Spatiotemporal expression of the selenoprotein P gene in postimplantational mouse embryos

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ABSTRACT Selenoprotein P (Sepp) is an extracellular glycoprotein which functions principally as a selenium (Se) transporter and antioxidant. In order to assess the spatiotemporal expression of the Sepp gene during mouse embryogenesis, quantitative RT-PCR and in situ hybridization analyses were conducted in embryos and extraembryonic tissues, including placenta. Sepp mRNA expression was detected in all embryos and extraembryonic tissues on embryonic days (E) 7.5 to 18.5. Sepp mRNA levels were high in extraembryonic tissues, as compared to embryos, on E 7.5-13.5. However, the levels were higher in embryos than in extraembryonic tissues on E 14.5-15.5, but were similar in both tissues during the subsequent periods prior to birth. According to the results of in situ hybridization, Sepp mRNA was expressed principally in the ectoplacental cone and neural ectoderm, including the neural tubes and neural folds. In whole embryos, Sepp mRNA was expressed abundantly in nervous tissues on E 9.5-12.5. Sepp mRNA was also expressed in forelimb and hindlimb buds on E 10.5-12.5. In the sectioned embryos, on E 13.5-18.5, Sepp mRNA was expressed persistently in the developing limbs, gastrointestinal tract, nervous tissue, lung, kidney and liver. On E 16.5-18.5, Sepp mRNA expression in the submandibular gland, whisker follicles, pancreas, urinary bladder and skin was apparent. In particular, Sepp mRNA was detected abundantly in blood cells during all the observed developmental periods. These results show that Sepp may function as a transporter of selenium, as well as an antioxidant, during embryogenesis.

KEY WORDS: selenoprotein P, quantitative RT-PCR, in situ hybridization, mouse embryo

Introduction

Selenium (Se) is an essential dietary trace element, which exerts important human health effects associated with immune response, cancer prevention, thyroid hormone metabolism, reproduction, and antioxidant defense functions, which are linked to these enzyme functions (Rayman, 2000). Se deficiency has been linked with cardiomyopathy, malignant tumors, deforming arthritis, immunological defects, and diseases of accelerated aging and infertility, including spermatogenesis disorders and spontaneous abortion (Rayman, 2000; Brown and Arthur, 2001).

Se exerts its biological functions via the encoding of specific tRNA^Sec (TrsP) by a UGA codon into selenoprotein (Berry et al., 1991). Twenty-five selenoproteins in humans or 24 selenoproteins in rodents have been identified thus far, including glutathione peroxidase (GPx), thioredoxin reductase, iodothyronine deiodinase, selenoprotein P (Sepp), selenoprotein W, and other selenoproteins (Kryukov et al., 2003). These selenoproteins have been shown to be capable of regulating the actions of antioxidation, redox status, thyroid hormone, glucose and Se metabolism, and sperm maturation (Stadtman, 1990; Beckett and Arthur, 2005).

Maternal Se depletion facilitates teratogenesis and death in embryos exposed to phenytoin (Ozolins et al., 1996). Male mice lacking Sepp were infertile due to specific flagellar structural defects in the mature spermatozoa. These results also appeared to be identical to Se-deficient mice (Olson et al., 2005). Targeting disruption of mouse Trsp induces early embryonic lethality, and the selective deletion of Trsp in endothelial cells, using foxP-Cre technology, has revealed that selenoprotein is essential to embryo...
Sepp is a Se-rich extracellular protein which harbors 50% of the total Se within the mouse plasma (Burk and Hill, 2005; Hill et al., 2007). Sepp is expressed in a variety of tissues, but the greatest quantities of Sepp are generated in the liver and secreted into the plasma (Carlson et al., 2004). Sepp knockout mice have been implicated in neurological dysfunctions, including motor abnormalities with severe spasticity induced by Se metabolism disorder (Hill et al., 2004; Shrimali et al., 2007). Although it has been postulated that Sepp may be an important material for Se metabolism during embryogenesis, more comprehensive details of the roles and functions of Sepp during embryogenesis are necessary. The principal objective of the present study was to assess the relative expression levels and to identify the specific localization of SeppmRNA during mouse embryogenesis for the first time. The analysis of SeppmRNA expression in normal embryonic tissues might help to elucidate the specific role of Sepp.

### Results

**Expression level of Sepp mRNA during postimplantational embryogenesis**

The temporal expression pattern of Sepp was assessed by a real-time RT-PCR analysis. As shown in Fig. 1, Sepp mRNA was detected in all the embryos and extraembryonic tissues on embryonic days (E) 7.5-18.5. In the embryos, SeppmRNA increased gradually during embryogenesis. Interestingly, expression in the extraembryonic tissues was higher than in embryos from E 7.5 to 13.5, but decreased suddenly at E 14.5. After E 16.5, SeppmRNA evidenced a similar expression level in embryos and extraembryonic tissues. GAPDH was utilized as an internal standard.

**Localization of Sepp mRNA in whole embryos**

The spatiotemporal expression pattern of SeppmRNA was investigated in the embryos at E 7.5-12.5 via whole mount *in situ* hybridization. On E 7.5, SeppmRNA was significantly expressed in the ectoplacental cone, trophoderm, and decidual cells of extraembryonic tissues, but was detected weakly in the neural ectoderm of embryo (Fig. 2A). On E 8.5, Sepp mRNA was principally expressed in ec-

### Table 1

**COMPARISON OF SEPP mRNA EXPRESSION IN DEVELOPING EMBRYONIC ORGANS**

<table>
<thead>
<tr>
<th>Organs</th>
<th>Embryonic day</th>
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<tr>
<td></td>
<td>13.5</td>
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<tr>
<td>Brain</td>
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<tr>
<td>Lung</td>
<td>++</td>
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<td>Liver</td>
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<td>Pancreas</td>
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<tr>
<td>Urinary bladder</td>
<td>NC</td>
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<td>Skin and hair follicle</td>
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<tr>
<td>Heart</td>
<td>+</td>
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<tr>
<td>Blood cells</td>
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* Signal intensity: - (absence), + (mild), ++ (moderate), +++ (strong), ++++ (very strong). NC (Not checked)

### Fig. 1

**Relative levels of Sepp mRNA expression in developing embryos and extraembryonic tissues.** Embryos and extraembryonic tissues were prepared at different developmental stages and total RNA was extracted and analyzed by quantitative RT-PCR. The expression of Sepp mRNA appears throughout all the embryonic stages, but shows a different pattern between embryos and extraembryonic tissues. Data represent means ± SD (n=5).
Expression of Sepp in mouse embryos

Expression of Sepp mRNA in mouse embryos on embryonic days (E) 7.5-12.5. Embryos were hybridized with digoxigenin-labeled antisense (A-G) or sense (H) probe for Sepp mRNA. (A) E 7.5, (B,C) E 8.5, (D) E 9.5, (E) E 10.5, (F) E 11.5, (G,H) E 12.5. On E 7.5-8.5, Sepp mRNA is mainly expressed ectoplacental cone (ec), neural ectoderm (ne), neural tube (nt), and neural fold (nf). On E 9.5-12.5, Sepp mRNA is highly expressed in the nervous system, e.g. in the prosencephalon (pe), telencephalon (te), mesencephalon (ms), metencephalon (mt) and dorsal neural tube (nt). The signal is greatly expressed in forelimb bud (fl) and hindlimb bud (hl) after E 10.5. am, amnion; ht, heart.

toplacental cone, neural fold, and neural tube (Fig. 2 B,C). In whole embryos after E 9.5, Sepp transcript was detected in the heart and central nervous system (CNS) including prosencephalon, mesencephalon, metencephalon, and dorsal neural tube (Fig. 2D). On E 10.5-12.5, Sepp mRNA was expressed in the developing limbs and CNS, including telencephalon, mesencephalon, metencephalon, and neural tube (Fig. 2 E,F,G). In addition, there were no apparent signals in in situ hybridization analysis using DIG-labeled Sepp sense probe (Fig. 2H).

Tissue-specific expression of Sepp mRNA in developing embryos

Sepp mRNA expression was assessed using tissue-sectioned in situ hybridization from E 13.5 to 18.5 during mouse development. In the developing nervous system, Sepp mRNA was expressed diffusely in the telencephalon, mesencephalon, and metencephalon, but it was primarily expressed at higher levels in the marginal zone of the cerebral cortex after E 15.5 (Fig. 3A). Sepp mRNA was expressed weakly in the developing hearts, whereas it was predominantly detected in the blood cells during all developmental periods (Fig. 3B). In the developing livers, Sepp mRNA was observed abundantly and diffusely on E 13.5-16.5, but the signal was reduced after E 17.5 (Fig. 3C). Also, Sepp mRNA was observed diffusely in the gastrointestinal tract during embryogenesis (Fig. 3D). In the developing lungs, the signal was detected at a higher level in bronchial epithelium than in the mesenchyme on E 13.5-16.5, but was diffusely expressed after E 17.5 (Fig. 3E). As shown in Fig. 3F, Sepp mRNA in metanephros was highly expressed in the metanephric corpuscles and tubules whereas it evidenced a weak expression level in the mesenchyme. Sepp mRNA expressions were gradually increased according to growth, and were higher in the acinar cells in the pancreas (Fig. 3G) and submandibular gland (Fig. 3H). Also, Sepp expression in the whisker follicles and skin were apparent on E 16.5-18.5 (Fig. 3I,J). In the developing urinary bladder, Sepp mRNA was observed at a higher level in the transitional epithelium than the muscle layer after E 17.5 (Fig. 3K). In addition, the signal was detected at a higher level in the digital part of hind limb buds on E 14.5 (Fig. 3L), the Leydig cells of the testes on E 15.5 (Fig. 3M), and sensory epithelium of the inner ear on E 16.5 (Fig. 3N). There was no apparent signal in the in situ hybridization analysis using a DIG-labeled Sepp sense probe (data not shown).
The relative expression levels of Sepp mRNA in the developing embryonic organs were summarized in Table 1.

**Discussion**

Sepp is an abundant extracellular glycoprotein that harbors one selenocysteine in the N-terminal domain and nine other selenocysteine residues in the C-terminal domain. Sepp has two principal functions that provide Se to the various tissues via the transport of Se and function as antioxidants in the extracellular space. The C-terminal domain could be critical for the maintenance of Se in the brain and testis, whereas the N-terminal domain could be ascribed to the antioxidant function and the maintenance of Se in the kidney (Ma et al., 2002; Burk et al., 2003; Hill et al., 2007). Se deficiency in women has been associated with infertility, spontaneous abortion, and retained placenta, thereby suggesting that Se may be necessary in mammalian embryonic development (Bedwal and Bahuguna, 1994).

During normal embryogenesis, the developing embryos generate reactive oxygen species (ROS) by utilizing both aerobic and anaerobic metabolic pathways, and require an antioxidant defense mechanism (Ornoy, 2007). Recently, we showed that antioxidant enzymes, including GPx1 and superoxide dismutase 1, were expressed throughout all mouse embryonic stages (Baek et al., 2005; Yon et al., 2008).

In the current study, we assessed the spatiotemporal expression pattern of Sepp mRNA at ontogenic stage of mouse embryo utilizing quantitative RT-PCR and in situ hybridization. Kasik and Rice (1995) reported that low Sepp mRNA expression in the mouse placenta begins to increase for 4 days before birth, and then becomes the maximum level at birth. In this real-time RT-PCR study, Sepp mRNA expression was detected in all embryos and extraembryonic tissues including placenta from E 7.5 to 18.5. Sepp mRNA was expressed abundantly in extraembryonic tissues rather than embryos on E 7.5-13.5. However, the SeppmRNA level in embryos was higher than in extraembryonic tissues on E 14.5, and then appeared at a similar level in both tissues until birth. According to the results of in situ hybridization, Sepp mRNA was significantly expressed in the ectoplacental cone, trophectoderm, and the extraembryonic tissues on E 7.5-8.5. Decidual tissues originate from the maternal endometrial fibroblast and differentiate into the structure surrounding the implanting embryos. Those are generally considered to form a barrier and to provide nutrition for embryo (Bell, 1983). Collectively, these findings indicate that Sepp may play a role in the transplacental transport of Se from maternal fluid to the embryo in the early and middle periods of gestation, and contributes to the protection of the conceptus in late gestational periods.

In the early developmental stage, CNS begins as a simple neural plate region of the ectoderm and then folds to form the neural fold and groove. The tube is formed by the dorsal fusion of the neural folds and differentiates into the spinal cord and the brain (Roberts, 1990). In the present study, Sepp mRNA was expressed abundantly in the neural ectoderm, neural fold, and neural tube on E 7.5-8.5. Also, on E 9.5-18.5, Sepp mRNA was abundantly expressed in the CNS including the prosencephalon, telencephalon, mesencephalon, metencephalon, and spinal cord. Fantel et al. (1995) reported that the limb bud and brain malformation of the rat embryos were induced by ROS generation during transient uteroplacental hypoperfusion. Although no embryonic
lethality was detected in the Sepp knockout mice, mice with the deleted Sepp gene have evidenced growth defect, motor-incoordination or ataxia, and severe neurological dysfunction (Hill et al., 2003; Schomburg et al., 2003). These facts show that Sepp may perform a crucial role for neuronal survival, and fulfill essential functions for Se maintenance in the brain during embryogenesis.

Sepp is synthesized principally in the liver. Plasma Sepp derived from hepatocytes is the principal transport form of Se which is supported to the kidney, testis, and brain (Renko et al., 2008). Accordingly, the absence of hepatic Sepp synthesis results in an increase in urinary Se excretion, which results in the depression of whole body Se concentration (Burk et al., 2006).

During the mid-gestational periods of mice embryos, high level of Sepp expression was observed in hepatocytes (Steinert et al., 1998). In the current study, Sepp mRNA expression was abundant in the developing livers during E 13.5-16.5. However, the signal was decreased slightly after E 17.5. The kidney performs an important role in the maintenance of Se status and takes up Sepp from the plasma. Sepp provides Se for the biosynthesis of selenoprotein, including plasma GPx (GPx3) (Lochitch, 1989; Schweizer et al., 2005). The present data indicated that the expression of Sepp mRNA in the developing kidney was abundantly detected in the metanephric corpuscles and tubules. Sepp is required for sperm development and is expressed predominantly in the interstitial Leydig cells in the rat and mouse testes (Koga et al., 1998; Steinert et al., 1998; Olson et al., 2005). In the present study, Sepp mRNA was expressed predominantly in the interstitial Leydig cells in the developing testes. Furthermore, Sepp mRNA was expressed predominantly in blood cells during embryonic development. GPx activity in blood cells is tightly associated with plasma Se concentration, and Sepp also harbors the largest proportion of plasma Se (Richard et al., 1991; Burk and Hill, 2005). In addition, Steinert et al. (1998) reported that Sepp was expressed in the hematopoietic cells clustered within the blood vessels on E 16.5. These results show that Sepp is an essential constituent in the Se-transporting pathway of developing tissues.

On the other hand, GPx3, another extracellular selenoprotein, was detected in the developing lung and intestinal epithelium. Sepp mRNA was also expressed within the gut epithelium on E 16.5 (Kinsley et al., 1998; Steinert et al., 1998). This study demonstrated a constant level of Sepp mRNA expression in the developing tissues.
ing lung and intestine at all embryonic stages. The fetal lungs are exposed to relatively hypoxic tensions which arise suddenly at birth, and this alternation may induce oxidative injury in neonates (Araujo et al., 1998). The diminution of Sepp expression in colon cancer may increase susceptibility to oxidative damage and tumor progression (Al-Taie et al., 2004). As shown in Fig. 3, we distinctly noted Sepp expression in the whisker follicle and skin. In particular, Sepp transcripts were upregulated between E 16.5 and E 18.5. UV light induces oxygen radicals to exert many adverse effects in the skin. Se exerts a protective effect in UV-A damage to cultured skin fibroblasts (Leccia et al., 1993). In addition, Sepp mRNA expression was detected at massive levels in epithelial tissues including the glandular epithelia of the pancreas and submandibular gland, transitional epithelium of urinary bladder, and the sensory epithelium of the inner ear (Fig. 3). The epithelia are specifically differentiated to perform functions for protection, absorption, secretion, excretion, and formation of a barrier for selective permeation (Dellmann and Eurell, 1998). These data show that Sepp may function as an antioxidant against excessive ROS in metabolically active sites during embryogenesis.

Sepp knockout mice lead to reduced Se content in plasma, kidney, testis and brain. Furthermore, Sepp-deleted male mice are infertile and Sepp deficiency leads to neurological impairment with ataxia and seizure (Hill et al., 2003; Schomburg et al., 2003; Olson et al., 2005). In this study, Sepp gene was observed spatiotemporally in the CNS, limb buds, blood cells, lung, liver, intestine, testis, and developing epithelia, as well as extraembryonic tissues, during organogenesis. These findings indicate that Sepp may have a pivotal function to protect the embryo against oxidative damages and perform a role in transplacental and/or within the embryonic tissues in the transport of Se as a necessary material for embryogenesis.

Materials and Methods

Animals

Male and female ICR mice (8 to 10 weeks old) were purchased from a commercial breeder, Biogenomics Co. (Seoul, Korea). One male and three female mice were mated overnight in our facilities, which were maintained at 21±1°C and 55±10% relative humidity on a 12h light/dark cycle. Pregnancy was confirmed the following morning by the presence of a vaginal plug or spermatozoa detected in the vaginal smear, which was considered as E 0.5. Under pentobarbital anesthesia, the pregnant mice were sacrificed and the embryos and extraembryonic tissues, including the placenta, were acquired from E 7.5 to 18.5. All procedures were maintained at 21°C ±2ºC and 55±10% relative humidity on a 12h light/dark cycle. Pregnancy was confirmed the following morning by the presence of a vaginal plug or spermatozoa detected in the vaginal smear, which was considered as E 0.5. Under pentobarbital anesthesia, the pregnant mice were sacrificed and the embryos and extraembryonic tissues, including the placenta, were acquired from E 7.5 to 18.5. All procedures were conducted in accordance with the "Guide for the Care and Use of Animals" (Chungbuk National University Animal Care Committee, NIH # 86-23).

Quantitative RT-PCR Analysis

Total RNA was extracted from the mouse embryos and extraembryonic tissues using the Trizol reagent kit (Invitrogen, U.S.A.). Two µg of total RNA was utilized for reverse transcription (RT) to generate cDNA using a cDNA synthesis kit (Bio-Rad, U.S.A.). The generated cDNA was employed as a template for PCR reactions. Quantitative RT-PCR reactions were conducted using the TaqMan Universal PCR Master Mix Kit (Applied Biosystems, U.S.A.). Sepp cDNA amplification was conducted using a Model 7500 Real-Time PCR System by Assay on Demand # Mm00486049 (Applied Bioscience). The Taqman probe was FAM-labeled. Each PCR program was initiated via 2 minutes of UNG (uracil-N-glycosylase) incubation at 50°C, followed by 10 minutes of incubation at 95°C. Reactions were conducted in 40 cycles for 15 seconds using a denaturation temperature of 95°C and for 1 minute with an annealing and extension temperature of 60°C. The data were acquired and analyzed with 7500 system SDS software (version 1.3.1.21). Amplification kinetics was recorded in real-time mode as sigmoid process curves, for which the fluorescence was plotted against the number of amplification cycles. GAPDH mRNA was utilized as an internal standard (Assay on Demand # 4352932E, Applied Bioscience) to normalize target transcript expression. The relative ratios of Sepp mRNA to GAPDH mRNA, which can be used to quantify precisely the levels of Sepp expression in embryos and extraembryonic tissues, were calculated with the standard curves. The data were analyzed for duplicates of three independent runs (means±SD).

Preparation of probe and in situ hybridization

The spatial expression patterns were determined by whole mount in situ hybridization (Baek et al., 2005; Yon et al., 2008) using a DIG-labelled antisense probe. An antisense probe for the in situ hybridization was transcribed with T7 from the full length cDNA in the original pGEM-T vector digested with SpeI (sense: SP8). For sections, mouse embryos (E 13.5-18.5) were embedded in paraplast and sectioned. In situ hybridization for tissue sections was conducted as previously described protocol (Baek et al., 2005; Yon et al., 2008).

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