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ORIGINAL PAPER

Tissue distribution of quiescin Q6/sulfhydryl oxidase (QSOX) in developing mouse

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Abstract Quiescin Q6/sulfhydryl oxidases (QSOX) are revisited thiol oxidases considered to be involved in the oxidative protein folding, cell cycle control and extracellular matrix remodeling. They contain thioredoxin domains and introduce disulfide bonds into proteins and peptides, with the concomitant hydrogen peroxide formation, likely altering the redox environment. Since it is known that several developmental processes are regulated by the redox state, here we assessed if QSOX could have a role during mouse fetal development. For this purpose, an anti-recombinant mouse QSOX antibody was produced and characterized. In $E_{13.5}$, $E_{16.5}$ fetal tissues, QSOX immunostaining was confined to mesoderm- and ectoderm-derived tissues, while in P1 neonatal tissues it was slightly extended to some endoderm-derived tissues. QSOX expression, particularly by epithelial tissues, seemed to be developmentally-regulated, increasing with tissue maturation. QSOX was observed in loose connective tissues in all stages analyzed, intra and possibly extracellularly, in agreement with its putative role in oxidative folding and extracellular matrix remodeling. In conclusion, QSOX is expressed in several tissues during mouse development, but preferentially in those derived from mesoderm and ectoderm, suggesting it could be of relevance during developmental processes.

Keywords Redox state · Mouse development · Quiescin sulfhydryl oxidase · Mesoderm

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Introduction

QSOX (quiescin Q6/sulfhydryl oxidase) comprises a family of multidomain proteins characterized by two thioredoxin (Trx) /protein disulfide isomerase (Pdi) domains at the N-terminal region, a spacer region and a C-terminal augmenter of liver regeneration protein (Alr)/essential for respiration and vegetative growth (Erv) domain, which contains the bound FAD and the active CXXC motif. QSOX was first described in rodent male reproductive system, due to its ability to insert disulfide bonds and produce hydrogen peroxide (Chang and Zirkin 1978; Ostrowski et al. 1979), and has been revisited since the recognition that quiescence-regulated quiescin Q6 (Coppock et al. 1993, 2000), bone-derived growth factor, cell growth inhibiting factor, chicken egg white sulfhydyl oxidase (Hoober et al. 1999a) and seminal fluid sulfhydryl oxidase (Chang and Zirkin 1978; Ostrowski et al. 1979; Benayoun et al. 2001) are structurally-related and founding members of QSOX superfamily (Thorpe et al. 2002). Then, several works have contributed to elucidate some biological and biochemical aspects of this enzyme. An increasing

amount of catalysis data was provided by Thorpe's group for the avian QSOX (Hoober et al. 1996, 1999b; Hoober and Thorpe 1999; Raje and Thorpe 2003). In addition to egg white (Hoober et al. 1996) and male reproductive tract (Chang and Zirkin 1978; Ostrowski et al. 1979; Benayoun et al. 2001), QSOX has already been found in diverse tissues, such as endometrium (Musard et al. 2001), nervous system (Mairet-Coello et al. 2002, 2004), epidermis (Matsuba et al. 2002), neuroblastoma (Wittke et al. 2003), fetal serum (Zanata et al. 2005), and several other secreting and non-secreting tissues (Coppock and Thorpe 2006; Tury et al. 2006). Two splice variants of the QSOX gene have been reported in human, mouse (Coppock and Thorpe 2006) and rat (Radom et al. 2006) tissues, in addition to the neuroblastoma QSOXN gene (Wittke et al. 2003). Exon 12 splicing gives rise to at least two products: a more abundant short isoform and a longer isoform containing a transmembrane domain. Recently, QSOX longer isoform was demonstrated to restore growth and disulfide bond formation in Ero-1p-deficient yeast strain, evidencing that it can also act as a thiol oxidase in vivo (Chakravarthi et al. 2007). However, in spite of all these data, little is still known about the physiological roles of QSOX. According to its localization in ER/Golgi (Thorpe et al. 2002; Wittke et al. 2003; Mairet-Coello et al. 2004; Chakravarthi et al. 2007) and expression pattern (Coppock et al. 1993, 2000; Musard et al. 2001), several hypotheses have been raised. It has been considered an important player in oxidative protein folding (Sevier and Kaiser 2006). In this scenario, while short QSOX has been reported to cooperate with Pdi (Hoober et al. 1999b; Thorpe and Coppock 2007), the long isoform was shown to be not as efficient as Ero-1-p to reoxidize Pdi (Chakravarthi et al. 2007). In addition, QSOX has been proposed to be involved in extracellular matrix remodeling (Coppock et al. 1998; Thorpe and Coppock 2007), since it is secreted (Chang and Zirkin 1978; Hoober et al 1996, Coppock et al. 2000; Matsuba et al. 2002; Amiot et al. 2004; Thorpe and Coppock 2007) and extracellular matrix proteins are rich in disulfide bonds. Additional participation in cell cycle regulation has also been considered, given that QSOX expression is up-regulated in G0-phase (Coppock 2000; Musard et al. 2001). In line with this finding is the recent report showing that QSOX knockdown increases endothelial cell proliferation and sprouting, indicating an anti-angiogenic role (Hellebrekers et al. 2007).

It is already known that redox state is implicated in development (Allen and Balin 1989; Das 2004). The importance of redox regulation is recognized in early embryo (Harvey et al. 2002), as well as in fetal development (Dennery 2004). Usually, a more oxidant environment is correlated to a differentiative phenotype (Allen and Balin 1989; Schafer and Buettner 2001). In this context, thiol-disulfide exchange reactions may contribute to maintain an adequate redox state to development. Indeed, several thiol proteins seem to be relevant in embryo and fetal stages. Thioredoxin and glutaredoxin expression is regulated during development (Kobayashi et al. 2000; Jurado et al. 2003) and thioredoxin knockout mouse embryo die early (Matsui et al. 1996). Since we have recently shown that fetal serum presents high levels of active QSOX, in contrast to post natal serum (Zanata et al. 2005), here we analyzed QSOX distribution in fetal and neonatal mouse tissues.

Material and methods

Animals

Swiss mice, male Wistar rats (3–4 months old) and New Zealand white rabbits were used in this study. All procedures related here were approved by the Ethics Committee from the Pontifícia Universidade Católica do Paraná (protocol numbers 42 and 76).

Cloning of QSOX cDNA from mouse cerebellum

Total RNA was isolated from adult mouse cerebellum using Trizol™ (Invitrogen) according to the manufacturer. Reverse transcription reaction was performed with 1 μg DNA-free RNA using Oligo d(T) and Superscript II reverse transcriptase (Invitrogen). PCR was performed using specific primers 5′-GG*GGTACC*TACTCGTCCTCTGAC-3′ (sense) and a 5′-CCC*AAGCTTTCA*AGAAGAGTCTATGACGAT-3′ (antisense) with restriction sites (italic) for *Kpn*I and *Hin*dIII, respectively. The PCR conditions were as follows: one cycle at 94°C, 5 min, 38 cycles at 94°C, 1 min, 52°C, 1 min, 72°C, 1 min, followed by 72°C, 6 min. PCR product of 1,533 bp was purified by agarose gel electrophoresis and cloned into a pET32a vector (Novagen). The plasmid pET-QSOX encodes a fusion protein containing mouse QSOX (NM_023268 coding sequence + 215 to + 1747 bp) preceded by a 6 histidines-tag (His_6-tag).

Expression of recombinant mouse QSOX

BL21(DE3)pLysS *E. coli* strain (Novagen) was grown under usual conditions and transformed with pET-QSOX vector by electroporation. Expression of His_6-QSOX protein was induced by IPTG (0.4 mM, 4 h). Extracts were prepared by French press lysis of cell suspensions in buffer A (200 mM Tris HCl, 500 mM NaCl, 10% glycerol, 1 mM

PMSF; pH 8.0) containing 8 M urea and 20 mM imidazole, followed by a centrifugation at 10,000×*g* for 30 min. His_6-QSOX was then purified from the supernatant with a Ni-NTA agarose resin (Novagen). Bound protein was eluted with buffer A containing 300 mM imidazole. Urea and the imidazole were finally removed by dialysis against PBS.

Antibody production

Polyclonal anti-QSOX antibodies were generated in New Zealand white rabbits (Harlow and Lane 1988). Briefly, animals were immunized with 200 µg of antigen at each injection. The first injection was performed in complete Freund's adjuvant, followed by injections in incomplete Freund's adjuvant. 6His-QSOX in buffer A with 1 M urea was mixed with adjuvant oil and $Al(OH)_3$ precipitates to form an emulsion. The resulting emulsions were injected subcutaneous and intramuscularly at 3-week intervals. Hyperimmune serum was submitted to affinity chromatography to purify IgG, employing protein A Sepharose CL-4B (GE Healthcare), according to the manufacturer's recommendations.

Western blotting

Seminal fluid from adult mice and rats were homogenized in buffer (50 mM Tris–HCl pH 7.4, 0.2% sodium deoxycholate, 0.5% Triton X-100, 0.5% NP-40, protease inhibitors (Roche)) at 4°C, centrifuged at 12,000×*g* and the supernatant was collected. Protein concentrations were determined by Bradford method. Seminal fluid proteins (35 µg) and recombinant mouse QSOX (200 ng) were submitted to a 10% SDS-PAGE and transferred to nitrocellulose membrane. After blocking the membranes with 5% non-fat milk, blots were incubated with antibodies (diluted 1:1,000 in TBST with 5% slim milk) overnight at 4°C. Pre-immune IgG, anti-QSOX IgG and anti-QSOX IgG pre-incubated with His_6-QSOX (14.7 µg recombinant QSOX/µg anti-QSOX IgG, 1.5 h at 4°C) were used. Membranes were then incubated with anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare; 1:10,000); and reaction was developed using the SuperSignal WestPico chemiluminescence kit (Pierce).

Tissues collection and processing

Male rats and mice were killed by cervical dislocation and their seminal vesicles were rapidly removed, fixed in 10% formalin in PBS and embedded in paraffin. Female mice were kept on a normal day/night cycle and received water ad libitum and commercial food. Day 0 of gestation was defined as starting at 1 a.m. on the day on which a vaginal plug was detected after a mating period of 16 h. At days 13.5 and 16.5 pregnant mice were killed by cervical dislocation. After dissection of the uterine horns, the fetuses were removed and fixed in 10% formalin in PBS. Newborn mice (P1) were killed by hypothermy at 4°C. Tissues were embedded in paraffin. Sections (4 µm) were prepared with a microtome Leica model RM2145 and mounted on organosilane-coated glass slides.

Immunohistochemistry

Sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked with 1–3% (v/v) hydrogen peroxide during 45 min. Sections were then treated with 1% bovine serum albumin (BSA) for 1 h. The anti-QSOX antibody was used at dilutions of 1:100 to 1:900 and was incubated overnight at 4°C. LSAB-HRP kit^{TM} (Dako) was used as secondary antibodies. Reactions were developed with diaminobenzidine (DAB), while counterstaining was obtained with hematoxylin. As negative controls, each immunoreaction was accompanied by a reaction omitting the primary antibody. In some sections, antibody specificity was tested by pre-incubating the antibody with recombinant protein during 3–4 h at 4°C, as described for Western blotting. Images were captured in an Olympus microscope model BX 50 coupled to a camera Sony CCD Iris using the image software Image ProPlus version 4.5.

Results

Polyclonal anti-QSOX antibody production and characterization

Since no commercial tools were available for QSOX studies, we first raised anti-mouse QSOX antibodies. Therefore, we cloned the QSOX coding sequence (nt + 215 to + 1747, Genbank accession number NM_023268) from mouse cerebellum cDNA into the cloning vector pET 32a. This fragment covers practically the full mature short protein and is common to both short and long isoforms. The expression product was a recombinant His_6-fusioned protein with the expected molecular mass of ca. 65 kDa, as observed in a 10% SDS-PAGE and Western blotting probed with an anti-His_5 tag antibody (data not shown). The purified recombinant His_6-QSOX was then used to immunize two rabbits. IgGs were purified from both pre- and hyper-immune sera and were used in all experiments. To examine antibody specificity, we employed seminal vesicle fluid as a source of endogenous QSOX (Ostrowski et al.

1979; Benayoun et al. 2001) in Western blotting assay (Fig. 1a). Mouse and rat seminal fluid proteins (35 μg) were electrophoresed and blotted onto nitrocellulose membranes, which were incubated with pre-immune IgG, anti-QSOX IgG or antigen-neutralized anti-QSOX IgG. While no reaction could be observed for pre-immune IgG (lanes 1 and 4) or antigen-neutralized anti-QSOX IgG (lanes 3 and 6), a specific 65 kDa band was evident with anti-QSOX IgG antibody (lanes 2 and 5). This result demonstrated that the raised antibody specifically recognized a seminal fluid protein which competes with recombinant His_6-QSOX for the anti-QSOX antibody (Fig. 1a). To investigate whether the antibody was appropriated for immunohistochemistry, we analyzed rat and mouse seminal vesicle sections. Photomicrographs confirmed that the antibody strongly stained the apical surface of epithelial cells from this tissue (Fig. 1b), in agreement with the expected localization of QSOX in adult secretory tissues (Chang and Zirkin 1978; Benayoun et al. 2001; Tury et al. 2006). These results clearly demonstrate that this antibody is a specific reagent for QSOX studies. Importantly, due to aminoacid sequence similarities between mouse and rat QSOX (Matsuba et al. 2002), our antibody was also able to specifically recognize rat QSOX. To assess the possibility of detecting the long QSOX variant, a Western blotting assay with mouse cerebellum and brain was performed, given that both rat and murine brain QSOX gene can be alternatively spliced (Radom et al. 2006). The result obtained showed three prominent bands with apparent molecular masses of ca. 65, 72 and 75 kDa (Fig. 1c), which seem to be the corresponding 63, 70 and 85 kDa QSOX polypeptides described for rat brain (Radom et al. 2006). These results indicate that our anti-QSOX is able to recognize the short (65 kDa) and probably the long (transmembrane, 78–85 kDa) QSOX isoforms. Finally, although we cannot assure, the heavier bands observed may be the long variant with two different post-translational modifications.

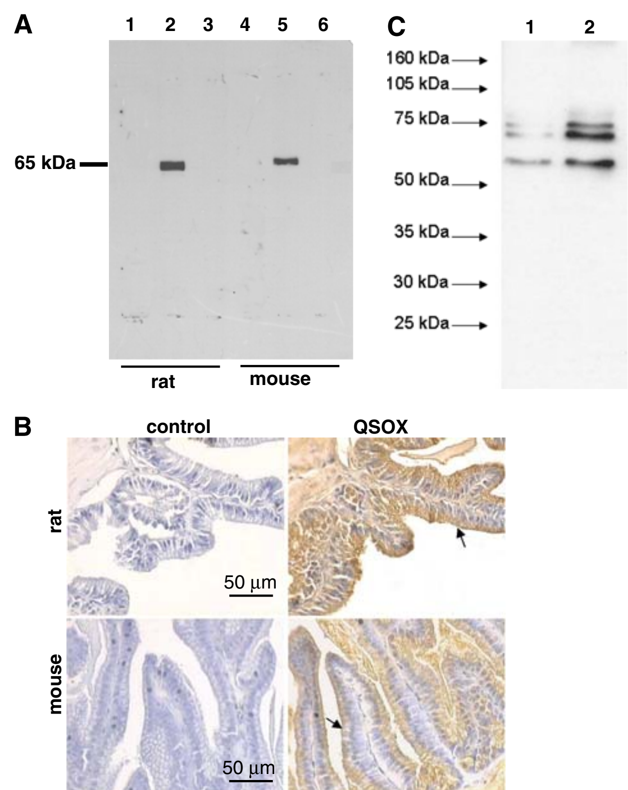

Fig. 1 Rabbit polyclonal anti-QSOX specificity. Western blotting of rat and mouse seminal fluid (35 μg) incubated with 1:1,000 dilution pre-immune IgG (lanes 1 and 4), hyperimmune IgG (lanes 2 and 5) or hyperimmune IgG pre-incubated with recombinant QSOX (lanes 3 and 6) (**a**). Immunohistochemistry of rat and mouse seminal vesicle, probed (QSOX) or not (control) with 1:100 dilution anti-QSOX IgG. Apical side of epithelial cell is stained (arrows). Magnification: 400× (**b**). Western blotting of 50 μg adult mouse cerebellum (lane 1) and brain (lane 2) protein extracts, incubated with 1:1,000 dilution anti-QSOX (**c**)

QSOX distribution in mouse fetal and newborn tissues

Sagittal and transversal sections of $E_{13.5}$, $E_{16.5}$ mouse fetuses and P1 neonate mice were immunostained with the produced antibody for QSOX distribution analysis. QSOX was present in tissues from all the three stages studied, although its distribution pattern showed germ layer specificity. In fetal stages, a remarkable positive staining was preferentially observed in both ectoderm- and mesoderm-derived tissues, in contrast to the absence of QSOX in tissues originated from the endoderm. At P1 neonatal stage, a weak QSOX expression was also detected in some endodermal-derived tissues. Data are summarized in Table 1. Among mesodermal tissues, QSOX was observed in paraxial mesoderm, outer bony cortex of the ribs, cardiac muscle and blood cells (Fig. 2a), blood vessels, skeletal muscle (result not shown), connective tissues, mesenchymal tissues from choroid plexus (Fig. 2c), kidney (Fig. 2e), intestine/mesentery (Fig. 2f), lung (Fig. 2g), adrenal gland, esophagus, liver (data not shown), dermis (Fig. 3a) and pancreas (Fig. 3c) in fetal and P1 newborn mice.

Ectoderm-derived tissues positively labeled for QSOX included cerebellar primordium (Fig. 2c) and brain (Fig. 2d) white matter, peripheral nervous fibers and spinal ganglia (data not shown). Neuronal cell bodies presented a weak QSOX expression at fetal stages (Figs. 2c and 3b) and an increased expression 1 day after birth (Table 1).

Tissues in which QSOX was always negatively stained include cartilage, retina, neuroepithelium, thyroid and thymus glands, glomerulus and olfactory epithelium. In spite of the slightly brownish staining at the epidermis (Fig. 3a), control sections also gave similar result.

Table 1 QSOX immunoreactivity in fetal and newborn mice tissues

Tissue	$E_{13.5}$	$E_{16.5}$	P1
Nervous system			
Neuroepithelium	–	–	–
Ectodermis	+	+	+
Brain white matter	+	+	+
Cerebellum white matter	+	+	+
Neuronal bodies	±	±	+
Spinal ganglion	+	+	+
Olfactory epithelium	–	–	ND
Peripheral nervous fibers	+	+	+
Choroid plexus	+	+	+
Skin			
Epidermis	–	–	–
Dermis	+	+	+
Epidermis basal lamina	–	–	–
Hair follicle			
Epithelial portion	–	–	–
Mesodermal papilla	–	+	+
Intestine (small and large)			
Epithelium	–	–	+
Muscular and mesenchymal tissues	+	+	+
Villi	–	–	+
Esophagus			
Epithelium	–	–	+
Muscular and mesenchymal tissues	+	+	+
Eye			
Retina	–	–	–
Salivar gland	ND	ND	–
Thyroid	–	–	–
Thymus	–	–	–
Heart			
Cardiomyocytes	+	+	+
Cardiomyocytes basal lamina	+	+	+
Cartilage	–	–	–
Skeletal muscle	+	+	+
Kidney			
Glomerulus	–	–	–
Urinary tubules	–	–	+
Mesenchyme	+	+	+
Liver			
Hepatocytes	–	–	+
Mesenchymal cells	+	+	+
Hepatocyte basal lamina	–	–	–
Pancreas			
Ácini	–	–	ND
Islets	–	–	ND
Mesenchyme	+	+	ND
Lung			
Parenchyma	+	+	–
Epithelium	–	–	+
Mesothelium and mesenchyme	+	+	+
Blood vessel			
Endothelium	+	+	+
Smooth muscle	+	+	+

ND, not determined; +, positively stained; –, negatively stained; ±, weakly stained

Therefore, it was considered negative. Tissues derived from endoderm such as pancreatic acinus and islets, digestive tract epithelia and hepatocytes were QSOX-negative in fetal stages. Visceral epithelial cells, such as those from small and large intestine, esophagus, bronchiole and kidney however, became weakly positive in newborn mice (Table 1).

Though the assays had not been designed for quantitative analysis, QSOX relative levels and distribution profiles did not vary significantly throughout the analyzed stages (Table 1). Staining specificity was assessed incubating some sections either with recombinant QSOX-neutralized antibody or omitting the primary antibody. While in the latter situation immunostaining is completely absent in all tissues analyzed (e.g. Fig. 2b), with the exception of epidermis, pre-incubation of the antibody with recombinant QSOX did not abolished the staining, but inhibited most of it (Fig. 2g). Residual immunostaining was mostly observed in strongly QSOX-positive sections, likely due to out-competition of the tissue QSOX for the residual free antibody. Expression was usually observed intracellularly, mainly at perinuclear regions of the cells (see for instance Fig. 3a), but in most cases distinction between intra, extracellular or even at cytoplasmic extensions staining was not straightforward (Figs. 2 and 3). A possible extracellular staining was more evident in some loose mesenchymal tissues (Fig. 3a, c). In addition, due to possibility of detecting both the short and long isoforms of QSOX, here we cannot determine which transcript(s) predominate(s) in each fetal tissue.

Discussion

We have recently demonstrated that QSOX is present in bovine fetal serum, but absent in adult sera, and the observed expression levels correlated with a sulfhydryl oxidase activity (Zanata et al. 2005). This led to the proposition that QSOX could be involved in the redox developmental program, corroborated by several lines of evidences. For instance, human QSOX mRNA was first

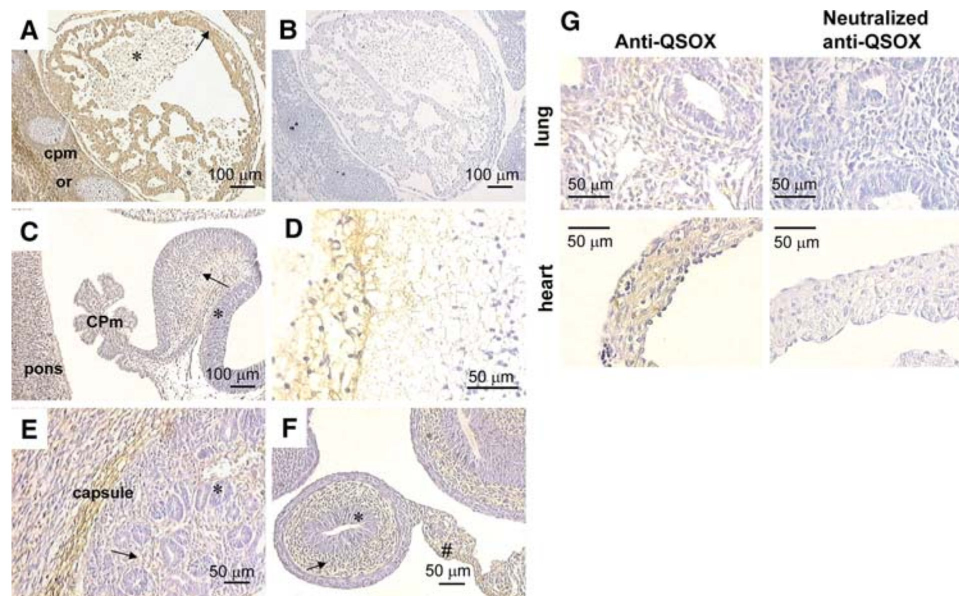

Fig. 2 Representative immunohistochemical localization of QSOX in $E_{13.5}$ mouse fetal tissues. Intense immunostaining is observed in myocardium (arrow), red blood cells (*), the outer bony cortex of ribs (or) and connective paraxial mesorderm (cpm), but is absent in cartilaginous ribs (**a**), while omission of the primary antibody completely blocks immunoreaction (**b**). Choroid plexus mesenchyme (CPm) and cerebellar primordium white matter (arrow) are weakly stained, and no QSOX is seen in neuroepithelium (*) (**c**). White matter is QSOX-positive (**d**). QSOX presence is recognized in renal mesenchyme (arrow) and connective tissue (capsule), but no staining is observed in epithelial cells (*) (**e**). Intestinal loop mesenchymal tissue (arrow) and mesentery (#) express QSOX, but no staining is observed in epithelial cells (*) (**f**). Anti-QSOX IgG neutralized with recombinant QSOX does not bind to $E_{13.5}$ lung and heart tissues as anti-QSOX (**g**). Magnifications: 100× (**a–c**), 200× (**e**, **f**), 400× (**d**, **g**). Anti-QSOX IgG was employed at 1:300 (**a–f**) and 1:800 (**g**) dilutions

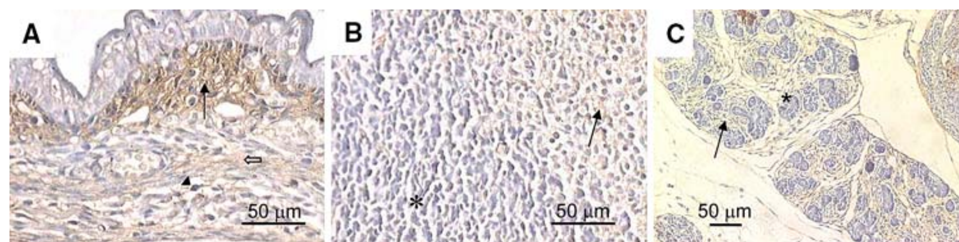

Fig. 3 Representative immunohistochemical localization of QSOX in $E_{16.5}$ mouse fetal tissues. Skin is strongly stained in dermis (arrow) and subcutaneous tissue. An extracellular (open arrow) and perinuclear (arrowhead) staining is evidenced (**a**). White matter is intensively positive (arrow), in contrast to gray matter (*) (**b**). QSOX is expressed in mesenchymal pancreatic tissue (arrow), but not in epithelia (**c**). Magnifications: 400× (**a**, **b**), 200× (**c**). Anti-QSOX IgG was employed at 1:300 dilution

described when human fetal lung fibroblasts entered a reversible quiescence (Coppock et al. 1993, 2000), ESTs from E_{10}–E_{12} mouse embryos (Genebank AK 012943 and AK083938) have been characterized as QSOX; and neuroblastoma QSOXN mRNA is more expressed in some fetal than in the corresponding adult tissues (Wittke et al. 2003). Here we investigated the expression profile of QSOX postnatally and during late organogenesis by immunohistochemistry. For this purpose, recombinant mouse QSOX was produced, purified and a polyclonal anti-QSOX antibody was raised. The anti-QSOX IgG specifically recognized both recombinant and endogenous QSOX. Additional results confirmed that the antibody recognized rat and mouse seminal fluid QSOX, by Western blotting and immunohistochemistry, and also mouse brain 65, 72 and 75 kDa proteins, indicating short and long QSOX isoforms detection.

With this reagent, we showed the expression pattern of QSOX in two stages of mouse late organogenesis: $E_{13.5}$ (Theiler stage 22), $E_{16.5}$ (Theiler stage 25) and postnatally at day 1 (P1). Our data show that QSOX expression was remarkably confined to ectoderm- and mesoderm-derived tissues at fetal stages, while at the P1 it is slightly extended to some endoderm-derived tissues (Table 1).

QSOX distribution has already been intensively investigated in other environments, such as rat (Mairet-Coello et al. 2004) and guinea pig (Amiot et al. 2004) central nervous system, rat peripheral tissues (Tury et al. 2006)

and during rat brain development (Mairet-Coello et al. 2005a, b). Interesting points emerged from such studies and data provided here. The first refers to QSOX expression by nervous system cells, in developing and adult brain. Mairet-Coello and coworkers have demonstrated that this oxidase presents a complex spatiotemporal expression pattern during rat brain development, until reaching the adult stage (Mairet-Coello et al. 2005a, b). According to those studies, most rat neurons turn on QSOX expression at E_{15}. From then on, the overall expression increases until postnatal day 30. Adult rat neurons still produce QSOX and ultrastructural studies showed that QSOX is localized at Golgi apparatus and/or secretory granules of several neuronal cells (Tury at al. 2004), with the highest levels found in disulfide peptides-secreting populations (Mairet-Coello et al. 2004, 2005a, b). Our results show that neuronal bodies from both mouse $E_{13.5}$ and $E_{16.5}$ (Fig. 3c), which correspond to rat E_{15} and E_{18}, respectively, are only slightly stained, and an increased expression is observed at newborn P1 mouse neurons (Table 1). These data suggest that our method sensitivity may be different from that of Mairet-Coello and collaborators. Although these authors had used immunofluorescence, probably less sensitive than signal-amplified immunoperoxidase used here, they had studied QSOX expression in frozen sections, that better preserve antigens, favoring their detection. Also, our antibody possibly does not recognize exactly the same epitopes as the anti-short QSOX antiserum (Benayoun et al. 2001; Mairet-Coello et al. 2005a, b). While short QSOX purified from seminal fluid was used as antigen to raise such antiserum (Benayoun et al. 2001), our immunization protocol employed a recombinant QSOX, whose nucleotide sequence is common to both short and long variants. A second interpretation is that the corresponding stages in mouse and rat may be redox distinct. An inter-species dissimilar expression pattern has been documented for thioredoxin in human and mouse cardiac muscle (Kobayashi et al. 2000). In any case, neuronal QSOX immunopositivity tended to increase as mouse development progresses, as reported for rat (Mairet-Coello et al. 2005a, b). In addition, our data show a considerable QSOX staining in white matter (Figs. 2b and 3b). White matter is composed by myelinated axons and glial cells. Since rat glial cells do not express QSOX at any developmental stages (Mairet-Coello et al. 2005a, b), we infered that the observed staining in mouse white matter (Figs. 2d and 3b) is in neuronal axons, probably in secretory granules, or at the myelin sheath. In agreement, peripheral nervous fibers are also QSOX-positive (Table 1). Interestingly, protein zero, the major protein of the myelin sheath in peripheral nerves (Spiryda 1998), exhibits an important disulfide bond at its immunoglobulin domain (Zhang and Filbin 1994; Pfend et al. 2001). Therefore, QSOX participation in the oxidative folding of the secretory pathway could be suggested in this context, particularly the Golgi-located transmembrane QSOX (Chakravarthi et al. 2007). Furthermore, the absence of QSOX in neuroepithelium agrees with previous data in the literature reporting down-regulation of this enzyme in proliferating cells (Coppock et al. 2000), such as neuroblasts.

A second point concerns the reported strong QSOX expression by epithelial cells, especially from secreting tissues like pancreas, some glands, intestines (Thorpe et al. 2002) and seminal vesicle (Thorpe and Coppock 2007) in adult organisms and by epidermal cells in neonates (Matsuba et al. 2002), while our data showed that fetal and P1 postnatal epithelia are not or only weakly stained, respectively. These differences suggest that QSOX is developmentally regulated, gradually increasing with tissue maturation. In addition, the inconsistency regarding QSOX expression in neonatal epidermis between our result and that from Matsuba and collaborators (2002) could be explained by the use of a more mature newborn mouse than the P1 postnatal animals used in this study. Accordingly, our data demonstrate that adult seminal vesicle from both rat and mouse presented an intense positivity in secretory epithelial cells. Other adult secreting tissues such as endometrium (Musard et al. 2001), and placenta (Thorpe and Coppock 2007) were also described to abundantly express QSOX.

Furthermore, fetal fibroblasts that do not express QSOX preferentially attach to the substratum compared to those which express, suggesting that QSOX may play a negative regulation in cell adhesion (Coppock et al. 1998). Because epithelial cell attachment to extracellular matrix is essential to prevent anoikis (Frisch and Francis 1994) and to promote cell morphogenesis, it is tempting to suggest that absence of QSOX in fetal epithelia could be a mechanism to promote appropriate fetal epithelial survival. Interestingly, a pro-apoptotic member of the QSOX superfamily, QSOXN, was described (Wittke et al. 2003). QSOXN was shown to sensitize neuroblastoma cells to INFγ-induced apoptosis, by still unknown mechanisms. In this context, absence of QSOX in fetal epithelia may prevent apoptosis.

Finally, our work disclosed an interesting pattern of QSOX expression in mesoderm-derived tissues. These are the main precursors of mesenchymal tissues, which are an important source of extracellular matrix proteins-secreting cells. This result is consistent with the initial hypothesis that QSOX might be involved in matrix (re)modeling (Coppock et al. 1998). Indeed, several secreted proteins are rich in disulfide bonds (Fahey et al. 1977; Frand et al. 2000), contributing to the mild oxidative extracellular environment (Moriarty-Craige and Jones 2004). Although the accepted view that disulfide bonds are exclusively introduced intracellularly in the ER (Sevier and Kaiser

2006), secretion of QSOX to medium by cultured cells (Coppock et al. 2000; Matsuba et al. 2002; Amiot et al. 2004), the presence of active QSOX in fetal sera (Zanata et al. 2005), and a positive immunoreactivity of QSOX in mesenchymal and connective tissues, suggesting an extracellular staining (this work), implies the idea that a sulfhydryl oxidase activity may be extracellularly relevant. In developmental context, extracellular matrix modeling is a primordial step to promote cell adhesion, migration and differentiation (Gullberg and Ekblom 1995). QSOX could be producing disulfides in specific environments, to control extracellular protein complex assembly. For instance, TGF-β has been related to actively participate in epithelial-to-mesenchymal transitions, a mechanism that requires a proper matrix support and the presence of adjacent mesenchymal cells (revised by Gullberg and Ekblom 1995). It is also known that TGF-β, a known anti-proliferative agent (Massagué and Gomis 2006), upregulates QSOX expression in fetal fibroblasts (Coppock et al. 2000). Therefore, it would be interesting to investigate the role of TGF-β and QSOX interaction in cell cycle control and appropriate matrix support preparation. Alternatively, QSOX could act as a switch on/off at cell surface environment. It is already known, for instance, that integrin activation can be mediated by thiol-disulfide exchange within the extracellular domain of beta subunit (Yan and Smith 2000, 2001; Jordan and Gibbins 2006), a process likely mediated by Pdi (Lahav et al. 2000, 2002). Because QSOX can oxidize Pdi in vitro (Hoober et al. 1999b), it could act on integrin activation either indirectly, via Pdi (re)oxidation, or directly.

In conclusion, data presented here demonstrated that QSOX is intriguingly expressed by mesoderm and ectoderm-derived tissues in mouse fetus suggesting it could be of relevance during mouse developmental processes. Our results point to a role in extracellular milieu, likely related to ECM modeling, while in the adult, it seems to be additionally involved in protein secretion.

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