The Wilms tumor suppressor gene *wt1* is required for development of the spleen

Ute Herzer, Alexander Crocoll, Debra Barton, Norma Howells and Christoph Englert

The Wilms tumor suppressor gene *WT1* (*wt1* in mouse) is unique among tumor suppressors because, in addition to its involvement in cancer [1,2] and various other diseases [3–6], it has an essential role in the development of certain organs. This is revealed by the phenotype of mice with inactivated *wt1* alleles [7]. These animals exhibit a complete failure of kidney and gonad development as well as abnormalities of the heart and mesothelial structures. On a C57BL/6 genetic background, *wt1*$^{-/-}$ animals die between day 13.5 (E13.5) and 15.5 (E15.5) of embryonic development [7]. We report here that crossing of the *wt1* mutation onto different mouse backgrounds delayed embryonic lethality until birth. In *wt1*$^{-/-}$ mice on these different genetic backgrounds, we observed a dramatic failure of spleen development, in addition to the well characterized phenotypic abnormalities. The spleen anlage formed at around E12 to E13 and involuted by the E15 stage, before the invasion of hematopoietic cells. The absence of proper spleen development in these *wt1*$^{-/-}$ embryos correlated with enhanced apoptosis in the primordial spleen cells. The expression of *hox11*, a gene that also controls development of the spleen [8,9], was not altered by the inactivation of *wt1*. *In situ* hybridization revealed that the two genes are regulated independently. These findings demonstrate that the penetrance of the *wt1*$^{-/-}$ phenotype depends on the existence of one or more modifier gene(s) and that *wt1* plays a pivotal role in the development of the spleen, thereby extending its role in organogenesis.

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Results and discussion

The phenotype associated with inactivation of *wt1* depends on the genetic background

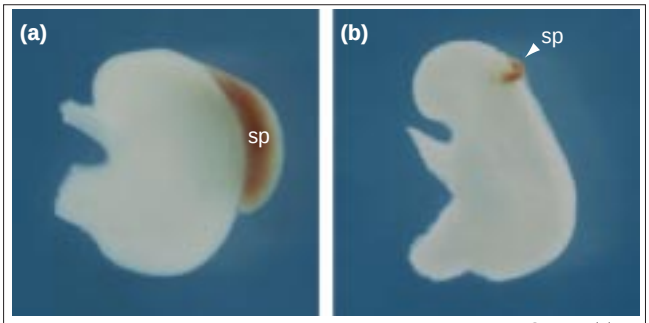
The *wt1* gene is expressed in the developing kidney, the stromal cells of the gonads and spleen and the mesothelial cells that line the heart, diaphragm and peritoneum [10–12]. All *wt1*-expressing tissues contain mesoderm-derived epithelium, and it has therefore been hypothesized that Wt1 exerts its role by mediating the differentiation from mesenchyme to epithelium [10]. How Wt1 fulfills this role in molecular terms is still unclear, but most evidence suggests that it works as a transcription factor [13]. The targeted inactivation of *wt1* leads to a lethal phenotype at E13.5–E15.5 [7] and, in the C57BL/6 (B6) mouse background that has been used so far for these experiments, no viable *wt1*$^{-/-}$ embryos could be found after day E15.5.

Prompted by the observation that the genetic background can dramatically influence the phenotypes that result from gene-targeting experiments [14,15], we bred the *wt1* mutation into the mouse strains MF1, Balb/c and C3H. Genotypic analysis of the offspring from intercrosses of heterozygous F_1 animals revealed that homozygous *wt1*$^{-/-}$ animals were born in each case. The mice died immediately after birth because they could not inflate their lungs. For all the subsequent typing and phenotypic analyses, we performed caesarian sections at E18.5 and focused on the MF1 background. Overall, viable (non-resorbed) *wt1*$^{-/-}$ embryos were found with a frequency of approximately 16% (11/67) on a mixed B6 × MF1 background. To discriminate between the influence of the robustness of MF1 mothers and the existence of a modifier gene, we transferred blastocysts from a pure B6 background as well as from the mixed MF1 × B6 background into MF1 foster mothers and analyzed the developing embryos. Whereas 25% of E18.5 embryos (4/12) from the mixed background were homozygous for the *wt1* mutation, no B6 *wt1*$^{-/-}$ embryos (0/20) of this age could be found. These results suggest the existence of one or more gene(s) modifying the penetrance of the *wt1* mutation.

Lack of spleen development in *wt1*$^{-/-}$ embryos

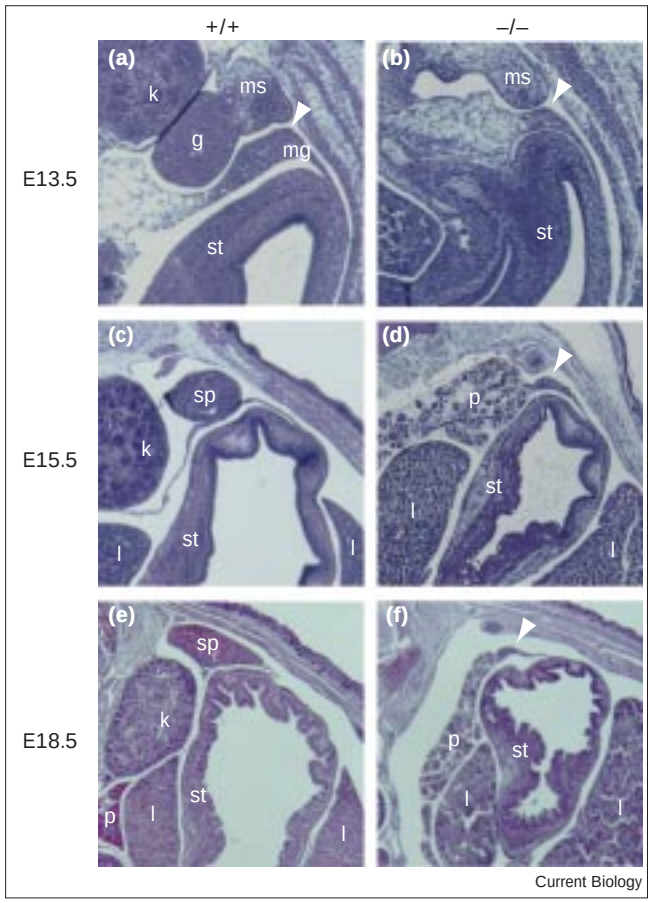
The longer lifespan of B6 × MF1 *wt1*$^{-/-}$ embryos enabled us to examine the *wt1*$^{-/-}$ phenotype in more detail. It has recently been reported that *wt1* is required for the development of the adrenal gland [16]. In agreement with that observation, the mutant E18.5 embryos on a mixed MF1 × B6 background lacked adrenal glands. In addition to the previously described developmental defects [7], we also found an almost complete absence of the spleen in *wt1*$^{-/-}$ animals (Figure 1). Organogenesis of the spleen involves a reciprocal interaction between the coelomic epithelium of the dorsal mesogastrium (tissue connecting the stomach with the body wall) and the

Figure 1



Whole-tissue preparation of stomach and spleen (sp) from (a) a wild-type and (b) a *wt1*$^{-/-}$ mouse embryo at stage E18.5. Although the stomach is comparable in size and appearance, only a remnant of the spleen can be observed in the preparation from a *wt1*$^{-/-}$ embryo. Note the red/brown color of the mutant spleen, which suggests normal colonization by hematopoietic cells. Original magnifications: ×10.

Figure 2



Histology of wild-type and *wt1*$^{-/-}$ embryos. (a) Transverse section through an E13.5 wild-type embryo, showing the spleen primordium (arrowhead) within the mesogastrium, the stomach, the gonad and the developing kidney. (b) Transverse section through an E13.5 *wt1*$^{-/-}$ embryo showing a local thickening of the mesogastrium (arrowhead). The gonad and kidney are absent. (c) Transverse section through an E15.5 wild-type embryo. The spleen has increased in size compared to (a). (d) Transverse section through an E15.5 *wt1*$^{-/-}$ embryo. Because of the absence of the kidney, the well-developed pancreas is located next to the spleen primordium (arrowhead), which has not developed further compared to (b). (e) Transverse section through an E18.5 wild-type embryo, showing a normally developed spleen. (f) Transverse section through an E18.5 *wt1*$^{-/-}$ embryo, revealing an almost complete absence of the spleen (arrowhead). Abbreviations: g, gonad; k, kidney; l, liver; mg, mesogastrium; ms, mesonephros; p, pancreas; sp, spleen; st, stomach. Original magnifications, (a,b) ×80, (c,d) ×40, (e,f) ×25.

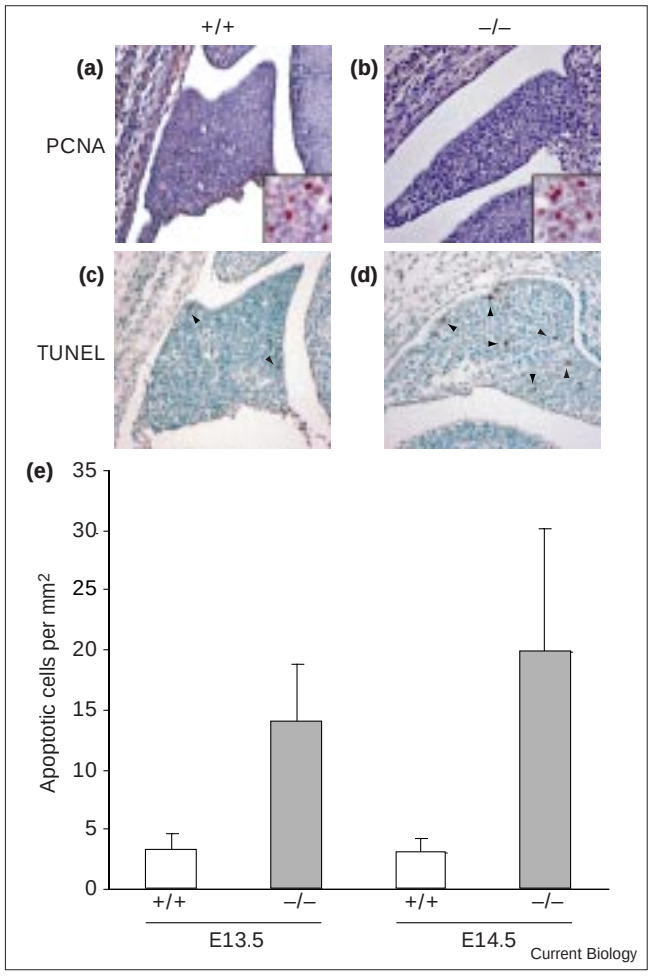
underlying mesenchyme [9,17]. The first morphological evidence of spleen development is a localized thickening of the mesogastrium, which also contributes to pancreas development. This spleen primordium is visible at E13.5 of mouse development. The first hematopoietic cells populate the spleen only after E15.5.

In order to examine the spleen defect in more detail, we analyzed histological sections at different stages of development. In sections through E13.5 wild-type mice, the spleen primordium was clearly visible (Figure 2a). In the mutant embryo, a comparatively smaller spleen primordium was present and the connection to the prospective pancreas was shortened (Figure 2b). The stomach and pancreas, however, developed normally in *wt1*$^{-/-}$ embryos. At E15.5, before hematopoietic cells invade the spleen, a well-developed spleen could be seen in the wild-type embryos (Figure 2c), whereas only some thickening of the mesogastrium could be observed in *wt1*$^{-/-}$ embryos (Figure 2d). This observation demonstrates that the defect in spleen organogenesis is not due to a lack of invasion of hematopoietic cells but to a failure of the stromal cells of the spleen to develop. In E18.5 *wt1*$^{-/-}$ embryos, the spleen did not develop any further (Figure 2f), whereas a normal organ could be observed in E18.5 wild-type embryos (Figure 2e).

Wt1 as an anti-apoptotic factor

To analyze whether the failure of proper spleen development in *wt1*$^{-/-}$ animals can be explained by either impaired proliferation or increased apoptosis, sections of the developing mesogastrium of wild-type and *wt1*$^{-/-}$ embryos were stained with an antibody against the proliferating cell nuclear antigen (PCNA; Figure 3a,b). This assay revealed the presence of a significant number of proliferating cells in several organs, including the kidney, the notochord and the liver. In the developing mesogastrium of E13.5 or E14.5 wild-type and *wt1*$^{-/-}$ embryos, however, no PCNA-expressing cells could be detected. These results have been confirmed independently by bromodeoxyuridine (BrdU) staining (data not shown). Thus, at this stage of spleen development, proliferation is not the major determinant of organogenesis. In contrast, the TUNEL assay showed a significant difference in the

Figure 3



Proliferation and apoptosis during early spleen development. (a,b) PCNA staining of the mesogastrium from (a) a wild-type and (b) a *wt1*$^{-/-}$ E13.5 embryo. No PCNA-expressing cells could be detected in either. The staining in the wild-type mesogastrium is not nuclear and is therefore non-specific. As a positive control, liver tissue from the same slides is shown (insets). (c,d) Apoptosis in the mesogastrium of (c) a wild-type and (d) a *wt1*$^{-/-}$ E13.5 embryo as determined by the TUNEL method. Apoptotic cells are marked by arrowheads. Original magnifications, ×20 (insets, ×40). (e) Quantification of apoptosis in wild-type and *wt1*$^{-/-}$ embryos at E13.5 and E14.5. The values were obtained by dividing the numbers of apoptotic cells by the area of the respective mesogastrium. The mean ± standard deviation of values from four different animals (or three E14.5 *wt1*$^{-/-}$ animals) are shown; at least four sections were counted from each animal.

amount of apoptosis between E13.5 wild-type and *wt1*$^{-/-}$ embryos. Whereas a small percentage of cells underwent apoptosis in the wild-type animals, a high degree of apoptosis was observed in the *wt1*$^{-/-}$ embryos (Figure 3c,d). Quantification showed that there was a 4–5-fold increase in the number of apoptotic cells in the spleen primordium of *wt1*$^{-/-}$ embryos compared with wild-type animals (Figure 3e). A similar difference was also seen at the E14.5 stage. Thus, the lack of spleen development in *wt1*$^{-/-}$ mice correlates with enhanced apoptosis rather than with a lack of proliferation.

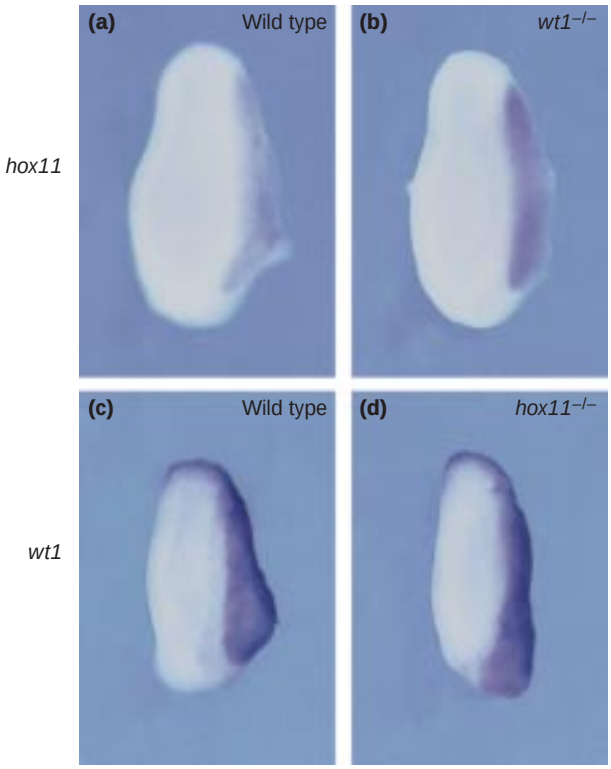
This situation is reminiscent of kidney development, during which the absence of *wt1* leads to apoptosis in the metanephric mesenchyme [7]. It is interesting to note in this context that Wt1 has been shown to have anti-apoptotic properties because it can inhibit p53-mediated apoptosis in osteosarcoma cells [18]. This seems to contradict the initial characterization of *WT1* as a tumor suppressor gene. However, the recent finding that a chromosomal translocation that juxtaposes the zinc finger domain of WT1 to the regulatory domain of the Ewing sarcoma gene product (EWS) is associated with desmoplastic small round cell tumors is an additional example of *WT1* also having oncogenic properties [19]. Given the suggested role of *wt1* in the differentiation of mesenchyme to epithelium differentiation, it is conceivable that the anti-apoptotic properties of *wt1* are indirect and that the enhanced apoptosis in *wt1*$^{-/-}$ embryos might be a consequence of the lack of proper differentiation signals.

The *wt1* and *hox11* genes are regulated independently during spleen development

The expression pattern of *wt1* (in stromal cells of the spleen [10,12]) and its mutant phenotype (defective spleen development) are very similar to those of the *hox11* gene [20]. This homeobox gene has been shown to be required for organogenesis of the spleen [8,9]. In order to analyze the relationship between *wt1* and *hox11*, we examined the expression pattern of *hox11* in wild-type and *wt1*$^{-/-}$ animals by *in situ* hybridization. In both wild-type and *wt1*$^{-/-}$ embryos, *hox11* expression was detected in a ridge of cells on the dorsal side of the stomach at E13.5 (Figure 4a,b) and E12.5 (data not shown). Thus, *hox11* expression appears to be unaffected by the *wt1* mutation. We then performed the reciprocal experiment to test whether the expression of *wt1* was affected by the *hox11* mutation. In *hox11*$^{-/-}$ animals, *wt1* expression could be observed in the developing mesogastrium at E12.5 (Figure 4c,d) and E11.5 (data not shown). These data suggest that, although *hox11* and *wt1* are coexpressed in the spleen primordium and show a similar phenotype with regard to spleen development, these genes do not seem to regulate each other. Although *wt1* and *hox11* appear to act genetically independently, Wt1 and Hox11 proteins could still form a biochemical complex. It will be challenging to identify the downstream targets of Hox11 and Wt1, in order to learn whether both proteins converge on the regulation of the same genes that are required for development of the spleen.

In summary, we have shown that the phenotypic consequences of *wt1* inactivation are influenced by modifier genes and that *wt1* is essential for development of the spleen. Interestingly, all the organs for which Wt1 function is required share a common feature: they are formed

Figure 4



Independent regulation of *hox11* and *wt1*. Whole-mount *in situ* hybridizations of (a) wild-type and (b) *wt1*$^{-/-}$ stomach and mesogastrium of stage E13.5 embryos with a *hox11*-specific probe. Staining of the mesogastrium on the dorsal side of the stomach can be observed in both. Whole-mount *in situ* hybridizations of (c) a wild-type and (d) a *hox11*$^{-/-}$ stomach and mesogastrium of stage E12.5 embryos with a *wt1*-specific probe. The expression pattern of the *wt1* gene is not altered by the *hox11* mutation. Original magnifications, ×25.

by interactions between epithelial and mesenchymal components. Originally, the role of Wt1 has been viewed as mediation of the 'one-way' transition from a mesenchymal cell to a well-differentiated epithelial cell. More recently, however, Wt1 has been suggested to enable cells to flip between epithelial and mesenchymal states [16]. Although the precise molecular mechanism of Wt1 function is still unclear, the data presented here contribute to the notion that Wt1 mediates a process that is fundamental to organogenesis.

Materials and methods

Mouse strains

A *wt1*$^{+/-}$ and a *hox11*$^{-/-}$ breeding pair were obtained from The Jackson Laboratory (Bar Harbor, USA). The former was genotyped according to the protocol provided. The *hox11* mutant animals were genotyped by PCR as well as Southern-blotting analysis as described [S1]. The inbred strains BALB/cOlaHsd, C3H/HeNHsd and C57BL/6JOlaHsd as well as the outbred strain HsdOla:MF1 are maintained as breeding colonies in-house.

Supplementary material

Supplementary material including additional methodological details of immunohistochemistry and *in situ* hybridization is available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

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Immunohistochemistry

Embryos were fixed in 10% formalin and routinely processed for paraffin embedding. Sections 6 μm thick were cut from paraffin blocks, deparaffinized and hydrated before immunostaining. PCNA staining was started with an incubation in 2 N HCl for 10 min followed by several blocking steps to prevent non-specific staining in 0.3% hydrogen peroxide, avidin, biotin, 10% normal horse serum and 0.5% mouse serum. PCNA antibody (DAKO) was applied in a 1:100 dilution followed by a biotinylated horse anti-mouse (1:50) secondary antibody. An avidin-linked peroxidase enzyme was used for detection using 3-amino-9-ethylcarbazole as a substrate. The sections were counterstained with hematoxylin and mounted under coverslips with aqueous mounting medium. Apoptotic nuclei were stained using the Apoptag kit (Oncor) following the manufacturer's protocol.

In situ *hybridization*

A *hox11*-specific *in situ* hybridization probe was generated by PCR amplification of a genomic fragment using the upstream primer 5′-AGAGGAACGTGAGGCCGAGA-3′ and the downstream primer 5′-GGATCCCAGAAGCCTTCCGG-3′ [S1] and subsequent TA-cloning into the pGEM-T vector (Promega). To generate the antisense and sense control probes, the recombinant plasmid was linearized with *Apa*I or *Bst*XI and used as a template for *in vitro* transcription with SP6 or T7 RNA polymerase. Antisense and sense control probes specific for *wt1* were generated by digestion of a pBluescript II SK plasmid containing codons 98 to 449 of *wt1* with *Eco*RI or *Hind*III and *in vitro* transcription with T7 or T3 RNA polymerase. Procedures for whole-mount *in situ* hybridization were followed as described elsewhere [S2] with the following modifications. Riboprobes were precipitated with LiCl, washed and resuspended in DEPC-treated water. Abdominal organs were dissected and fixed in 4% paraformaldehyde in PBS at 4°C overnight. The organs were then treated with proteinase K (4.5 μg/ml) in PBST (PBS + 0.1% Tween-20) for 4 min at room temperature. The final washing steps after prehybridization and hybridization of the tissues were done in maleic acid buffer (100 mM maleic acid pH 7.5, 150 mM NaCl) at room temperature (2 × 10 min), at 70°C (2 × 30 min), and finally in PBS at room temperature (2 × 10 min). Tissues were stained in BM purple substrate (Roche) and signals became visible after 3.5 h (*wt1*) or 5 h (*hox11*). The specificity of the probes was confirmed using the respective sense probes, which led to the detection of no specific signals.

Supplementary references

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