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Targeted disruption of the *Wnt2* gene results in placentation defects

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SUMMARY

***Wnt* genes have been implicated in a range of developmental processes in the mouse including the patterning of the central nervous system and limbs. Reported here for the first time is the expression of *Wnt2* in the early heart field of 7.5-8.5 dpc (days post-coitum) mouse embryos, making *Wnt2* a potentially useful gene marker for the early stages of heart development. Expression was also detected in the allantois from 8.0 dpc and at later stages in the placenta and umbilicus. Mice deficient in *Wnt2*, generated by gene targeting, displayed runting and approximately 50% died perinatally. Histological analysis revealed alterations in the size and structure of placentas from these mice from 14.5 dpc. The placental defects were associated primarily with the labyrinthine zone and included oedema and tissue disruption and accumulation of maternal blood in large pools. There was also an apparent decrease in the number of foetal capillaries and an increase in the amount of fibrinoid material in the *Wnt2* mutant placentas. These results suggest that *Wnt2* is required for the proper vascularisation of the mouse placenta and the placental defects in *Wnt2*-deficient mice result in a reduction in birthweight and perinatal lethality.**

Key words: *Wnt2*, mouse, embryogenesis, allantois, gene targeting, placental pathology, perinatal death, IUGR

INTRODUCTION

Members of the *Wnt* gene family have been implicated in a number of developmental processes in the mouse as well as in a range of other species. The first *Wnt* gene identified, *Wnt1*, was isolated as a candidate oncogene involved in mammary tumours of mice (van Ooyen and Nusse, 1984). The *Drosophila* orthologue of *Wnt1* is the segment polarity gene *wingless* (*wg*), a gene known to play a number of important roles during the development of the *Drosophila* embryo (Rijsewijk et al., 1987; Chu-LeGraff et al., 1993; Couso et al., 1993; Wu et al., 1995; Skaer et al., 1992). Human *WNT2* was identified by chance during a chromosome walk while searching for the gene defective in cystic fibrosis (Wainwright et al., 1988). This was followed soon after by the homology cloning of the mouse *Wnt2* (*mirp*) cDNA (McMahon and McMahon, 1989). To date at least 20 distinct *Wnt* genes have been identified in *Drosophila*, zebrafish, *Xenopus*, chick, mouse and man.

While *Wnt* genes are expressed in a range of sites in the developing mouse embryo, the most common sites of expression are the developing limb and/or the developing central nervous system (Gavin et al., 1990; Parr et al., 1993; Christiansen et al., 1995) In some cases, the expression patterns of certain *Wnt* genes coincide, suggesting that there may be a degree of functional redundancy between genes with overlapping expression (McMahon and Bradley, 1990; McMahon et al., 1992). Null mutations have been generated for a number of *Wnt* genes and, in all cases, the genes have been demonstrated to be essential for the correct development of the mouse embryo. *Wnt1* 'knockout' mice have defects in the cerebellum often with the complete loss of the midbrain structures and these mice rarely survive birth (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). *Wnt3A* mutants exhibit defects in tail development, including absence of the caudal somites, a disrupted notochord and failure of tailbud formation as well as CNS defects such as incomplete closure and 'kinking' of the neural tube (Takada et al., 1994). In *Wnt4* mutant mice, the kidneys fail to develop normally and as a result these mice die soon after birth (Stark et al., 1994). Mutation of the *Wnt7A* gene results in a dorsal-to-ventral transformation of the limbs of these mutant mice with foot pads and other ventral structures appearing on the dorsal surface of the limbs (Parr and McMahon, 1995).

Despite the fact that *Wnt2* was the second *Wnt* gene identified, its function is still poorly understood. Apart from mice and humans, the only other species from which possible *Wnt2* orthologues have been reported are rat (Levay-Young and Navre, 1992), sea urchin (partial cDNA with a low level of homology (Sidow, 1992)) and leech for which a *Wnt* cDNA fragment was obtained that demonstrated higher homology with *Wnt2* than any of the other *Wnt* genes (Kostriken and Weisblat, 1992). There is very little known about the function of either human or mouse *Wnt2*. *Wnt2*, like all *Wnt* genes, is expressed in a restricted pattern during mouse embryogenesis

(McMahon and McMahon, 1989; Bellusci et al., 1996). It has also been implicated in normal mammary gland differentiation (Buhler et al., 1993) and has been indirectly implicated in the formation of mouse mammary tumours in vivo (Roelink et al., 1992).

In order to gain some understanding of the role played by *Wnt2* in the development of the mouse we have analysed the embryonic expression of *Wnt2* in embryos from 7.5 dpc to 10.5 dpc using whole-mount in situ hybridisation. In addition to confirming the pattern of *Wnt2* expression reported previously (McMahon and McMahon, 1989), we observed that *Wnt2* is expressed in the precardiac mesoderm from 7.5 dpc and in the foetal side of the developing placenta from 8.5 dpc until at least 11.5 dpc. The functional role played by *Wnt2* during mouse embryogenesis was addressed by generating mice that possess a targeted disruption in the *Wnt2* gene. This mutation, which is predicted to result in the absence of any functional WNT2 protein, causes structural defects in the placenta, which in turn result in runting and perinatal death.

MATERIALS AND METHODS

Whole-mount in situ hybridisation

Whole-mount hybridisation and subsequent sectioning of embryos was performed according to the procedure described in Christiansen et al. (1995). An adaptation of this method was used for the whole-mount in situs of placentas where the proteinase K treatment was increased 10-fold to allow efficient probe penetration. The efficiency of probe penetration using this method has been established using probes that detect cells throughout the depth of the placenta (D. J. Pennisi unpublished). For the detection of *Wnt2* expression, two *Wnt2* probes were subcloned from a near full-length *Wnt2* cDNA clone isolated from a C57Bl6/CBA adult mouse lung library. One probe was from within the coding region and the other from the 3′ untranslated region. Both probes gave the identical staining pattern and so only the probe that gave the strongest signal was used in subsequent experiments. *Wnt2* sense probes were used as a negative control and no staining was observed with these probes (not shown).

Digoxigenin in situ hybridisation of placental sections

In situ hybridisation to placental sections were performed as detailed in Schaeren-Wiemers and Gerfin-Moser (1993).

Electroporation of ES cells

Electroporation of ES cells was performed essentially according to the method of Wurst and Joyner (1993). Colonies were picked between 7 and 10 days after G418 and FIAU selection.

Morula aggregation

Morula aggregation was performed essentially according to the tetraploid aggregation method of Nagy and Rossant (1993) with the exception that only one 8-cell diploid embryo was used per aggregation with a clump of 10-15 ES cells. 8-cell embryos obtained from superovulated CD1 females were cultured and transferred to host CD1 pseudopregnant females as described in Nagy and Rossant (1993).

PCR genotyping

Two sets of primers were used in PCR genotyping reactions. One set amplified the DNA from within the *neo* coding sequence. The other set of primers amplified the region within exon 2 of the *Wnt2* gene across the site of the *neo* insertion. Only DNA that does not contain a *neo* gene in exon 2 is amplified by these primers. PCR reactions were performed in accordance with the protocols supplied by the manufacturers of the Taq polymerase used (Boehringer-Mannheim). Primers employed were: *neo* primers: 5′-ATCTCCTGTCATCTCACCTTGC-3′ and 5′-CAAGCTCTTCAGCAATATCACG-3′. *Wnt2* primers: 5′-GGTGGTACATGAGAGCTACAGG-3′ and 5′-CTACTTCGGAGGAGGACC-3′. The amplification conditions were 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute.

RT-PCR

3 μg of total or cytoplasmic RNA was incubated with 100 U of MMLV reverse transcriptase (BRL) in 10 mM Tris pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 0.01% Tween-20, 0.01% NP-40 in the presence of 1 mM dNTPs for 10 minutes at 25°C followed by 1 hour at 42°C. 1/5 of this reaction was heated to 95°C for 5 minutes then PCR amplified in the same buffer containing 2 μM of each primer and 1.5 U of Taq polymerase. Three separate primer pairs were used, one pair to amplify within the *HPRT* cDNA as a control (5′-CCTGCTGGATTACATTAAAGCACTG-3′ and 5′-GTCAAGGGCATATCCAACAACAAAC-3′) and two *Wnt2* gene-specific primer pairs; exon 1-3 (5′-GGGGTATGAACGTCCCTCTCGGT3′ and 5′-TCCTTGGCTACAGGCCCTGGTG-3′) and exon 3-4 (5′-CGGCCTTTGTTTACGCCATC-3′ and 5′-TGAATACAGTAGTCTGGAGAA-3′). The amplification conditions were 35 cycles at 94°C for 30 seconds, 57°C for 45 seconds and 72°C for 1 minute.

Caesarean section and fostering

Caesarean sections were performed on 18.5 dpc pregnant females essentially according to the method of Nagy and Rossant (1993). Following dissection of neonates from the uterus and removal of foetal membranes, the umbilicus was cut and a segment was retained for genotyping. The neonates were then placed under a heat lamp on a damp tissue and were prodded gently at intervals until they had begun to breath normally. They were then fostered with a female that had given birth in the past three days and observed at regular intervals for the next 10-12 hours.

Histology

Both embryos and other tissues (eg placenta) were fixed overnight at 4°C in 4% paraformaldehyde in PBS. The tissue/embryo was then embedded in paraffin and either 7 μm (for tissues) or 10 μm (for embryos) sections were cut. Haematoxylin and eosin was used to stain placental sections.

RESULTS

Embryonic expression of *Wnt2* by whole-mount in situ hybridisation

No *Wnt2* expression was detected prior to about 7.5 dpc (pre-headfold) using whole-mount in situ hybridisation (data not shown). At 7.5 dpc *Wnt2* expression was detected in a faint crescent-shaped pattern, which corresponds to the region of the heart primordium (Fig. 1A). At approximately 7.75 dpc when the headfold has become elevated, the foregut involutes and the differentiation of the heart precursor cells has begun, *Wnt2* expression had increased and was seen in a crescent surrounding the foregut involution (Fig. 1B). Sectioning of these embryos showed *Wnt2* expression was restricted to the mesodermal component of the heart field (Fig. 1D,E). Strongest expression in these embryos was detected in the lateral arms of the crescent, the prospective sinus venosus, and was weaker and more diffuse in the medial part of the crescent. Expression of *Wnt2* in 8.0 dpc embryos continued to be

detected in the crescent shaped cardiac mesoderm (Fig. 1C). At this stage, significant structural rearrangements have begun to occur at the midline where an anterior protrusion consists of differentiating myocardial and endocardial cells. *Wnt2* expression was weaker in this region of differentiating cells and strongest in the lateral arms the crescent. At later stages of development, *Wnt2* expression continued in the regions posterior to the heart such as the septum transversum and sinus venosus but was much weaker in the heart proper (Fig. 2A-C).

Other sites of *Wnt2* expression include tissues involved in foetal maternal communication. At 8.0 dpc, *Wnt2* expression was faintly detected in the allantois (Fig. 1C) and by 8.5 dpc (around the time that the allantois fuses with the chorion to form the chorioallantoic plate) the level of expression in this region increased (Fig. 2A). *Wnt2* expression is also detected in the chorioallantoic plate region once allantoic fusion has occurred (Fig. 2A). The *Wnt2* expression in the placental region at 9.5 dpc and 10.5 dpc continues to be confined to the chorioallantoic plate region (Fig. 2B,C). Whole-mount in situ hybridisation of 11.5 dpc placentas demonstrates that *Wnt2* is also expressed in the foetal blood vessels of the placenta (Fig. 2D,E). No expression was seen in any of the placenta that is derived from foetal trophoblasts.

Targeted disruption of the *Wnt2* gene in ES cells

A targeting vector was constructed to allow disruption of the *Wnt2* gene in ES cells by homologous recombination. The strategy outlined inFig. 3A utilises a positive/negative replacement targeting construct (Mansour et al., 1988) that contains approximately 10 kb of *Wnt2* genomic sequence including exons 1-3. Following homologous recombination at the *Wnt2* locus, exon 2 is disrupted by 1.8 kb of *neomycin phospho - transferase* (*neo*) gene driven by the *phosphoglycerate kinase-1* (*pgk*) promoter. The insertion of the *pgk* promoter and *neo* gene in exon 2 introduces at least one in frame stop codon preventing the production of full-length WNT2 protein from the targeted locus.

Following electroporation of the linearised targeting vector into R1 ES cells, G418- and FIAU-resistant ES cell colonies were screened for homologous recombination by Southern blotting. The screening strategy involved probing *Sac*I-digested genomic ES cell DNA with a 3′ external *Wnt2* probe (probe 1) shown in Fig. 3A. A representative Southern blot used to screen ES cell colonies is shown in Fig. 3B. Of the 96 ES cell colonies picked and screened, 8 were targeted at the *Wnt2* locus. *Sac*I digests of genomic DNA from these 8 clones and some of the non-targeted clones were also screened with an internal probe (probe 2, Fig. 3A) and, in all cases, the same band pattern was seen as for the external probe (data not shown) indicating that no random, additional insertion of the targeting vector had occurred in any of these ES cell clones.

Generation of *Wnt2*-deficient mice

Six of the eight targeted ES cell lines were successfully used to generate chimeric mice using diploid morula aggregation. 3 male and 1 female chimeras (each derived from different targeted ES cell line) when crossed to CD1 mates transmitted their ES-derived genome to their offspring as determined by coat colour. *Wnt2* heterozygous offspring from these matings were identified by a PCR genotyping strategy, which showed complete agreement with that used for Southern analysis (data not shown). From 21 intercross matings, 194 offspring have been genotyped at weaning (Table 1) and of these 22 (12 males and 10 females) were $Wnt2^{-/-}$. There were no deaths observed either before or after weaning in any of these litters. The distortion in the segregation of genotypes is highly significant ($P<0.0001$) for these intercrosses and is significant ($P=0.03$) for the $Wnt2^{+/-} \times Wnt2^{-/-}$ crosses. No significant ($P\geq0.1$ and $P=1$) segregation distortion was seen for litters genotyped prior to birth (Table 1) indicating that the $Wnt2^{-/-}$ mice died between birth and when the litter was first observed 12-24 hours after birth. Given that the number of $Wnt2^{-/-}$ expected according to Mendelian inheritance should be the same as the number of wild-type animals (59) in the case of the intercrosses or the same as the number of $Wnt2^{+/-}$ animals in the case of the $Wnt2^{+/-} \times Wnt2^{-/-}$ crosses, the survival rate for the $Wnt2^{-/-}$ animals is approximately 50%.

As a number of $Wnt2^{-/-}$ animals subsequently survived to adulthood, we examined the possibility that functional *Wnt2* transcripts were being made from the targeted allele. Reverse transcriptase PCR (RT-PCR) was performed on RNA extracted from the lungs and hearts of wild-type, $Wnt2^{+/-}$ and $Wnt2^{-/-}$ adult mice. *Wnt2* primer pairs were used to amplify across two regions of the *Wnt2* mRNA and *HPRT* gene primers were used for each of the RNA samples as positive controls (Fig. 3C). *HPRT* control primers amplified bands in all samples as did the *Wnt2* exon 3-4 primer pair. The *Wnt2* exon 1-3 primer pair only amplified a 401 bp product corresponding to wild-type *Wnt2* transcript from the $Wnt2^{+/+}$ and $Wnt2^{+/-}$ tissues and not from either of the $Wnt2^{-/-}$ tissues. A band of approximately 250 bp was visible in some of the tracks containing the exon 1-3 amplification reaction. Southern blotting indicated this was a non-specific amplification product not derived from the *Wnt2* gene (data not shown). This results indicates that no wild-type *Wnt2* transcript was present in the tissues from $Wnt2^{-/-}$ animals. The presence of transcripts containing exon 3 and 4 in $Wnt2^{-/-}$ tissues indicates either that *Wnt2* transcripts are being made that contain the *neo* in exon 2, or that transcription is initiating downstream of the *neo* gene or that exon skipping has removed exon 2 (and therefore the *neo* insertion). In all cases, given the highly

Table 1. Genotype of offspring from $Wnt2^{+/-} \times Wnt2^{+/-}$ and $Wnt2^{+/-} \times Wnt2^{-/-}$ matings

	Wnt2 genotype			
	+/+	+/–	–/–	*P*
28 days				
+/– × +/– mating	59	113	22	<0.0001
+/– × –/– mating	0	61	39	0.03
≤ 18.5 dpc				
+/– × +/– mating[a]	19	55	16	0.1
+/– × –/– mating†	0	18	19	1

*Ages ranging from 9.5-18.5 dpc
†Ages ranging from 14.5-18.5 dpc
P values calculated using χ^2 goodness of fit test for actual genotype segregation versus predicted.

conserved nature and functional importance of cysteine residues distributed throughout WNT proteins, it is highly unlikely that such transcripts would produce functional protein.

Perinatal death and runting of *Wnt2*$^{-/-}$ mice

To observe animals at and soon after birth when it appeared some lethality was occurring, litters were delivered from their mothers at 18.5 dpc by Caesarean section. The details of the genotypes of the 37 animals delivered by Caesarean section in these experiments are presented in Table 2. Although all *Wnt2*$^{-/-}$ neonates survived Caesarean section, they took longer to settle into a regular breathing pattern than their litter mates, remaining grey for many minutes. Some after continual prompting turned pink and began breathing regularly but two of these when placed with foster mothers did not appear to suckle, and died soon after. Of 9 *Wnt2*$^{-/-}$ mice delivered, only 5 survived the first 24 hours. Although mutant animals on dissection appeared anatomically normal, with subsequent histological examination showing no gross abnormalities, they were often runted (Fig. 4). Animals were weighed at various ages both before and after birth and Table 3 shows the mean weights of *Wnt2*$^{-/-}$ animals and controls. The difference in the average weights of the two groups becomes apparent at 14.5 dpc but does not become significant until 17.5 dpc. At birth the average weight of the *Wnt2*$^{-/-}$ mutants was 67% of the average weight of the controls and at weaning age both the male and female *Wnt2* mutants weighed 85% of their normal littermates. There was no difference between the average weights of wild-type animals and *Wnt2* heterozygotes.

To date 63 *Wnt2*$^{-/-}$ mice have been observed from birth to adulthood. None of these outbred mutant homozygote (31 male or 32 female) animals have exhibited any apparent physiological or anatomical defects. The oldest of these, which at writing are nearly 2 years old, remains apparently healthy and fertile. All 8 male and 8 female *Wnt2*$^{-/-}$ mutants tested were fertile and, in matings to heterozygotes, the phenotype of control and mutant offspring was indistinguishable to that of animals produced from intercross matings. Dissections of 2 adult *Wnt2*$^{-/-}$ animals have revealed no obvious abnormalities in terms of gross anatomy, organ size or organ appearance. Similarly when the *Wnt2*$^{-}$ mutation was crossed on to 129/Sv background although a similar level of perinatal lethality occurred a proportion of homozygous animals survived to adulthood and appear normal.

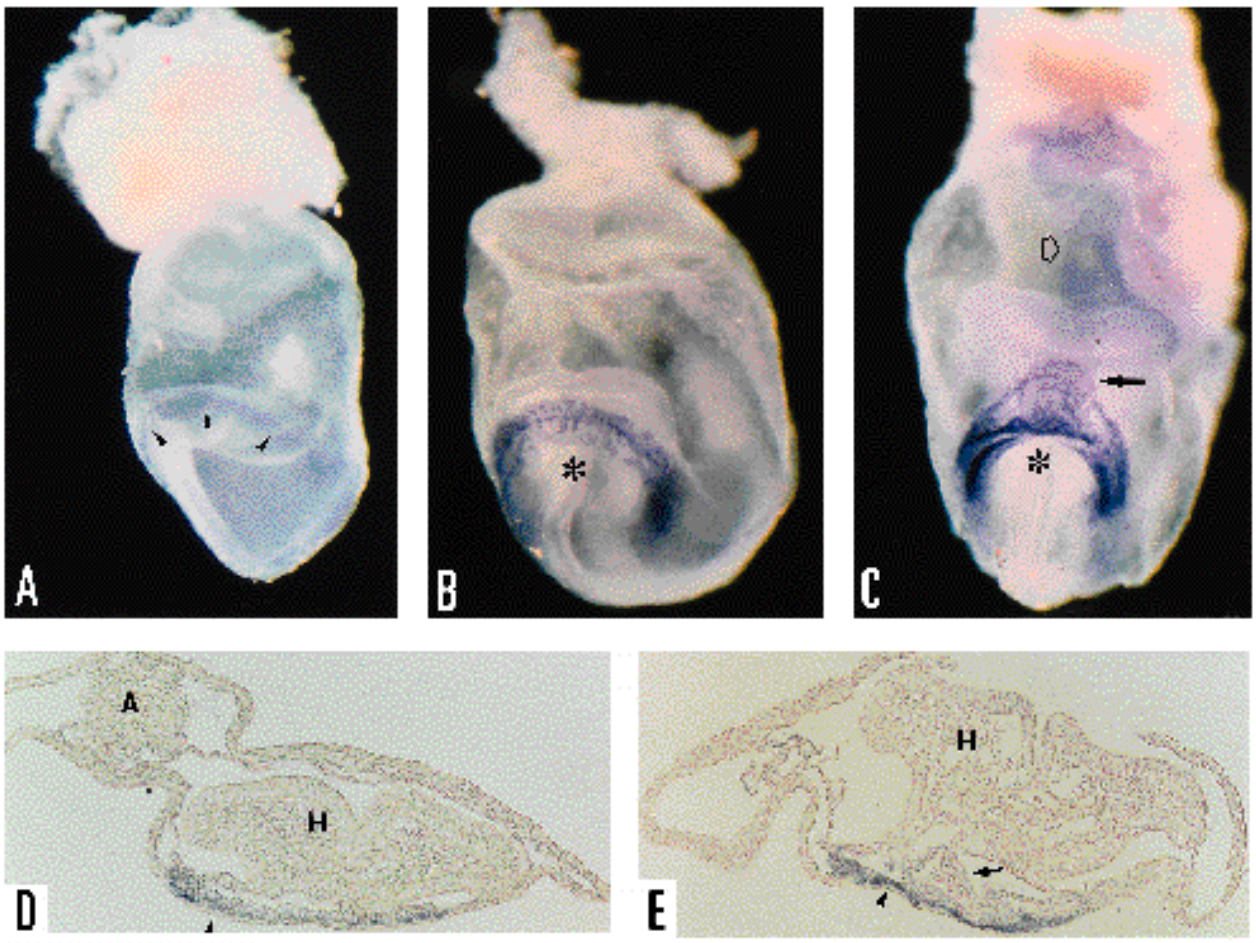


Fig. 1. Analysis of *Wnt2* expression in 7.5-8.0 dpc embryos. (A-C) Whole-mount in situ analysis of a 7.5 dpc embryo (A), a 7.75 dpc embryo (B) and an 8.0 dpc embryo (C). Arrowheads, precardiac mesoderm; asterisk, foregut involution; open arrow, allantois; arrow, protrusion of differentiating cardiac cells. (D,E) Transverse section through 7.75 dpc (D) and 8.0 dpc (E) *Wnt2* whole-mount embryo. Arrowheads, splanchnic mesoderm; arrow, myocardial cells; A, allantois; H, head.

Defects in the placentas of *Wnt2*$^{-/-}$ mice

During Caesarean sections, it was observed that some of the *Wnt2* mutant placentas exhibited visible haematomas on the foetal side of term placentas (Fig. 4D). To investigate possible defects in placentation, the placentas from both *Wnt2* mutant and control mice were collected at various stages throughout development and were examined histologically. No significant abnormalities were observed in 12 mutant placentas prior to 14.5 dpc. Between 14.5 dpc and 18.5 dpc, a number of defects were observed although the severity of pathology observed varied between mutant placentas. In a pairwise blinded comparison to placentas from control siblings, approximately 85% of 22 mutant placentas showed at least one defect including oedema and general disruption in the labyrinthine zone, the presence of abnormally large and/or numerous maternal blood pools and/or a reduction in the size and proportion of the labyrinthine zone and the chorioallantoic plate region (Fig. 5). An increase in the incidence of blood pools and a decrease in the incidence of oedema was observed as the placentas became successively older (compare Fig. 5B and I). In some of the more severe cases, there was often an increase in the amount of acellular material (often containing dead cells), possibly fibrinoid, in the labyrinthine zone (Fig. 5G,L). There was an also apparent reduction in the number of foetal capillaries when compared with control placentas (compare Fig. 4B,C). There was no evidence of necrosis or apoptosis in any of the placentas studied. The severity of pathology in the nine 18.5 dpc placentas of mutant animals delivered by Caesarean sections showed a close correlation with the degree of runting and length of survival after birth.

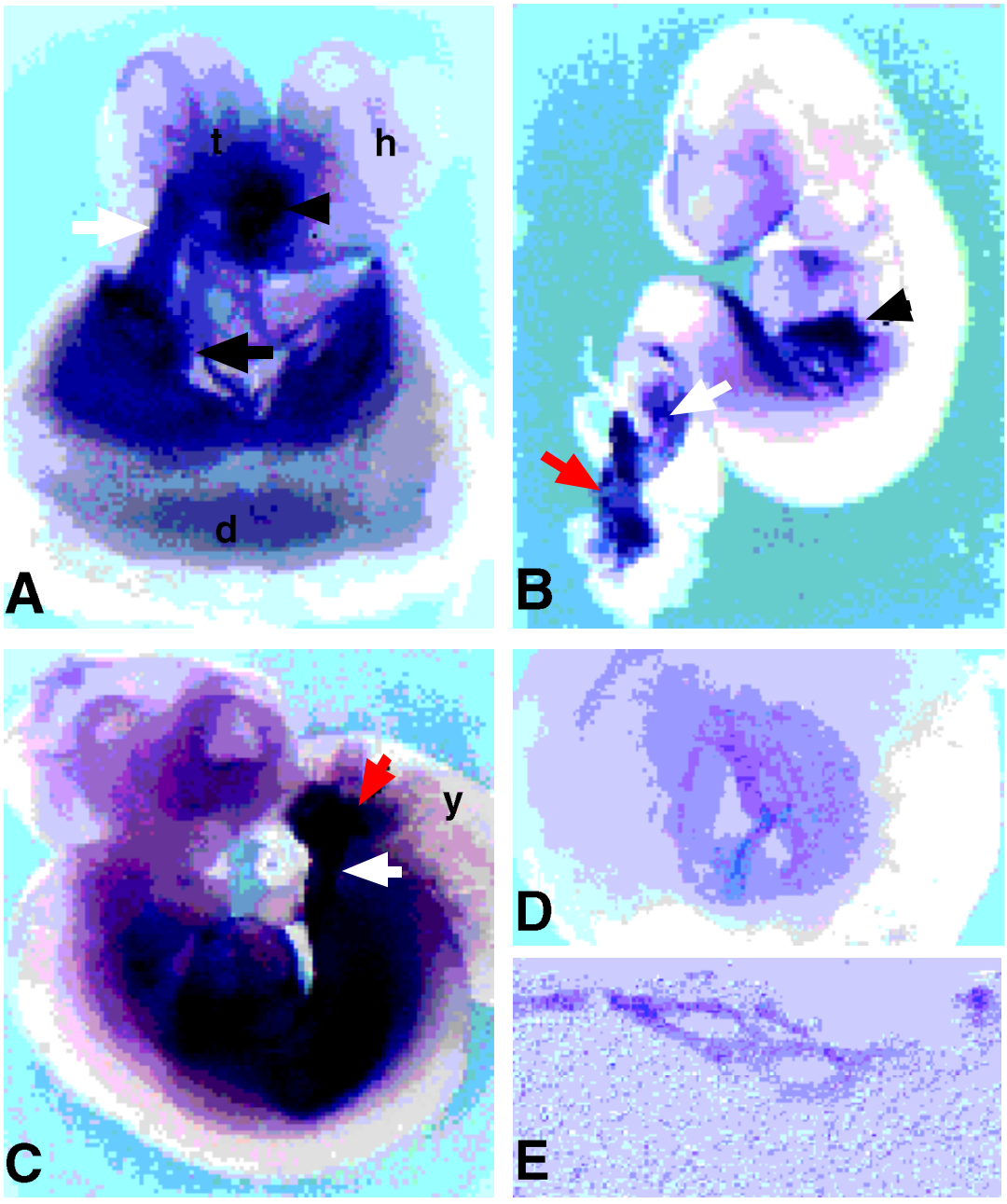


Fig. 2. Whole-mount in situ analysis of *Wnt2* expression in 8.5 dpc (A), 9.5 dpc (B), 10.5 dpc (C) embryos, 11.5 dpc placenta (D). (E) A section of the placenta shown in D showing expression localised to the blood vessels of the foetal circulation. The 8.5 dpc embryo in A is still attached to the decidua (d) via the allantois (white arrow). Arrowhead, heart; white arrow, allantois/umbilicus; red arrow, chorioallantoic plate; d, decidua; t, tail; h, head.

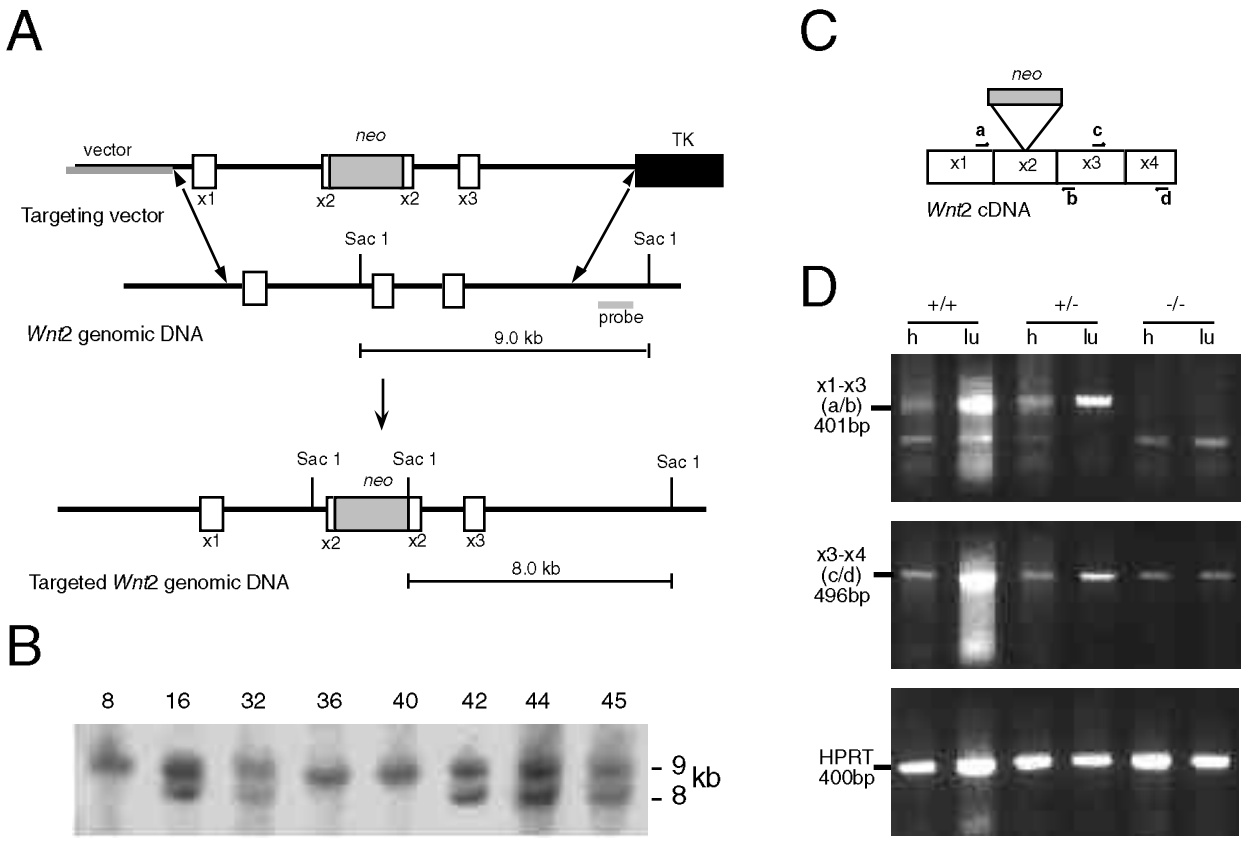


Fig. 3. Targeting of the *Wnt2* locus. (A) Schematic representation of the targeting strategy showing the targeting vector (top). Homologous recombination between the targeting vector and the *Wnt2* genomic DNA (middle) results in a targeted *Wnt2* genomic locus (bottom). (B) Identification of ES cell lines that carry the targeted *Wnt2* locus by Southern blot. The ES cell genomic DNA was digested with *Sac*I and probed with the external probe shown in A. A 9 kb band indicates an untargeted allele while an 8 kb band identifies the targeted allele. (C) RT-PCR analysis of *Wnt2* transcripts from heart and lung of wild-type ($Wnt2^{+/+}$), $Wnt2^{+/-}$ and $Wnt2^{-/-}$ mice. Schematic representation of processed *Wnt2* RNA showing positions of primers used in RT-PCR. In the case of targeted alleles the 1.8 kb *neo* gene is inserted in exon 2. (D) RT-PCR prducts analysed on agarose gels. Top, RT-PCR performed using primers a and b to amplify from exon 1 to exon 3. Middle, RT-PCR performed using primers c and d to amplify from exon 3 to exon 4. Bottom, RT-PCR performed using *HPRT* primers as a positive control. h, heart RNA; lu, lung RNA.

Placentas from $Wnt2^{-/-}$ animals have a normal number and distribution of trophoblasts

To examine the effect of the *Wnt2* mutation on the general organisation of the mouse placenta, we used in situ hybridisation to detect two different populations of placental trophoblasts in both control and mutant placentas. *Mouse placental lactogen II* (*mpl II*) was used to detect the trophoblast giant cells while clone 4311 hybridises specifically to spongiotrophoblast cells. Placental sections from various developmental stages from 10.5 to 16.5 dpc were used and, while there was no discernible difference in the pattern of 4311 expression between mutant and control placentas at any stage (Fig. 6A-D and data not shown), there were slight differences in the distribution of the *mpl II*-expressing giant cells. This was first observable at 14.5 dpc where in mutants placentas *mpl II*-positive cells were detected all the way to the foetal side of the placenta (Fig. 6G,H). In control placentas however there are very few *mpl II*-positive cells towards the foetal side of the placenta and none in the region of the chorioallantoic plate (Fig. 6E,F).

DISCUSSION

Wnt2 in heart and lung development

Wnt2 is expressed in a temporally and spatially restricted pattern during embryogenesis but, unlike all other *Wnt* genes thus far identified, it is not expressed in either the developing limbs or CNS. *Wnt2* has previously been shown to be expressed in the heart pericardium and regions associated with foetal-maternal communication (McMahon and McMahon, 1989) and more recently in the lung mesenchyme of 11.5 and 13.5 dpc embryos (Bellusci et al., 1996). Reported here for the first time is *Wnt2* expression in the regions of the embryo associated with early heart development. The expression of *Wnt2* in precardiac mesoderm makes it a potentially useful marker for the initial stages of heart development and suggests a possible role for *Wnt2* in this process. Using gene targeting, *Wnt2* knockout mice have been generated that as adults lack any detectable expression of the wild-type *Wnt2* transcript. No heart defects were observed in the *Wnt2* mutant mice at any stage of development. Hearts of mutant homozygous mice began beating at the normal time (8.0-8.5 dpc) and appeared

Table 2. Survival data on animals delivered by Caesarean section

	Wnt2$^{+/+}$	*Wnt2*$^{+/-}$	*Wnt2*$^{-/-}$
Birth	3	25	9
1 dpp	3	24	5
Difference	0	1(4%)	4(44%)

to continue to develop normally. The gross anatomy of the hearts of adult *Wnt2* mutants was indistinguishable from control littermates.

In all other *Wnt* knockouts reported to date, there have been examples of tissues or regions of the embryo that express the particular *Wnt* gene but remain unaffected by the null mutation (McMahon and Bradley, 1990; Stark et al., 1994; Parr and McMahon, 1995; Takada et al., 1994). For example, in the case of the extensively characterised *Wnt1* knockouts, there is an absence of phenotype in the spinal cord and myelencephalon, both of which express this gene (McMahon and Bradley, 1990). There is some evidence that *Wnt* 3A which has an overlapping expression domain to *Wnt1* may functionally compensate for *Wnt1* in these regions (Augustine et al., 1993, 1995). Functional redundancy may explain the lack of *Wnt2* phenotype in the heart and lung, although there is no other *Wnt* gene known to be expressed in the embryonic precursors to these organs. While *Wnt11* is expressed in the bulbus cordis/truncus arteriosus (outflow tract) of mouse embryos at 8.5-9.5 dpc (Christiansen et al., 1995) and *Wnt5A* and *Wnt5B* are both expressed in the adult heart (Gavin et al., 1990), none of the murine *Wnt* genes identified to date are expressed in an overlapping pattern with *Wnt2* either in the heart or the lung. It may be that such genes exist and have not yet been cloned. Alternative possibilities are that either there may be a minor and as yet undetected phenotype in the *Wnt2* mutant mice or that neither *Wnt2* or any other *Wnt* gene is required for either heart or lung development.

In addition to the embryonic expression, *Wnt2* is also known to be expressed in at least three adult tissues of the mouse, the heart, lungs and in the developing mammary gland (Wainwright et al., 1988; Weber-Hall et al., 1994; McMahon and McMahon, 1989). While it has been demonstrated that the adult *Wnt2* mutant mice do not express wild-type *Wnt2* RNA in either their lungs or heart, these animals are apparently healthy indicating that *Wnt2* is not required for the normal function of these organs. Although *Wnt2*$^{-/-}$ female mice appear to be able to rear pups normally, it is possible that the mutation may have effects on certain stages of mammary gland differentiation and this is currently being investigated.

Wnt2 and placentation

The generation of *Wnt2* null mice has demonstrated a role for this gene in the development of the haemochorial mouse placenta. When the embryo implants, the maternal decidua and uterine blood vessels are initially invaded by foetal trophoblasts, which break down the maternal vessels producing blood sinuses that are surrounded by trophoblast cells. It is these trophoblasts that come into direct contact with the foetal capillaries, which develop from the allantois. As the placenta develops, the foetal vessels and capillaries invade further into the placenta establishing the labyrinth-like network of foetal capillaries interspersed with maternal blood sinuses. It is in these regions where the foetal capillaries and maternal blood are separated by only a few cell layers that nutrient exchange occurs (Ellington, 1985; Amoroso, 1952).

Histopathology observed in the *Wnt2* mutant placentas between 14.5 dpc and 18.5 dpc included a reduction in size and proportion of the labyrinthine zone with tissue disruption presumably resulting from severe oedema, an increase in the acellular fibrin deposits in the labyrinthine zone and regions of abnormal maternal blood accumulation. All these defects can be attributed to inadequate circulation of either the maternal and/or the foetal blood. Inefficient establishment of the foetal capillary network could result in the oedema due to back pressure or vessel leakage. The later appearance of the maternal blood pools and the reduction in labyrinthine oedema during the development of mutant placentas would suggest that the oedema is replaced by the blood pools. Regions of acellular material containing dead cells also appear to have been deposited in spaces caused by the earlier oedema. This acellular material is possibly fibrin-type fibrinoid which is derived primarily from a blood clot of either foetal or maternal blood (Frank et al., 1994). In many cases, there also appears to be a reduction in size or a disorganisation of the chorioallantoic plate region. This is seen both in the H & E-stained placental sections and also in the *mpl II* in situs. Aside from this, there appears to be no difference in the number or distribution of the trophoblast giant cells or the spongiotrophoblast cells.

Wnt2 expression is confined to the allantoic component of the mouse haemochorial placenta, which is the precursor of foetal blood vessels and capillaries. As WNT2 is a secreted factor probably requiring direct cell-cell contact, it is possible that it is required in mediating the interaction between the foetal blood vessels and other cells of the placenta. Despite the fact that *Wnt2* expression is detected in the allantois prior to its fusion with the pre-placenta at 8.5-9.0 dpc and in some of the subsequently derived foetal vessels, no histopathology is observed until 14.5 dpc and significant runting is not detected for a further three days. This suggests WNT2 is not required in the initial allantoic invasion but is required to mediate or maintain the interaction between the foetal vessels and the trophoblasts surrounding the maternal blood sinuses. Several other mouse gene knockouts have been reported recently that result in placental defects or pathology: HGF/SF (Uehara et al.,

Table 3. Average body weights of *Wnt2*$^{-/-}$ and controls

	Average body weights(g) +/– s.e.m.		
Age	Controls†	*Wnt2*$^{-/-}$	*P* values*
10.5 dpc	0.028±0.002	0.029±0.001	>0.1
14.5 dpc	0.291±0.009	0.278±0.009	>0.1
15.5 dpc	0.443±0.036	0.413±0.020	>0.1
17.5 dpc	1.13±0.03	0.98±0.02	<0.001
C-section(18.5 dpc)	1.48±0.03	1.09±0.05	<0.001
28 day females	18.43±0.67	15.33±0.56	<0.02
28 day males	21.89±078	18.79±0.83	<0.01

*P values were calculated using the Mann-Whitney test.
†Both *Wnt2*$^{+/-}$ and *Wnt2*$^{+/+}$

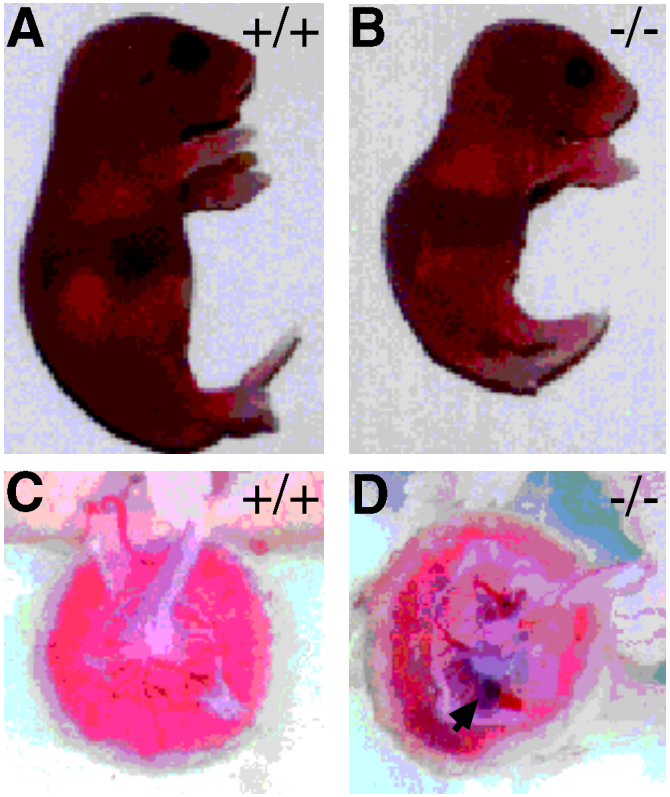


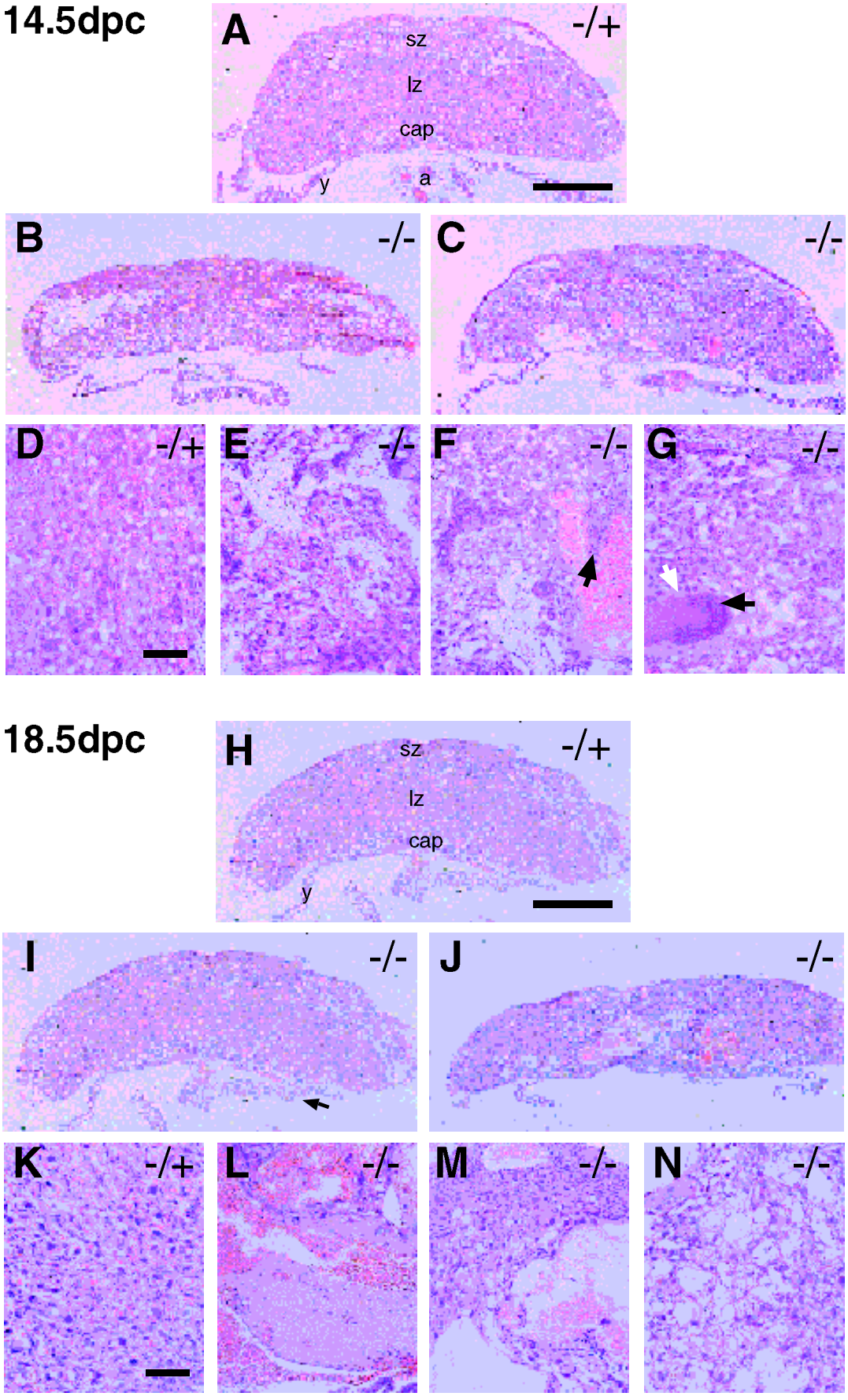
Fig. 4. Example of runting in *Wnt2*$^{-/-}$ mice. A wild-type neonate (A) and a *Wnt2*$^{-/-}$ neonate (B) that died within 24 hours of birth. Pathology evident in a *Wnt2* mutant term placenta (D) with a normal term placenta shown for comparison (C). The largest of a number of haematomas visible on the foetal side of the mutant placenta is arrowed.

1995), VCAM1 (Gurtner et al., 1995), α4 Integrins (Yang et al., 1995), *Mash*-2 (Guillemot et al., 1994) and LIFR (Ware et al., 1995). Disruption of VCAM1 and the α4 Integrin genes results in defects in the fusion of the allantois to the chorionic plate resulting in death of the embryo due to lack of formation of the chorioallantoic placenta. In support of a role for *Wnt2* in the consolidation of the foetal vessel network, but not in its initial establishment, such a fusion defect was not been observed in the *Wnt2* null mice. Only disruption of the low affinity leukemia inhibitory factor receptor (LIFR) shows pathology similar to *Wnt2* mutants. All LIFR mutant animals die perinatally at various times within the first 24 hours of delivery and there is some indication that they are smaller than their normal littermates. It is difficult to correlate the perinatal lethality with the placental pathology since there are a number of other potentially lethal defects found in these embryos (Ware et al., 1995).

Perinatal death and runting of *Wnt2* mutant animals

Approximately half the *Wnt2* homozygous mutants born die perinatally and many have a lower than average body weight. Since no other gross defects were apparent and there was a close correlation in the mutants between severity of placental defect and the degree of runting and viability, it seems likely that placentation defects are the cause of the these phenotypic effects. The placental defects are apparent approximately 3 days prior to the time when the runting of the embryos becomes significant, which supports this cause-effect relationship. Defective placental structure and function may reduce the birth weight of *Wnt2* mutant mice by impairing nutritional uptake or by affecting the placenta's hormonal regulation of foetal growth retarding the maturation of the embryo. In humans there is evidence for a link between placental pathology and low birthweight babies in a clinical outcome of pregnancy termed intrauterine growth retardation (IUGR) (Redline, 1995). IUGR results in low birthweight for gestational age babies and is often associated with a number of clinical and environmental factors such as preeclampsia, hypertension, maternal vascular disorders, infection and smoking; although, in many cases, the aetiology of IUGR remains unknown. Pathology associated with IUGR includes intervillous fibrin, abnormal placental blood flow (both maternal and foetal), maternal floor infarction and a reduction in villous surface area (equivalent to reduced foetal vascularisation) (Redline, 1995). There is disagreement as to the relationship between some of these pathologies and IUGR, although a reduction in villous surface area clearly correlates with IUGR in at least one study (Boyd and Scott, 1985). While there are certain structural differences between the placentas of mice and humans, they contain essentially the same basic elements (Ramsey, 1975). The reduction in birthweight of *Wnt2* mutant mice could be attributed to some of the placental defects seen in these mice (including apparent reduction in vascularisation and an increase in fibrin) which approximate the human pathologies

Fig. 5. Placentas from *Wnt2* mutant homozygous (–/–) and *Wnt2* heterozygote (+/–) mice. (A,D) Placentas from normal (heterozygote) 14.5 dpc embryos, D is a higher magnification of the labyrinthine zone of A. Note the even cellular distribution and compact organisation of the labyrinthine zone (lz). No differences in pathology were noted between wild-type and hetrozygous placentas. (B,C,E-G) Placentas from 14.5 dpc mutant homozygote embryos. E and F are higher magnifications of the labyrinthine zone of C and D respectively. The placenta in B is much thinner than the control and shows evidence of oedema and tissue disruption in the labyrinthine zone (seen at higher power in E). The placenta in C has an accumulation of large blood pools. The walls of these blood pools are disorganised often with regions of trophoblast cells protruding into the blood mass (arrow in F). The absence of nucleated blood cells in the blood of these pools indicates it is of maternal origin. In G a region of acellular fibrin is visible (white arrow) with a number of giant cells at the periphery (black arrow). (H,K) Placentas from 18.5 dpc heterozygote embryos. K is a higher magnification of the labyrinthine zone of H. The labyrinthine zone (lz) is organised and compact although, at this age, the labyrinthine zone makes up a larger proportion of the placenta than at 14.5 dpc. (I,J,L-N) Placentas from 18.5 dpc mutant homozygote embryos. L and M are higher magnifications of the labyrinthine zone of I and J respectively. The placenta in I contains a number of abnormally large blood pools and regions of fibrin deposit (arrow). The region of fibrin is seen at higher power in L where cells of various types are distributed throughout the acellular material. The placenta in J has a much reduced labyrinthine zone due to the occlusion of this region with blood pools and oedema (M). N shows an example of severe oedema in the labyrinthine zone of an 18.5 dpc mutant placenta. sz, spongiotrophoblastic zone; lz, labyrinthine zone; cap, chorioallantoic plate; u, umbilicus; y, yolk sac. A-C, H-J scale bar 1 mm; D-G, K-N scale bar =100 μm.


14.5dpc
A
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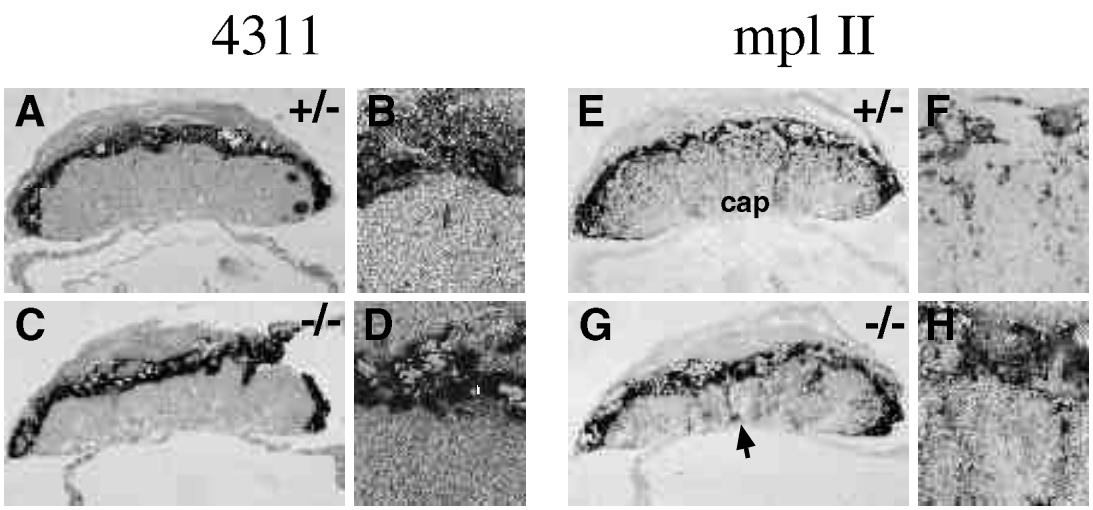

Fig. 6. In situ hybridisation analysis of the expression of trophoblast markers in 14.5 dpc placentas. A-D were probed with clone 4311. B and D are higher magnifications of the central regions of A and C respectively. Cells detected by this probe can be seen to be localised almost exclusively to the spongiotrophoblastic zone. There is no difference in the expression pattern of this marker in mutants (C,D) compared with heterozygote controls (A,B). E-H were probed with *mpl II*. F and H are higher magnifications of the central region of E and G respectively. Again there is no difference in the marker expression in the spongiotrophoblast zone of mutants (G,H) compared with heterozygote controls (E,F). There is a difference between mutants and controls in the distribution of the *mpl II*-positive giant cells in the labyrinthine zone with more of these cells being found towards the foetal side of the placenta (arrow) in the mutant while in the control there are progressively less of the mpl II-positive cells towards the chorioallantoic plate (cap) and none in the chorioallantoic plate itself.

often associated with IUGR. If this is the case the *Wnt2* mice generated in this study may be of value in understanding the relationship between placental defects and IUGR.

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