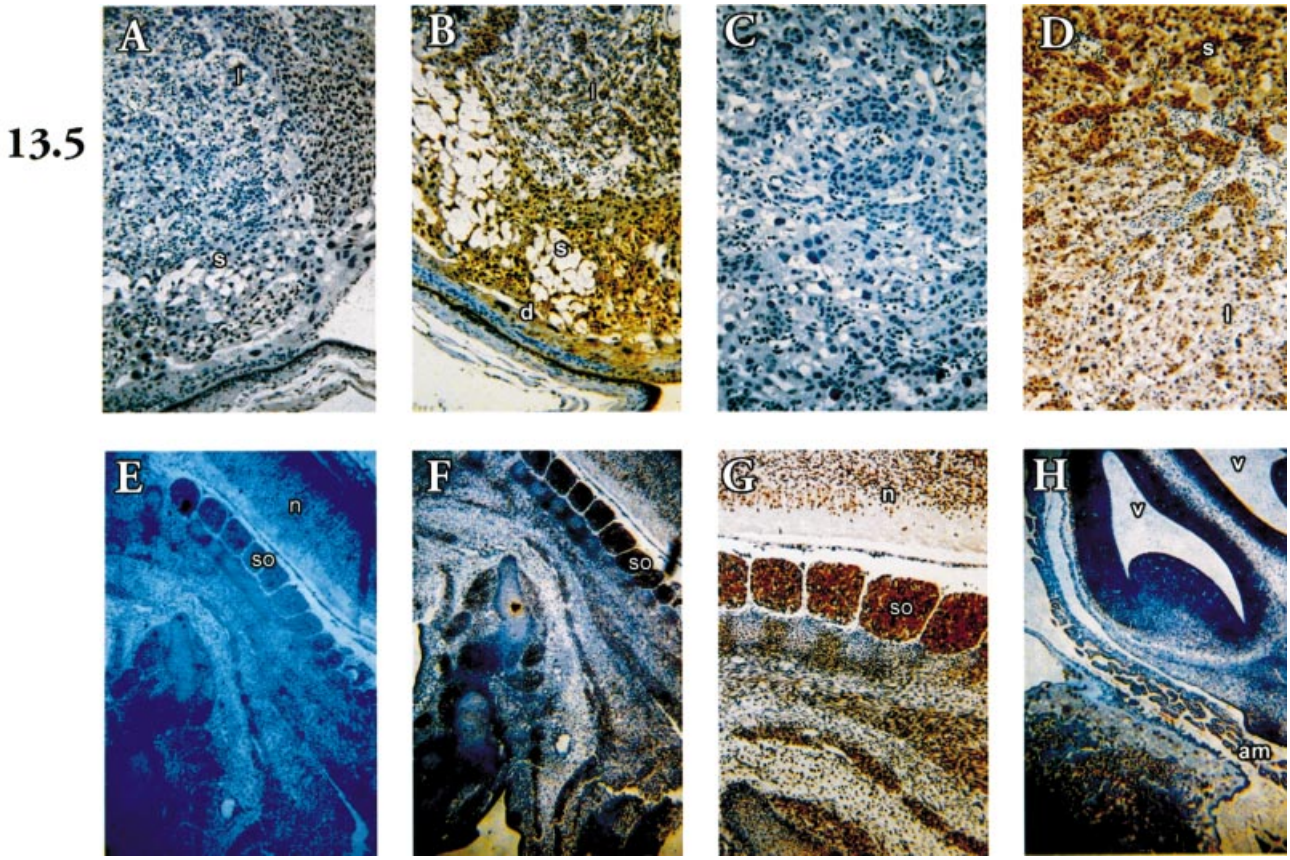

Figure 4. Fgl2 staining on day 13.5 of CBA×DBA/2 mating. (**A–D**) Placenta; (**A** and **C**) negative staining controls. **A**, **B**: magnification ×40; **C**, **D**: magnification ×100. (**E–H**) Selected areas of embryo; (**E**) negative control. (**F**) (magnification ×40) and (**G**) (magnification ×100) Staining of somites (so), and adjacent neural tube (n). (**H**) Layered staining of neural tissue enclosing developing ventricles (v); am = amniotic membranes.

Table I. Deficient production of fgl2 –/– and +/– offspring in heterozygous fgl2 +/–×+/– matings

Generation[a] (litter size)	Genotype	Total no. pups genotyped (%)	No. expected (%)	Male pups	Female pups	Male:female ratio
N2 + N3 (7.7)	+/+	302 (33)	302[b] (25)	152	150	1.0
	+/–	451[c] (49)	604[b] (50)	217	234	0.9
	–/–	171[d] (18)	302[b] (25)	81	90	0.9
N9 + N10 (8.7)	+/+	32[e] (41)	32[b] (25)	14	18	0.8
	+/–	38[f] (49)	64[b] (50)	20	18	1.1
	–/–	8[g] (10)	32[b] (25)	3	5	0.6

[a]N2 + N3 represent pooled data from the second and third matings of the mixed background mice carrying the transgene inactivating fgl2; N9 + N10 represent a pool from the 9th and 10th backcross of the mixed background mice to C57Bl/6.
[b]Assuming Mendelian genetics, this number of +/+ represents expected number of –/– and ×2 represents expected number of +/– offspring, as explained in Materials and methods. The data were pooled from two breeding generations.
[c]Less than expected by $P < 0.005$ based on number of +/+ pups. $\chi^2 = 16.5$. Parentheses show value as % of total number of pups typed.
[d]Less than expected by $P < 0.005$ based on number of +/+ pups. $\chi^2 = 31.4$.
[e]In N2 + N3 breeding generation there were 95 litters with 660 pups and no neonatal loss, of which 638 could be genotyped, and 29 litters with losses leaving 292 pups of which 286 could be genotyped. In the N9 + N10 generations there were 26 litters with 227 pups of which 146 survived and 78 were successfully genotyped.
[f]Less than expected $P < 0.025$ based on number of +/+ pups. $\chi^2 = 4.76$.
[g]Less than expected $P < 0.005$ based on number of +/+ pups. $\chi^2 = 12.5$.

after day 7.5 of gestation and most of the loss was complete by day 10.5–11.5 of gestation. During this time window, classical resorptions were not seen. The upper 95% confidence limit of the mean implantation number on day 10.5 + 11.5 was 9.6, and did not overlap the mean number of implantations on days 7.5 or 8.5 of gestation. Some resorptions were seen at later times (e.g. day 13.5, 4/26 = 15%), and if resorptions were subtracted from the total number of implantations shown in Figure 5 on day 13.5, the number of viable implantations per mouse approximated the litter size determined from N3 generation included in Table I. All of the spontaneous resorptions genotyped as +/–, for reasons set out in the Discussion. The larger variance of the number of implantations on days 7.5 and 8.5 shown in Figure 5 indicated significant heterogeneity in the timing of onset of pathology.

We have previously reported that embryo loss in the N3 heterozygote matings is typified by appearance of haemorrhage between fetal trophoblast cells and Reichert's membrane at the mesometrial pole of the embryo (Marsden *et al*., 2003). Data shown in Figure 6A are representative. In this day 8.5 embryo, there has been massive extravasation of maternal erythrocytes between trophoblast and Reichert's membrane, sparing only the mesometrial attachment of the embryo to the decidua. By immunostaining, occurrence of haemorrhage was associated with absent staining of fgl2 protein in trophoblast, by contrast to positive staining of trophoblast from a

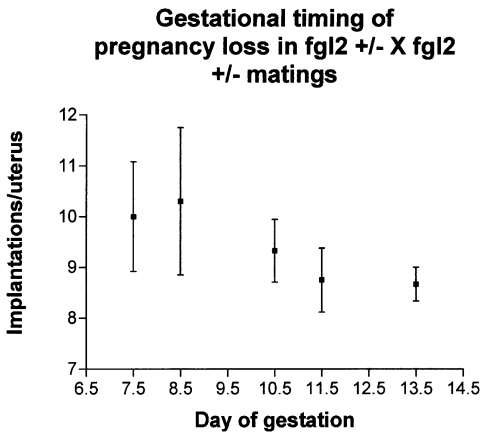

Figure 5. Gestational timing of pregnancy loss in fgl2 +/–×fgl2 +/– matings. The mean + SEM is shown for mice killed at different times after mating.

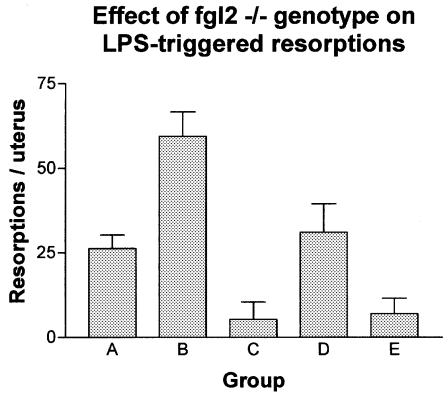

Figure 7. Effect of fgl2 –/– genotype on lipopolysaccharide (LPS)-triggered resorptions (abortions). Group A: CBA/J×DBA/2 matings given 0.1 ml phosphate-buffered saline (PBS) i.p. on day 6.5 of gestation. Group B: CBA×DBA/2 matings given 0.1 ml PBS containing 1 μg *S. enteritidis* LPS on day 6.5 of gestation. Group C: N3 +/+×+/+ control matings given PBS i.p. on day 6.5 of gestation. Group D: N3 +/+×+/+ matings given 5 μg *S. enteritidis* LPS i.p. on day 6.5 of gestation. Group E: N3 fgl2 –/–×fgl2 –/– mated mice given 5 μg *S. enteritidis* LPS i.p. on day 6.5 of gestation. The mean % resorptions + SEM on gestation day 13.5 is shown. LPS significantly increased the abortion rate in pregnant CBA/J and N3 +/+ females (B versus A, D versus C). The resorption rate in the fgl2 –/–×fgl2 –/– matings given LPS was not different from untreated N3 +/+×+/+ matings.

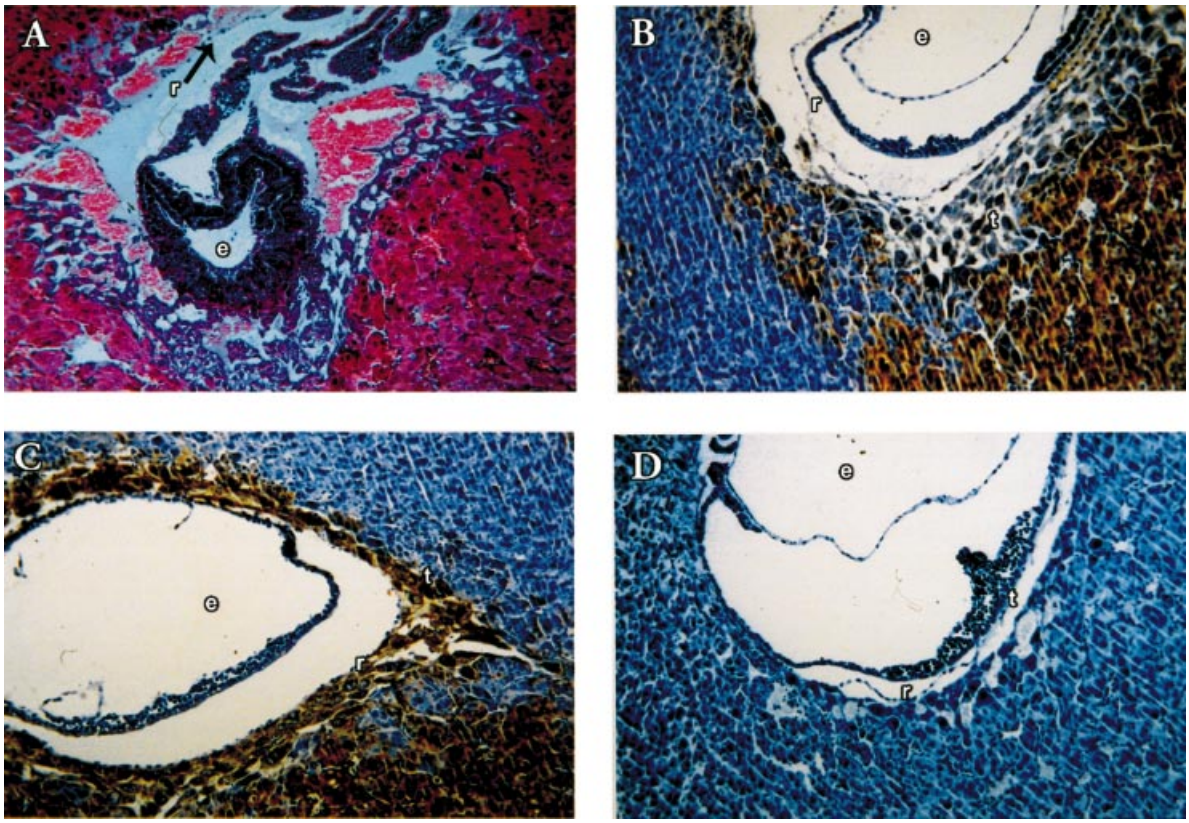

Figure 6. Typical embryonic histology on gestation day 8.5 of a heterozygous mating. (**A**) Masson's trichrome-stained section of an embryo showing extensive haemorrhage (arrow) between trophoblast, and Reichert's membrane, sparing the mesometrial pole; magnification ×100; e = embryo; t = trophoblast; d = decidua; r = Reichert's membrane. (**B**) Anti-fgl2-stained section of an embryo showing haemorrhage between trophoblast and Reichert's membrane (arrow); magnification ×200. (**C**) Anti-fgl2-stained section from an embryo in the same uterus lacking the haemorrhage shown in **A** and **B**; magnification ×100. (**D**) Negative control IgG staining of decidua and embryo; magnification ×100.

'normal' embryo, with no sign of haemorrhage and detachment of Reichert's membrane from trophoblast, in the same uterus (Figure 6B compared with C). Figure 6D shows non-staining in the rabbit IgG control, and a similar result was obtained using normal rabbit serum 1/10 000 (not shown); note that here the adjacent decidua was negative, consistent with the studies of the CBA×DBA/2 system.

N3 fgl2 –/– progeny that achieved sexual maturity were mated to generate pregnancies where no fgl2 expression would occur. Figure 7 shows that an i.p. injection of *S. enteritidis* LPS on day 6.5 of gestation generated increased numbers of abortions in CBA×DBA/2 matings, and was used in this study due to the ability to abort mice with the B6 background (Clark *et al*., 2003a). This type of LPS was able to boost the abortion rate of N3 +/+×+/+ matings, but had no significant effect on matings of N3 generation –/–×–/– mice. These data were consistent with the hypothesis that fgl2 plays a key role in LPS/cytokine-triggered abortions/resorptions.

Discussion

The kinetics of expression of fgl2 protein in maternal and embryonic tissues in a low abortion rate group of CBA×DBA/2 matings indicates that the fgl2 molecule can play a physiological role in early as well as late pregnancy. Low abortion rates in the CBA×DBA/2 model occur when there is minimal stimulation of TNF-α and IFN-γ production by tlr signals, such as LPS, provided by the endogenous flora. The expression of fgl2 in the embryo and decidua in early pregnancy is likely physiological rather than pathological. The physiological function could, of course, be due to antagonism of the procoagulant/proinflammatory function of fgl2 by co-presence of CD200 (OX2), and that remains to be investigated (Clark *et al*., 2001a, 2003b). It is also apparent that fgl2 protein is expressed within the embryo in a variety of organs undergoing development. It is unknown whether the functional activity of the protein is that of a prothrombinase/serine protease, immunosuppressive factor or represents some other functions (Chan *et al*., 2002, 2003). Further functional studies will be required to characterize the putative pleiotropic role of the fgl2 protein *in vivo*. As fgl2 –/– mice could develop normally and reach sexual maturity, an obligatory role for fgl2 in embryogenesis *per se* seems unlikely. However, generation of optimal litter size did require fgl2.

The spontaneous pregnancy losses of fgl2-deficient embryos shown in Figure 5 have a timing which has been equated to 'occult' (peri-implantation) failures; 'occult' losses arise by immunobiological mechanisms distinct from those causing spontaneous abortions/resorptions (Clark *et al*., 2001b). The latter, which are readily seen in CBA×DBA/2 matings, and in B6×B6 matings treated with *Salmonella* LPS, appear to arise by activation of pathological levels of fgl2 expression such that on gestation day 8.5, there is contact between fgl2HI trophoblast and fgl2HI decidua (that did occur in the low abortion rate mice in Figure 2), and down-regulation of the CD200 (OX-2) molecule which counters thrombin-triggered inflammation (Clark *et al*., 2001a). By contrast, the 'occult' losses of fgl2-deficient embryos (Figure 6 and Table I) were linked to a lack of physiological expression of fgl2. Curiously, from PCR typing, resorptions only appeared to affect embryos which had one intact fgl2 allele. This finding was likely an artefact resulting from the fact that following embryonic demise, maternal cells of the fgl2 +/– genotype invade the dead and dying embryonic tissue. Preliminary PCR typing of day 13.5 embryos has shown the same pattern of deficient –/– and +/– genotypes in both the N3 and N10 generation mice, consistent with embryo loss in early pregnancy and the only difference at the N9-10 generation breeding result in Table I was the unexpected high loss of neonates before PCR typing could be done. The mechanism of this loss is currently under investigation.

'Occult' losses can be generated via activation of T cell receptor αβ$^+$ T or NKT cells, or by antagonizing indoleamine 2,3-dioxygenase (IDO) which allows maternal αβ T cells to spontaneously activate in response to paternal transplantation antigens expressed by the semi-allogeneic embryo, and cause pregnancy loss (Clark *et al*., 2001b; Mellor *et al*., 2001). Recently, soluble fgl2 (also known as fibroleukin), which lacks prothrombinase activity, has been found to be a potent immunosuppressor of classical T cell activation (Chan *et al*., 2003). It is tempting to speculate that 'occult' pregnancy failures illustrated in Figure 5 might have been caused by loss of local suppression of classical T cells with the αβ T cell receptor. If correct, there would be a second immunoregulatory molecule besides IDO that is relevant to success during this phase of pregnancy. The presence of intense expression of fgl2 by day 5.5 of normal CBA×DBA/2 pregnancy is compatible with this hypothesis. Immunostaining does not, however, identify the soluble fibroleukin. After nine or ten generations' backcross of the mixed background fgl2$^{+/-}$ mice to C57Bl/6 (denoted as N9 and N10), heterozygosity between the mice should have been sufficiently reduced (from a mixture of strain 129 and B6 genes to >99.2% B6) to improve survival if losses of the fgl2-deficient embryos were due to maternal T cells as these embryos would lack foreign paternal histocompatibility antigens. This was not evident in the data. However, immunological events could play a secondary role in accelerating elimination of embryos; inbreeding in mice is associated with infertility, and residual heterozygosity (and hence potential antigen-immune system interactions) has been viewed as beneficial. It follows that the presence of fibroleukin and paternal antigen molecules might be required to ensure that residual immune reactivity to the implanting embryo is deviated towards being 'helpful' rather than being 'harmful'. Recent identification of implantation-'helpful' cytokines appears to be providing evidence for the immunotrophic hypothesis, and provide a basis for further study of the knockout model (Chaouat and Menu, 1997; Ghazeeri et al., 2001). Contribution of fgl2 deficiency to a critical defect in embryogenesis in mice has also not been excluded as a basis for loss of a proportion of embryos. Further studies will be required to address these issues.

A variety of deletions of coagulation factor and angiogenesis factor genes have been described that are relevant to understanding our results, and the essential features are summarized in Table II. In many instances, a similar timing of loss was seen as in the fgl2-deficient embryos (Figure 5). However, where haemorrhages occurred at the feto-maternal interface, in the examples shown in Table II, bleeding always occurred at the mesometrial pole. Absence of some haemostatic factors also led to post-natal losses, not unlike the findings in Table I, although as mentioned, bleeding did not seem to explain the deaths of fgl2 –/– neonates, especially in the N9 + N10 generations. In most instances, pregnancy failures seen in the models in Table II required a complete gene product deletion in the embryo or mother, unlike our results with fgl2-deficient embryos. A partial deficiency of a non-fgl2 coagulation factor that caused loss of –/– embryos rarely affected the success of +/– embryos; only prothrombin deficiency (factor II) seemed to affect +/– embryos. In one study, lack of tissue factor seemed to reduce the success of +/– embryos, but due to the small number of embryos genotyped, this did not reach statistical significance, and was not reproduced in two independent studies cited in Table II. By contrast, two independent reports of vascular endothelial growth factor (VEGF) knockout mice show 100% loss of both +/– and –/– embryos, consistent with a critical requirement for normal levels (Carmeleit *et al*., 1996b; Dowd *et al*., 1996; Ferrara *et al*., 1996). VEGF is important for angiogenic events that lead to formation of a placenta during early pregnancy. A variety of critical steps in vascularization of the embryo take place between

Table II. Effect of knockout of coagulation factors and angiogenesis factors on embryonic survival in heterozygous +/–×+/– matings

Knockout	No. of progeny of genotype			Timing of losses (gestation day start→end)	Reference(s)
	+/+	+/–	–/–		
Tissue factor	30	49* (60)[a]	0* (30)[a]	gd 10.5→1.5	Carmeliet *et al.* (1996a)
	19	35 (36)	0* (19)	gd 10.5→11.5	Bugge *et al.* (1996)
	58	133 (118)	21* (58)	gd 10.5→11.5	Toomey *et al.* (1996)
Factor VII	'Normal Mendelian distribution'			Post-natal deaths only	Rosen *et al.* (1997)
Factor V	89	176 (178)	32* (89)	gd 10.5→11.5	Cui *et al.* (1996)
Factor II	223	378 (446)*	19* (223)	$\frac{1}{2}$ < gd 8.5, remainder lost gd 14.5→18.5	Xue *et al.* (1998), Sun *et al.* (1998)
Fibrinogen Aα	71	157 (142)	81 (71)	31% of –/– females died by gd 10.5, bled into uterine lumen and placental labyrinth	Suh et al. (1995)
Fibrinogen Fγ	'Normal Mendelian distribution'			Pregnant –/– females bled and died gd 6.5→10.5; embryonic arrest gd 9	Ploplis *et al.* (2000), Iwaki *et al.* (2002)
Thrombin receptor	41	110 (82)	24* (41)	gd 8.5→9.5	Darrow et al. (1996), Connolly et al. (1996)
Thrombomodulin	56	96 (112)	0* (56)	gd 8.5→10.5	Healey *et al.* (1995), Weiler-Guettler *et al.* (1996)
Factor X	77	182 (154)	0* (77)	gd 11.5→12.5	Dewerchin *et al.* (2000)

[a]Expected number = 2 times the number of +/+ embryos. For –/–, the expected number is same as number of +/+ embryos.
*$P < 0.05$ less than number expected from Mendelian prediction by χ^2. The same method was used to calculate expected number as in Table I.

gestation days 7.5 and 11.5, and haemostasis must be carefully regulated as maternal blood vessels are being invaded and eroded by fetal trophoblast cells. One may suggest that normal levels of fgl2, and possibly factor II and tissue factor, may be important for reproductive success. However, a dominant positive–negative effect has also not been formally excluded (i.e. one defective fgl2 allele might interfere with the normal allele); loss of fgl2 function was not found in macrophages from fgl2 +/– progeny, but all tissues were not tested (Marsden *et al*., 2003). Studies of the level of phenotypic expression of functional fgl2 protein in the fgl2 +/– embryo trophoblast have not been completed, and we do not yet know if every embryonic trophoblast cell of an fgl2 +/– embryo expresses 50% of normal functional fgl2 levels, or if there is an admixture of normal and totally deficient trophoblast (due to expression of one or other of the fgl2 alleles).

A significant proportion of fgl2 –/– embryos survive in –/– mothers, and this suggests that unlike many of the coagulation factor deficiencies in Table II where all homozygotes failed, there is either compensation for lack of fgl2 by other coagulation factors or the role of fgl2 in early pregnancy concerns embryogenesis and not coagulation. Indeed, embryonic demise of tissue factor and fibrinogen –/– embryos could be prevented by deliberately increasing levels of these factors in the mother (Parry *et al*., 1998; Iwaki *et al*., 2002). In our studies, the number of –/– embryos surviving to day 13.5 in –/– and +/– mothers did not appear different; this suggests that the early embryonic demise from gd 7.5 to 11.5 was due entirely to the embryonic fgl2 phenotype. Indeed, this is likely true for other haemostatic factor knockouts listed in Table II.

If the –/– embryonic genotype for most coagulation factors leads to >80% demise, as listed in Table II, then the partial loss of fgl2 –/– embryos suggests that fgl2 is not a critical haemostatic factor and might, in fact, play a different role in early pregnancy. When fgl2 levels are up-regulated by cytokines, however, there is coagulation-dependent demise of the embryo, and these events occur on the mesometrial side of the embryo where fetal and maternal tissues interact (Clark *et al*., 2001b). LPS induces expression of proinflammatory cytokines such as TNF-α and IFN-γ that up-regulate fgl2 in both maternal decidua and fetal trophoblast so as to trigger classical abortions/resorptions (Clark *et al*., 2001b). As shown in Figure 7, it was not possible to induce classical abortions/resorptions with LPS in matings where embryonic and maternal fgl2 were absent, consistent with the prediction that fgl2 up-regulation plays a key role in initiating resorptions in certain situations (Ding *et al*., 1997). In fgl2 –/– mice, a systemic dose of LPS does induce shock, and up-regulation of tissue factor expression on macrophages (Marsden *et al*., 2003); the tissue factor pathway acts via factor X and is distinct from fgl2-induced coagulation which is factor X-independent (Chan *et al*., 2002). If LPS enhanced expression of tissue factor by maternal macrophages at the feto-maternal interface in pregnant fgl2 –/– mice, this up-regulation was clearly insufficient to induce resorptions. A lack of coagulation factor X (Table II) can cause resorptions of –/– embryos, which occur due to bleeding between gestation day 11.5 and 12.5 (strongly suggesting that factor X is present and plays an important role in haemostasis at the feto-maternal interface at this stage of pregnancy). If fgl2 were crucial for haemostasis during the placental phase of pregnancy (gd 9.5 to 21.5), one might have expected a high spontaneous abortion rate. However, both fgl2 +/– and –/– genotype embryos can survive to birth. It is tempting to speculate that factor X might compensate in part for the fgl2 –/– state at days 11.5–13.5 of pregnancy; the coagulant activity of fgl2 is independent of factor Xa, although once thrombin has been activated by fgl2 *in vivo*, feedback activation of other haemostatic factors could lead to a contribution by factor X-dependent pathways, which, unlike fgl2, are sensitive to inhibition by heparin-activated anti-thrombin III (Clark *et al*., 1999a,b; Chan *et al*., 2002). Factor X may not compensate completely for the fgl2 –/– or +/– state, given the 15% resorption rate we noted at day 13.5 in heterozygous matings, which is higher than the residual 2–6% expected on the basis of embryonic chromosome abnormalities (Clark *et al*., 2001a).

In Table II, one notes that early embryo loss occurred with deficiency of tissue factor which requires factor X, but not with factor X deficiency or factor VII deficiency. This is puzzling. It may be noted, however, that defects in embryonic development point to a role of certain coagulation factors in embryonic developmental processes independent of haemostasis (Suh *et al*., 1995; Erlich *et al*., 1999; Iwaki *et al*., 2002). (Loss of factor X –/– and VII –/– progeny does occur after birth, however, and here deaths *are* caused by bleeding.) In early

embryo–maternal tissue interactions there is enzymatic tissue digestion at the interface as part of the process of trophoblast invasion to establish an interface with model maternal blood at the mesometrial embryonic pole where a placenta is formed in secondary maternal decidua. It has been suggested that coagulation factors could play a role in these processes (Carmeleit *et al*., 1996a; Cui *et al*., 1996). It is tempting to speculate that fgl2 protein may play a similar role at the anti-mesometrial pole of the embryo where implantation of the mouse embryo in primary decidua occurs. Further studies will be required to elucidate these interactions and to understand haemostatic mechanisms that appear to follow different pathways than the role predicted for various coagulants by the standard model of thrombosis.

We used two different strain combinations of mice in the present study, and the purist might think it necessary to cross the fgl2 knockout gene onto the CBA/J and DBA/2 backgrounds to show that fgl2 was necessary for classical abortions in this system. Since specific anti-fgl2 antibody blocks abortions in the CBA×DBA/2 model (Clark *et al*., 1998), we do not believe such an expensive and time-consuming effort is required to draw firm conclusions with respect to the role of fgl2 in classical abortions. It would be of interest, however, to know if eliminating early expression of fgl2 in the peri-implantation period would convert the CBA×DBA/2 model into one of 'occult' loss. It is not evident that neutralizing antibody given in early pregnancy will be effective (Clark *et al*., 1998), perhaps due to problems with access to the fgl2 molecule. Similarly, the purist might want to see immunostaining of fgl2 on days 5.5–8.5 of pregnancy in fgl2 –/– and fgl2 +/+ mice on the B6 (N10) background. These studies are in progress. It is clear from Figure 6 that our antibody can specifically identify fgl2 + and fgl2 – tissues, in agreement with previous studies of mouse and human tissues (Knackstedt *et al*., 2002; Marsden *et al*., 2003).

From the standpoint of confirming fgl2 as a mechanism for the miscarriage of otherwise normal pregnancies, the most important findings in this paper are resistance to LPS-induced abortions in mice of the fgl2 –/– genotype, and occult losses where trophoblasts lack fgl2 expression. We believe these findings are relevant to the investigation of normal and abnormal human pregnancy. The mouse has a haemochorial placenta, albeit slightly different from that of human, and a majority of the immunobiological findings in mice have been reproduced when human pregnancy has been studied (Clark, 1999; Clark *et al*., 1999b, 2001a, 2003a,b; Knackstedt *et al*., 2002). 'Occult' loss, often perceived as infertility or implantation failure, is a particularly vexing problem since it is more frequent than spontaneous clinical miscarriage, and the underlying mechanisms are poorly understood in most cases (Clark, 2003). It has recently become possible to identify embryos in the process of an 'occult' failure (Coulam and Roussev, 2002). When these tissues are studied, it will be important to test for deficiencies of fgl2 as described in this paper, and the related haemostatic factors listed in Table II. There may be potential clinical application of therapy with soluble fgl2 (which has been made as a recombinant protein) should it prove able to prevent occult embryo loss in the fgl2-deficient mouse model or in other models of immunologically triggered occult failure (Clark *et al*., 1993; Chan *et al*., 2003).

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