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Selenoprotein expression is essential in endothelial cell development and cardiac muscle function

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Abstract

LoxP-Cre technology was used to remove the selenocysteine tRNA gene, *trsp*, in either endothelial cells or myocytes of skeletal and heart muscle to elucidate the role of selenoproteins in cardiovascular disease. Loss of selenoprotein expression in endothelial cells was embryonic lethal. A 14.5-day-old embryo had numerous abnormalities including necrosis of the central nervous system, subcutaneous hemorrhage and erythrocyte immaturity. Loss of selenoprotein expression in myocytes manifested no apparent phenotype until about day 12 after birth. Affected mice had decreased mobility and an increased respiratory rate, which proceeded rapidly to death. Pathological analysis revealed that mice lacking *trsp* had moderate to severe myocarditis with inflammation extending into the mediastinitis. Thus, ablation of selenoprotein expression demonstrated an essential role of selenoproteins in endothelial cell development and in proper cardiac muscle function. The data suggest a direct connection between the loss of selenoprotein expression in these cell types and cardiovascular disease.

Keywords

Cardiovascular disease; Conditional knockout; Endothelial cells; Heart muscle; Selenocysteine; Selenocysteine tRNA; Selenoproteins

1. Introduction

There are 24 known selenoproteins in rodents and 25 in humans [1]. Some, but not all of these selenoproteins, have an essential role in mammalian development. The targeted removal of different selenoprotein genes in mice have shown that glutathione peroxidase-4 (GPx4) [2], selenoprotein P [3,4], thyroid hormone deiodinase 2 [5–8], cytosolic thioredoxin reductase [9] and mitochondrial thioredoxin reductase [10] appear to have essential roles, since their knockout is lethal or results in abnormal phenotypic changes. On the other hand, glutathione peroxidase-1 (GPx1) [11] and glutathione peroxidase-2 [12] appear to have non-essential roles, since their knockout results in little or no phenotypic change. Those selenoproteins that are

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non-essential to the life of the animal may provide protection from environmental stress, since such animals do not cope as well as their wild type counterparts to certain traumas [13,14]. Interestingly, many selenoproteins that apparently have a role in stress-related phenomena appear to be dependent on the presence of a 2'-O-methyl group on the ribosyl moiety at position 34 (designated Um34) in selenocysteine (Sec) tRNA^{[Ser]Sec} [15]. That is, rescue of the selenoprotein population in a standard knockout of the Sec tRNA^{[Ser]Sec} gene (*trsp*) with a mutant transgene whose product cannot synthesize Um34 results in the animal's failure to express several selenoproteins including GPx1, glutathione peroxidase-3, SelR, SelT and SelW. The rescued animals appear to be phenotypically normal with the exception of reduced fertility in males and a reduced litter size in females.

A unique feature of selenoproteins is their absolute dependence on the presence of Sec tRNA^{[Ser]Sec} for their synthesis. Removal of *trsp* from the mouse genome is embryonic lethal [16,17] resulting in death within the first few days post-coitum (dpc) [16]. These data further demonstrate that selenoproteins have a major role in mammalian development. However, the mechanism of how and when selenoprotein expression is required in the development of various tissues and organs is largely unknown. Generation of a conditional knockout of *trsp* using *loxP-Cre* technology [17] has provided an important tool for carrying out a more detailed analysis of the role of selenoproteins in specific tissue and organ development. For example, targeted removal of *trsp* from mammary epithelium has shown that selenoprotein expression is altered when *Cre* is under the control of the mouse mammary tumor virus long terminal repeat promoter, but that there were no observed phenotypic changes [17]. Targeted removal of *trsp* from adult liver, however, demonstrated an essential requirement for selenoprotein expression in liver function following complete removal of the synthesis of this selenium-containing protein class [18]. These studies prompted us to examine the role of selenoproteins in the development of other tissue types by perturbing their expression using *loxP-Cre* technology to selectively remove *trsp* and to provide models for examining the role of selenoproteins in disease prevention.

In the present study, we have examined the targeted removal of floxed *trsp* with *Cre* recombinase in two different cell types. These studies were designed to elucidate the role of selenoproteins in cardiovascular disease since both cell types are directly related to heart development and function. In one cell type, *Cre* recombinase is under the control of the *Tie2* promoter that is highly specific to endothelial cells [19,20], and in the other, the recombinase is under the control of the muscle creatine kinase (*MCK*) promoter that is highly specific to skeletal and cardiac muscle [21]. Removal of *trsp* in endothelial cells resulted in embryonic death, and the affected fetuses had multiple abnormalities compared to their wild type siblings at 14.5 dpc. Removal of *trsp* in skeletal and heart muscle resulted in abrupt death at about 12 days after birth and the abnormality appeared to be a result of the loss of selenoprotein expression in cardiac muscle. The results of these observations are described herein.

2. Materials and methods

2.1. Mice

Floxed *trsp* (designated *trsp^{fl}*) mice in strain C57BL6 have been described [17]. Heterozygous *Tie2Cre* mice in strain C57BL6 wherein the *Cre* recombinase is driven by the endothelial-specific promoter/enhancer metallothionein-1 poly A signal sequence [20] were obtained from Frank Gonzalez at NCI/NIH with permission from M. Yanagisawa. *MCKCre* mice in strain FVB wherein the *Cre* recombinase is driven by the muscle specific promoter/enhancer of the *mck* gene [21] were from Taconic, USA with permission from C.R. Kahn.

2.2. Matings, embryos and offspring

Male mice that were heterozygous for either *Cre* gene (designated *Tie2Cre*^{+/-} or *MCKCre*^{+/-}) were bred to females that were homozygous for floxed *trsp* (designated *trsp*^{fl/fl}). Male offspring that were *trsp*^{fl/+}-*Tie2Cre*^{+/-} or *trsp*^{fl/+}-*MCKCre*^{+/-} were bred to females that were *trsp*^{fl/fl}. Carrying out breedings in this manner assured us of not having homozygous *Cre* offspring as mice encoding both copies of either *Tie2Cre* [20] or *MCKCre* [21] produce a characteristic phenotype. Since offspring with genotype *trsp*^{fl/fl}-*Tie2Cre*^{+/-} were suspected of being embryonic lethal, females were monitored for the presence of a vaginal plug (VP) in order to determine the first day of gestation. Those females manifesting a VP on the first morning after mating were designated as dpc 1. Embryos at dpc 10.5, 12.5, 14.5, 16.5, 18.5 were examined by euthanizing dams with CO₂ (dams carrying embryos greater than 14 days of age were anesthetized with Avertin IP prior to euthanasia), and the ventral abdomen was then opened to retrieve the uterine horn with implanted embryos. Embryos were placed in sterile 1×PBS and washed in the same solution to remove cellular debris. Prior to fixation, the limb of the fetus was removed for genotyping. Fetuses were fixed in Bouin's solution and then transferred to 70% ethanol.

Pups that died suddenly on or about day 12 from matings between male *trsp*^{fl/+}-*MCKCre*^{+/-} and female *trsp*^{fl/fl} mice were fixed in 10% neutral-buffered formalin within 5 min following death after opening the abdomen and thoracic and cranial cavities. Comparable aged siblings were euthanized and fixed in formalin following the same procedures and used as controls. Tails of 6–8 days old mice were clipped for isolating genomic DNA to determine genotypes. Necropsy and histopathologic evaluation were performed by a veterinary pathologist (GFM), board certified by the American College of Veterinary Pathologists. Tissues were routinely processed and embedded in paraffin. Five-micron-thick sections were prepared and stained with hematoxylin and eosin (H&E). Photomicrographs were taken using an Axiophot (Zeiss) microscope, and a Coolsnap HQ (Photometrics) digital camera.

Animal care was in accordance with the National Institutes of Health guidelines.

2.3. Genotyping by PCR

Genotypes of embryos from matings between *trsp*^{fl/+}-*Tie2Cre*^{+/-} males and *trsp*^{fl/fl} females and of 6–8 days offspring from matings between *trsp*^{fl/+}-*MCKCre*^{+/-} males and *trsp*^{fl/fl} females were analyzed by PCR using genomic DNA isolated from limbs and tails, respectively. *Tie2Cre* [20] and *MCKCre* transgenes [21] were detected by PCR as described. Genotyping of the *trsp* region was performed by PCR as previously described [17].

2.4. Embryo processing/sectioning

Fixed 14.5 gestation day old embryos were processed for routine light microscopic evaluation (Histoserv, Inc., Germantown, MD) in paraffin block for sagittal sections. Five-micron-thick step sections, taken at 50 µm intervals, were cut on a microtome, and stained with H&E. Sections were examined using an Olympus BX41 microscope. Photomicrographs were taken as described above.

2.5. Neonatal processing/sectioning

Six day old, 10 day old and about 12 day old freshly deceased mutant, neonatal mice and the corresponding age-matched controls that were from matings between male *trsp*^{fl/+}-*MCKCre*^{+/-} and female *trsp*^{fl/fl} mice were dissected by a veterinary pathologist (GFM), and organs were embedded in paraffin, sectioned 5- µm-thick, stained with H&E, coverslipped, and examined using an Olympus BX41 microscope. In addition, to detect occurrence of muscle fibrosis and deposition of amyloid in skeletal muscle tissue, Massons's Trichrome and Congo

red staining were carried out, respectively. The following organs were examined: heart, brain, spinal cord, liver, spleen, kidney, lung, thymus and skeletal muscle.

3. Results

3.1. Targeted removal of *trsp* in endothelial cells

3.1.1. Phenotypes and genotypes—Examination of the offspring from matings between *trsp^{fl/+}-Tie2Cre^{+/-}* male and *trsp^{fl/fl}* female parents revealed that none of the pups were *trsp^{fl/fl}-Tie2Cre^{+/-}*. We therefore turned our attention to the embryonic stages. Embryos were retrieved at different periods of gestation. A total of 10 *Tie2Cre* knockout embryos and an equal number of littermate controls were analyzed. Two mice of E 10.5, three of E 12.5, three of E 14.5 and one each of E 16.5 and E 18.5 were examined. At day 10.5, the embryos were small and no phenotypic or pathological differences between wild type and knockout siblings were apparent. By day 12.5, some embryos appeared to be developing more slowly, but no significant differences were found between these and wild type embryos by pathological analysis (see below). However, by day 14.5 dpc, striking differences were found among embryos (see 14.5 dpc siblings in Fig. 1A). Affected E 14.5 embryos were smaller in size, more fragile, had a poorly developed vascular system, underdeveloped limbs and tails and smaller heads than their normal siblings. The skull of affected embryos was malformed as can be visualized in the figure. By day 16.5, affected embryos began undergoing major disintegration, and by day 18.5, they had been virtually reabsorbed.

The genotypes of affected embryos and their siblings were determined (Fig. 1B). All E 14.5 embryos homozygous for *trsp^{fl/fl}* and heterozygous for *Tie2Cre^{+/-}* exhibited the same pathological phenotype. As *Tie2Cre* was not expressed in the limb tissue (which is composed of less than 2% endothelial cells) and only *trsp* was targeted for removal in endothelial cells, the genotype shown in the figure contained intact *trsp^{fl/fl}*. Normal (control) embryos that were used for pathological analysis were heterozygous for floxed *trsp* and *Tie2Cre* (Fig. 1B) or were heterozygous only for floxed *trsp* (data not shown). No phenotypic differences were observed among control embryos even though their genotypes were different. Pathological analysis was then carried out on affected (*trsp^{fl/fl}-Tie2Cre^{+/-}*) and unaffected embryos (either *trsp^{fl/+}-Tie2Cre^{+/-}* or *trsp^{fl/+}* [see below]).

3.2. Pathological analysis

Three each of 12.5 and 14.5-gestation-day-old embryos, *trsp^{fl/fl}-Tie2Cre^{+/-}*, *trsp^{fl/+}* and *trsp^{fl/+}-Tie2Cre^{+/-}*, were examined microscopically. Overall, in the case of E 12.5 embryos there were no significant differences found in the various tissues and organs examined, including brain (Fig. 2A), spinal cord (Fig. 2B), subcutaneous region (Fig. 2C), endothelial cells lining the aorta (Fig. 2D) and erythrocytes, even though this cell type was found to be nucleated in both wild type and knockout embryos at this developmental stage (Fig. 2E). Abnormal changes were found only in the E 14.5 embryos carrying *trsp^{fl/fl}-Tie2Cre^{+/-}*. The parenchyma of the brain and spinal cord was largely necrotic (compare knockout and control embryo brain and spinal cord sections in Fig. 2F and G, respectively). Derivative of the central nervous system (dorsal root ganglia, eyes, optic nerves), however, were largely unaffected (not shown). There was extensive multifocal subcutaneous hemorrhage (Fig. 2H) and endothelial cells lining the aorta were hypertrophied (Fig. 2I) in affected embryos compared to control embryos (*trsp^{fl/+}* and *trsp^{fl/+}-Tie2Cre^{+/-}*) as shown by the arrows in the respective panels. Erythrocytes were less mature than those in control embryos (Fig. 2J). As expected, only few erythrocytes in the control embryos retained nuclei, which were small and hyperchromatic. In contrast, most erythrocytes in affected embryos had large nuclei as shown by the arrow in the knockout panel in the figure. The thymus was either not present or was disorganized in affected embryos, preventing its recognition. There was abundant cellular debris within the tongue,

consistent with cell lysis (not shown). Since endothelial cells line the blood vessels, gene inactivation within this cell line will most certainly not be restricted to any specific tissue and thus will lead to widespread effects. No differences were observed between *trsp*^{fl/+} and *trsp*^{fl/+} -*Tie2Cre*^{+/-} control embryos.

3.3. Targeted removal of *trsp* in skeletal and heart muscle

3.3.1. Phenotypes and genotypes—The offspring from matings between *trsp*^{fl/+}-*MCK-Cre*^{+/-} male and *trsp*^{fl/fl} female parents did not manifest any apparent differences phenotypically (i.e., no external morphological differences) until about day 12, when some of the neonatal mice died suddenly. The ill mice exhibited labored breathing and reduced mobility (weakness) 4–5 h before death.

Genotyping 6–8 days old offspring from *trsp*^{fl/+} -*MCKCre*^{+/-} and *trsp*^{fl/fl} matings revealed that the litters contained each of the expected genotypes in targeted tissues and in control organs and tissues (Fig. 3). *trsp*^{fl/fl} was partially removed in skeletal and heart muscle DNA, while it was unaffected in tail, kidney, liver, spleen, brain and lung. Only partial loss of *trsp* was expected in targeted tissues since the *MCKCre* is specific to myocytes and both of these tissues are made up of less than 50% myocytes. Each tissue contained the *MCKCre* transgene (Fig. 3). The only neonatal mice to become ill were those containing the *trsp*^{fl/fl}-*MCKCre*^{+/-} genotype, and we therefore took 10 day old mice of all genotypes for pathological analysis. Neonates with genotype *trsp*^{fl/fl} or genotype *trsp*^{fl/+}-*MCKCre*^{+/-} showed no differences in the tissues and organs examined.

3.4. Pathological analysis of affected and unaffected neonatal offspring

To investigate the cause of death, affected neonatal mice were stored in formalin shortly after death and pathological analysis performed. Unaffected siblings of the same age were sacrificed and also examined pathologically as controls. No gross changes were found in the fixed carcasses. Samples of heart, lung, liver, spleen, thymus, pancreas, kidneys, skeletal muscle and bone were collected and examined microscopically. In affected mice, the myocardium was multifocally infiltrated by mixed inflammatory cells (~90% neutrophils, ~5% lymphocytes and ~5% macrophages), with degeneration and loss of myofibers as compared to controls (see Fig. 4A). In several mice, the inflammation extended into the mediastinum. Cause of death was apparently acute myocardial failure. No degenerative or inflammatory changes were found in other examined tissues or in the control mice. To examine the pathogenesis of these inflammatory and degenerative changes, a total of three muscle-specific *trsp* knockout pups, one 6-day and two 10-day old with an equal number of control pups were euthanized and a full necropsy performed. Gross changes were not found in the following tissues examined microscopically: thymus, kidney, adrenal, spleen, liver, stomach, intestine, pancreas, skeletal muscle, bone, lung, trachea, esophagus, tongue, brain and skull. The mediastinum of the 6-day-old muscle specific knockout mouse was infiltrated, however, by small numbers of inflammatory cells, primarily neutrophils (see arrows in left panel, Fig. 4B), while no changes were found in the age-matched control (right panel, Fig. 4B). Myocarditis, myocardiofiber degeneration, pulmonary arteritis/ periarteritis, and mediastinitis were found in the 10-day-old muscle-specific *trsp* knockout mouse (see arrows in left panel, Fig. 4C), but no changes were found in the age-matched control (right panel, Fig. 4C). No significant differences were found in the skeletal muscle tissue of wild type and knockout animals on H&E staining (Fig. 4D). In addition, Masson's Trichrome staining for increased muscle fibrosis and Congo red for deposition of amyloid in skeletal muscle tissues did not show any significant difference between wild type and knockout animals (data not shown).

4. Discussion

The standard knockout of *trsp* in mammals is embryonic lethal demonstrating that selenoprotein expression is essential in mammalian development [16,17]. Removal of *trsp*, therefore, does not permit us to assess the manner in which selenoprotein expression is essential. By producing the conditional knockout of *trsp* [17] and then targeting specific tissues and organs, we can begin to decipher how these tissues and organs are dependent on selenoprotein expression in development and proper function and the possible role of selenoproteins in disease prevention. In the present study, we find that endothelial cells, which play a major role in the development of the vascular system, are dependent on selenoprotein expression for development and proper function. The *Cre* recombinase that is responsible for targeting the removal of *trsp*^{fl/fl} is under the control of the *Tie2* promoter. The *Tie2* promoter normally governs the expression of the *tie* gene that encodes the receptor tyrosine kinase, which is specific to endothelial cells and is first detected at day 8 of embryogenesis ([19] and references therein). Embryos from matings between male and female mice with genotypes *trsp*^{fl/+}-*Tie2Cre*^{+/-} and *trsp*^{fl/fl}, respectively, showed no differences in phenotype at day 10. However, by 12 dpc, some of the embryos were slower developing, and by day 14.5, these embryos were much smaller with numerous phenotypic abnormalities. Pathological analysis showed several severely affected organs and tissues. For example, necrosis of the parenchyma of the brain and spinal cord was evident and endothelial cells lining the aorta were hypertrophic. Erythrocytes in affected embryos were less mature and had large nuclei. There was extensive multifocal subcutaneous hemorrhage in the affected embryos.

Studies involving mammalian endothelial cells in culture have provided insights into which selenoproteins are expressed in this cell type that include GPx1, GPx4, thioredoxin reductase-1 and SelP [22,23]. These studies were prompted by the fact that selenium appears to protect endothelial cells from oxidative damage which has been implicated in cardiovascular diseases, including atheroma ([22,23] and references therein). In the present study, our data provide strong evidence that selenoproteins play a major role in the development and proper function of endothelial cells. It seems likely, therefore, that the beneficial effect of selenium in protecting endothelial cells from oxidative damage is through the action of selenoproteins.

We also examined the role of selenoproteins in skeletal and heart muscle development and function. The *Cre* recombinase was under the control of the *MCK* promoter that normally encodes the *mck* gene which is highly specific to skeletal and cardiac muscle [21]. Interestingly, neonatal mice that carried a knockout of *trsp* manifested no phenotypic differences compared to their siblings carrying wild type *trsp* until about day 12 after birth. Those neonates with the *trsp* knockout became severely ill manifesting rapid breathing 4–5 h before dying. Pathological analysis suggested that the mice died from acute myocardial failure and that they manifested moderate to severe lesions in heart, but not in the skeletal muscle. In mice, *MCK* expression is complete by day eight after birth in heart muscle, whereas its expression at maximal levels in skeletal muscle is not as clearly defined (C.R. Kahn, personal communication). Thus, it seems that in heart the loss of selenoprotein expression presumably by day eight after birth requires additional time to manifest itself as the lack of selenoproteins in heart muscle is severe leading to sudden cardiac arrest at day 12. Possible explanations for skeletal muscle being normal is that the animals do not live long enough to exhibit gross abnormalities in skeletal muscle tissue or that *MCKCre* is not fully expressed as early in skeletal as in heart muscle. The effect of the lack of selenoproteins in heart muscle is severe leading to sudden cardiac arrest and the animal dies before effects can be manifested in skeletal muscle. As noted in Section 3, the heart lesions consisted predominantly of neutrophils, macrophages and lymphocytes as an inflammatory infiltrate. The most likely cause of inflammation in the heart muscle of the *trsp* knockout mice is oxidative stress due to the depletion of selenium-containing antioxidant enzymes in the myocardium [24] as removal of *trsp* will deplete the entire class of

selenoproteins in myocytes. Several selenoproteins are known to be important antioxidants and redox regulators in mammals protecting cells from endogenous and exogenous reactive oxygen species. Selenium at low doses has been reported to provide significant protection of the human coronary artery endothelium against damage by oxidative stress [24]. Various chemotactic factors released by such damaged cells could lead to widespread inflammation. It is common during acute inflammation that the major invading cell type is the neutrophil followed by monocytes, both of which respond to chemotactic signals such as the release of cytokines, e.g., interleukin-1. Thus we presume that necrosis of the myocytes occurs first and further leads to inflammation.

Characterization of the Sec tRNA^{[Ser]Sec} population in endothelial cells and myocytes was not possible in the present study due to the low abundance of the targeted *trsp* knockout cell type. Furthermore, there are technical limitations for demonstrating reduced selenoprotein expression as antibodies against many selenoproteins are not available and many of those antibodies that are available cross-react with other proteins yielding substantial backgrounds rendering them unusable for immuno-staining. It should also be noted that only small amounts of the total cell population in embryos consist of endothelial cells and that less than 50% of the cell population in cardiac muscle consists of myocytes. Thus, acquiring a pure population of these cell types is a limiting factor. Genotyping affected embryos demonstrated that they contained floxed *trsp* and the appropriate *Cre*, while genotyping cardiac muscle in affected animals demonstrated that less than 50% of floxed *trsp* was removed by the encoded *Cre*. In both cases, the observed level of *trsp* removal was expected from the known amounts of the targeted cell type.

Selenium has been known for many years to have a role in heart disease prevention [25] and it seems from the present study that selenoproteins are the effective agents. It should also be noted that a link between selenium deficiency, Cocksackie virus infection, myocarditis and the development of dilatative cardiomyopathy (Keshan disease) has been observed in children residing in selenium deficient regions in China [25]. Selenium supplementation in the diet of individuals residing in areas of China deficient in this trace element have virtually eradicated Keshan's disease. Interestingly, Beck and collaborators [26] have shown that mice fed a selenium-deficient diet developed myocarditis when inoculated with a strain of Cocksackie virus that was non-pathogenic in mice fed a diet with adequate selenium providing an excellent model for studying the molecular biology of the role of selenoproteins in viral expression and heart disease. Interestingly, another selenoprotein, selenoprotein N, has been found to be associated with three congenital myopathies including desmin-related myopathy with Mallory body-like inclusions, rigid spine muscular dystrophy and multiminicore disease ([27] and references therein) providing additional links between selenium and muscle disorders.

Since the targeted removal of selenoproteins in endothelial cells is embryonic lethal and their removal in myocytes results in premature death due to cardiac failure in newborn mice, it is unlikely that these approaches will provide a model for specifically identifying those selenoproteins that play a major role in cardiovascular disease prevention. However, expression of the selenoprotein population can be perturbed using specific Sec tRNA^{[Ser]Sec} mutant isoforms [28] in such a manner that only a portion of the protein population can be rescued [15] or partially replaced in tissues in which the selenoprotein population has been targeted for removal (B.A. Carlson, M.E. Moustafa, V.N. Gladyshev, D.L. Hatfield, unpublished). By preserving expression of selenoproteins involved in housekeeping and other essential functions, and disrupting gene expression of selenoproteins involved in stress-related phenomena, we can begin assessing the roles of the latter selenoproteins in cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.nmd.2006.10.006.

Fig 1.

Phenotypes and genotypes of embryos with and without *trsp* in endothelial cells. In (A), 14.5 day old embryos lacking *trsp* (left panel) or encoding *trsp* (right panel) were photographed (10×magnification). In (B), DNA was extracted from the limbs of embryos, digested and analyzed with either the probe for *trsp* (upper panel) or *Tie2Cre* (lower panel) as given in Section 2. The 1.1 bp fragment contains *trsp*^{fl/fl} while the 900 bp fragment encodes *trsp*. The 100 bp fragment indicates the presence of *Cre*.

Fig 2.

Pathological analysis of embryos with and without *trsp* in endothelial cells. Left panels show tissues of affected embryos (designated knockout) and right panels the corresponding tissues of unaffected embryos (designated wild type). In E 12.5 embryos, tissues and organs analyzed and the fold magnifications of each were: (A) brain (20×); (B) spinal cord (20×); (C) subcutaneous region (20×); (D) endothelial cells lining the aorta (40×); and (E), RBC's (100×). In E 14.5 embryos, tissues and organs analyzed and the fold magnifications of each were: (F) brain (20×); (G) spinal cord (20×); (H) subcutaneous region (20×); (I) endothelial cells lining the aorta (40×); and (J) RBC's (100×). Panel descriptions are given in the text. Staining was carried out as given in Section 2.

Fig 3.

Genotyping of neonatal mice. The 1.1 kb fragment from the various tissues or appendages shown encodes *trsp*^{fl/fl}, while the 450 bp fragment in heart and muscle indicates the loss of *trsp*. The 100 bp fragment indicates the presence of *Cre*.

Fig 4.

Pathological analysis of neonatal mice with and without *trsp* in muscle. Left panels show tissues and organ from affected pups (designated knockout) and right panels, the corresponding tissues and organ from unaffected pups (designated wild type). Tissue analyzed, ages of neonatal mice, and the fold magnifications were: (A) heart, 10 days (40 $\times$); and letters with arrows designate: L, lymphocyte; N, neutrophil; M, macrophage; (B) mediastinum, 6 days (20 $\times$); (C) mediastinum, 10 days (20 $\times$) and (D) skeletal muscle, 10 days (40 $\times$). Panel descriptions are given in the text. Staining was carried out as given in Section 2.