The Role of Phospholipid Hydroperoxide Glutathione Peroxidase Isoforms in Murine Embryogenesis*

Astrid Borchert‡, Chi Chiu Wang§, Christoph Ufer†, Heike Schiebel*, Nicolai E. Savaskan‡, and Hartmut Kuhn*‡

From the ‡Institute of Biochemistry, University Medicine Berlin-Charité, Monbijoustrasse 2, D-10117 Berlin, Germany, §Li Ka Shing Institute of Health Sciences and †Department of Obstetrics and Gynaecology, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, and the ‡Division of Cellular Biochemistry, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Phospholipid hydroperoxide glutathione peroxidase (GPx4) is a selenocysteine-containing enzyme, and three different isoforms (cytosolic, mitochondrial, and nuclear) originate from the GPx4 gene. Homozygous GPx4-deficient mice die in utero at midgestation, since they fail to initiate gastrulation and do not develop embryonic cavities. To investigate the biological basis for embryonic lethality, we first explored expression of the GPx4 in adult murine brain and found expression of the protein in cerebral neurons. Next, we profiled mRNA expression during the time course of embryogenesis (embryonic days 6.5–17.5) and detected mitochondrial and cytosolic mRNA species at high concentrations. In contrast, the nuclear isoform was only expressed in small amounts. Cytosolic GPx4 mRNA was present at constant levels (about 100 copies per 1000 copies of glyceraldehyde-3-phosphate dehydrogenase mRNA), whereas nuclear and mitochondrial isoforms were down-regulated between E14.5 and E17.5. In situ hybridization indicated expression of GPx4 isoforms in all developing germ layers during gastrulation and in the somite stage in the developing central nervous system and in the heart. When silenced expression of GPx4 isoforms during in vitro embryogenesis using short interfering RNA technology, we observed that knockdown of mitochondrial GPx4 strongly impairs segmentation of rhombomeres 5 and 6 during hindbrain development and induced cerebral apoptosis. In contrast, silencing expression of the nuclear isoform led to retardations at atrium formation. Taken together, our data indicate specific expression of GPx4 isoforms in embryonic brain and heart and strongly suggest a role of this enzyme in organogenesis. These findings may explain in part intrauterine lethality of GPx4 knock-out mice.

Phospholipid hydroperoxide glutathione peroxidase (phGPx or GPx4) is an intracellular antioxidant enzyme (1) that directly reduces peroxidized phospholipids even if they are incorporated in biomembranes and lipoproteins (2–4). In addition, the enzyme has been implicated in sperm maturation (5, 6) and appears to be essential for regular murine embryogenesis (7, 8). There are three different isoforms of GPx4 (cytosolic isoform (c-GPx4), mitochondrial isoform (m-GPx4), and nuclear isoform (n-GPx4)), but all of them derive from a single gene, which is located on human chromosome 19 (9) and in a sentential region of murine chromosome 10 (10, 11). The start codons for the m- and c-GPx4 isoforms as well as the targeting sequence that directs the mitochondrial enzyme into the mitochondrion are localized in the first exon of the GPx4 gene. Expression of the 34-kDa n-GPx4 (nuclear isoform) involves transcription of an alternative first exon (12). The three phGPx isoforms are expressed at low to medium levels in most mammalian cells and have been implicated in expression regulation of redox-sensitive genes (13), in inflammation (14), in modulation of programmed cell death (15), and in oxidative injury. High concentrations of GPx4 were found in testis (16), and low level expression of the protein has been related to male infertility (17). Homozygous GPx4-deficient mice are not viable (7, 8), and embryos die in utero at midgestation. In contrast, mice in which expression of the n-GPx4 was selectively silenced were viable and surprisingly also fully fertile (18). These data suggest that m- and/or c-GPx4 are more important for murine embryogenesis than n-GPx4. More detailed studies on intrauterine development of GPx4−/− mice indicated that the embryos fail to develop embryonic cavities but are characterized by enlarged Reichert’s membranes. At stage E7.5, when normal embryos have completed gastrulation, homozygous GPx4-deficient mice still resemble pregastrulation embryos showing primitive signs of endod-, meso-, and ectoderm differentiation. At later stages (E8.0–8.5), when normal embryos undergo organ development, GPx4-deficient individuals enter intrauterine resorption. Heterozygous GPx4-deficient mice are viable and fertile and develop normally (7, 8). However, these animals exhibit a reduced survival in response to γ-irradiation, suggesting an impaired antioxidant capacity of the individuals. In fact, cell lines derived from GPx4−/− mice were markedly more sensitive to inducers of ox-
dative stress as compared with cell lines derived from wild-type control littermates (7).

Taken together, these data indicated that expression of mitochondrial and/or cytosolic GPx4 isoforms is required for normal embryogenesis, whereas the nuclear isoform may not be essential. Unfortunately, the biological reasons for premature embryonic lethality remain unclear. To address this question, we studied expression of the three GPx4 isoforms during normal embryogenesis and explored the impact of silencing expression of these isoforms during in vitro embryogenesis. At late embryonic stages, we observed abnormal development of hindbrain (m-GPx4 silencing) and heart (n-GPx4 silencing). These data may explain in part the intrauterine lethality of GPx4-deficient mice.

MATERIALS AND METHODS

Chemicals—The chemicals used were from the following sources: Superscript III reverse transcriptase and RNaseOUT from Invitrogen (Karlsruhe, Germany); BD Advantage 2 Polymerase Mix from BD Biosciences (Pharminingen, Germany); dNTPs from Carl Roth GmbH (Karlsruhe, Germany); DNA molecular weight markers (100 bp, 1 kb) from New England Biolabs GmbH (Schwabach, Germany); and the QuantiTect SYBR Green PCR Kit from Qiagen (Hilden, Germany). PCR primers were custom-synthesized by BIOTEZ (Berlin, Germany).

**TABLE 1**

| Gene product | Direction | Primer Sequence | Size (bp) |
|--------------|-----------|-----------------|-----------|
| RT-PCR       |           |                 |           |
| GAPDH        | Forward   | 5'-CCA TCA CCA TCT TCC AGG AGC GA -3' | 447       |
|              | Reverse   | 5'-GGG TGA CCT TGC CCA CAG CCT TG -3' |           |
| m-c-GPx4     | Forward   | 5'-ACG CAG CCG TTC TTA TCA ATG AGA A -3' | 467       |
|              | Reverse   | 5'-GAG ATG AGC TGG GGC GCT CTG A -3' |           |
| m-GPx4       | Forward   | 5'-ACG CAG CCG TTC TTA TCA ATG AGA A -3' | 531       |
|              | Reverse   | 5'-AGT TCC TGG GCT TGT GTG CAT CC -3' |           |
| n-GPx4       | Forward   | 5'-ACG CAG CCG TTC TTA TCA ATG AGA A -3' | 458       |
|              | Reverse   | 5'-GAT TAC TTC CTG CCT GCC TC -3' |           |

In situ hybridization probes

- m-GPx4 (exon 1A) Forward 5'-CTC GGC CTC GCG CGT CCA TTG -3' | 146
- Reverse 5'-TGG TGC CTG CCA GAC CAG CGG -3'
- m-GPx4 (exon 1B) Forward 5'-AGC GGG GAC GCT GCA GAC AGC -3'
- Reverse 5'-CAA GCC CAG GAA CTC GGA GCT G-3'
- All isoforms (exons 2–3) Forward 5'-GTG CAT CCC GCG ATG ATT GGC G -3'
- Reverse 5'-GAT TAC TTC CTG CCT GCC TC -3'

**SCHEME 1.** Design of PCR primers and oligonucleotide probes used for in situ hybridization. A, structure of the GPx4 gene. B, localization of the PCR primers for isoform-specific amplification. C, localization of the probes used for in situ hybridization.
Preparation of Whole Murine Embryos and of Various Embryonic Tissues—All animal experiments were performed in strict adherence to the guidelines for experimentation with laboratory animals as set by the Chinese University of Hong Kong. Inbred Institute for Cancer Research pregnant mice were obtained from an animal house, and embryos in different developmental stages (E6.5 to postnatal day 5 (N5)) were separately dissected. Different embryonic tissues from the same litter were pooled, and at least three dams were collected independently. Tissue samples were kept in RNA later solution (Qiagen, Hilden, Germany) at 4 °C overnight and were then stored at −80 °C prior to RNA extraction.

RNA Extraction and Reverse Transcription—Total RNA was extracted from the embryonic tissues using the RNeasy mini kit (Qiagen, Hilden, Germany). It was reverse transcribed into the corresponding cDNA using oligo(dT)$_{15}$ primer and SuperScript III reverse transcriptase (Invitrogen) according to the vendor’s instructions.

Semiquantitative RT-PCR—The PCR samples (total volume of 25 μl) consisted of 40 μM Tricine buffer, pH 8.7, containing 0.5 μl of RT reaction, 15 mM potassium acetate, 3.5 mM magnesium acetate, 3.75 μg/ml bovine serum albumin, 0.005% Tween 20, 0.005% Nonidet P-40, 200 nM forward and reverse primers (Table 1), 200 μM dNTPs (final concentrations are given), and 0.5 μl of 50× BD Advantage 2 Polymerase Mix. The following PCR protocol was used to amplify the target gene products: preconditioning phase of 90 s at 95 °C; denaturing phase of 30 s at 95 °C followed by an annealing/extension phase of 60 s at 68 °C. After 35 cycles of amplification, a final extension phase of 30 s at 95 °C followed by an annealing/extension phase of 15 min at 68 °C was run, and the samples were stored at 0.1% diethyl pyrocarbonate for separation of extraembryonic tissues and the embryo proper. These preparations were carried out under a stereo microscope (Olympus, New York). At later developmental stages, embryonic brain and heart (from E10.5 to N5) were separately dissected. Different embryonic tissues from the same litter were pooled, and at least three dams were collected independently. Tissue samples were kept in RNA later solution (Qiagen, Hilden, Germany) at 4 °C overnight and were then stored at −80 °C prior to RNA extraction.

Quantitative Real Time RT-PCR—Real time PCR was carried out with a Rotor Gene 3000 (Corbett Research, Mortlake, Australia) using the QuantiTect SYBR Green PCR Kit from Qiagen (Hilden, Germany). The primer combinations specified in Table 1 were used, and the following PCR protocol was applied: 15-min hot start at 95 °C, followed by 40 cycles of denaturation (30 s at 94 °C), annealing (30 s at 65 °C), and synthesis (30 s at 72 °C) in a total volume of 10 μl. Homogeneity of the amplified PCR products was tested, recording the melting curves. For this purpose, the temperature was elevated slowly from 60 to 99 °C. Data were acquired and analyzed with the Rotor-Gene Monitor software (version 4.6). The amplification kinetics were recorded in real-time mode as sigmoid process curves, for which the fluorescence was plotted against the number of amplification cycles. To generate standard curves for exact quantification of gene expression levels, specific amplification curves for each target gene and GAPDH were used as external standards for each target gene and for GAPDH. The initial amplicon concentrations were set to values varying between 5 × 10$^5$ and 3 × 10$^6$ copy numbers. GAPDH mRNA was used as an internal standard to normalize expression of the target transcripts (m-GPx4, m+c-GPx4, and n-GPx4). Absolute ratios of the target mRNA species and the GAPDH mRNA, which exactly quantify the cellular expression levels of the target genes, were calculated using these standard curves. All RNA preparations were analyzed at least in triplicates, and means ± S.D. are given.

Discrimination between Various GPx4 Isoforms in RT-PCR and in Situ Hybridization—Since GPx4-deficient mice die in utero at midgestation, we explored expression of different GPx4 isoforms during murine embryogenesis. Unfortunately,
on the protein level, c-GPx4 and m-GPx4 cannot be distinguished, since the mitochondrial targeting sequence is cleaved off after mitochondrial import. However, such differentiation is possible on the mRNA level. We established isoform-specific real time PCR systems suitable for quantifying the expression kinetics of the different GPx4 mRNA species during the time course of murine embryogenesis. The reverse primer used for amplification of the three different GPx4 mRNAs was identical (Scheme 1B), but the forward primer for the m-GPx4 was placed inside the mitochondrial targeting sequence. In contrast, the forward primer for amplification of the n-GPx4 was placed in the alternative first exon (E1b), and both positions ensure selective amplification of the two isoforms. Selective quantification of the c-GPx4 mRNA was more complicated. To amplify this mRNA species, we placed the forward primer in exon E1a immediately after the transcriptional start site for the c-GPx4 (10). Unfortunately, this primer combination did not allow separate quantification of the m-GPx4 and c-GPx4 mRNA; in fact, the sum of m-GPx4 and c-GPx4 mRNA species (m + c-GPx4) was amplified. However, we separately amplified m-GPx4 mRNA using the above mentioned m-GPx4-specific primer combination, and thus, we were able to calculate the c-GPx4 copy numbers using the following formula: \[ \frac{\text{[c-GPx4]}}{\text{[m + c-GPx4]}} = \frac{\text{[m-GPx4]}}{\text{[m-GPx4]}} \].

**Immunohistochemistry**—For immunohistochemical staining, murine brains were perfused with ice-cold 4% (w/v) paraformaldehyde solution and were immersion-fixed over night. Sections were prepared on a McIlwain vibratome, quenched with NH₄Cl for 15 min, and blocked with 10% goat serum plus 0.1% Saponin in PBS for 1 h at room temperature. Blocked sections were exposed to the primary monoclonal anti-GPx4 antibody (1:200 diluted) overnight at 4 °C. After washing in 0.1% Saponin/PBS, sections were incubated at +4 °C with a fluorescent anti-mouse IgG (diluted 1:500; Molecular Probes, Inc., Leiden, The Netherlands). Counterstaining of nuclei was carried out using the HOECHST dye (Roche Applied Science). Control staining was performed appropriately using a diluted preimmune serum.

**In Situ Hybridization**—The expression of GPx4 isoforms at different developmental stages was studied by whole mount in situ hybridization. For this purpose, the whole embryos (E6.5 to E10.5) were fixed in 4% paraformaldehyde (in PBS) overnight, dehydrated in methanol, and stored at −20 °C prior to hybridization. The riboprobes were labeled with digoxigenin-11-UTP (Roche Applied Science) using the AmpliScribe kit (Epicenter Technologies). Whole mount in situ hybridization in the developing embryos was performed according to Wilkinson's methods (19) with minor modifications. In both, the prehybridization and hybridization solution SDS was used instead of CHAPS, and RNase treatment of the samples was omitted. For the posthybridization washes, formamide was not included in the washing solution. Both antisense and sense probes for each mRNA species were prepared from individual plasmids cloned into the PCR 2.1-TOPO vector (Invitrogen). For in situ hybridization, it was impossible to separately stain for c-GPx4 (Scheme 1C). In fact, the probe we used was placed in exon 2–3 and thus indicated expression of all three GPx4 isoforms.
(m+c+n-GPx4). However, the probes selected for m- and n-GPx4 were isofrom-specific.

Short Interfering RNA (siRNA) Experiments—m-GPx4 and n-GPx4 targeting siRNA probes were prepared from mouse cDNA plasmids. Antisense and sense RNA were separately amplified by T7 RNA polymerase using MEGAscript Kit (Ambion, Austin, TX). Annealed double-stranded RNA (15 µg) was digested with RNase III (15 units) for 1 h at 37 °C using the Silencer siRNA mixture kit (Ambion). Purified and concentrated siRNAs (1.0 µg/µl) were first mixed with 0.01% Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions and then microinjected with an ASTP (application solution transgenic platform) micromanipulator (Leica, Wetzlar, Germany) into the amniotic cavity of the developing embryos at early head-fold stage (E7.5). Sham-operated controls were injected with Lipofectamine alone without siRNA. Embryos were placed in a whole embryo culture roller incubator (BTC Engineering, Cambridge, UK) and allowed to develop for 72 h in 100% heat-inactivated rat serum with a continuous flow of gas mixtures (20).

Detection of Apoptotic Cells (TUNEL Assay)—After in situ hybridization, the siRNA-treated embryos and corresponding controls were fixed in 4% paraformaldehyde, washed at 4 °C in PBS, dehydrated, embedded in paraffin wax, and cut into 5-µm sections. Apoptotic cells were stained by the standard TUNEL technique. For this purpose, the tissue sections were incubated with the Tdt enzyme and conjugated with anti-digoxigenin peroxidase (Chemicon) for color development.

RESULTS

Expression of GPx4 in Adult Murine Brain—Homozygous GPx4 knock-out mice die in utero during a time window that is important for early development of the central nervous and cardiovascular systems. To investigate whether or not retardations in brain development might contribute to embryonic lethality, we first tested expression of GPx4 in adult murine brain. Immunohistochemical staining with an anti-GPx4 antibody, which was raised against the pure full-length recombinant human enzyme (Sec-Cys mutant expressed in Escherichia coli) indicated that cortical neurons express GPx4 at high levels (Fig. 1). Furthermore, pyramidal neurons were highly immunopositive for GPx4. Interestingly, both the neuronal cell bodies and the corresponding dendrites were stained positive. Moreover, leptomeninges expressed GPx4, whereas white matter regions, glia cells, and astrocytes largely remained immune negative (Fig. 1). Since our antibody does not distinguish between the different GPx4 isoenzymes, isoform-specific differences could not be investigated. These data indicate for the first time expression of GPx4 protein in cortical neurons, suggesting a biological role of the enzyme for neuronal function and/or neuron development.

Expression Kinetics of GPx4 Isoforms during Murine Embryogenesis—If GPx4 is important for neuronal function, systemic silencing of the enzyme during embryogenesis might contribute to premature lethality. To obtain evidence for GPx4 expression during the time course of murine embryogenesis, we first profiled expression kinetics of GPx4 mRNAs by semi-quantitative RT-PCR (Fig. 2) and found that mRNA species encoding for all three GPx4 isoforms were continuously expressed. At late gestation (E14.5 and later), expression of m- and n-GPx4 mRNA was apparently somewhat reduced, and a similar decrease was observed for m+c-GPx4 (Fig. 2A). Exact quantification of the copy numbers of the different mRNA species indicated that during midgestation, c- and m-GPx4 were expressed at similar levels. From Fig. 2B, it can be seen that about 100–200 copies of the c-GPx4 mRNA and 100–200 copies of the m-GPx4 were found per 10^3 copies of GAPDH mRNA. In contrast, expression of n-GPx4 was significantly lower (30–60 mRNA copies per 10^3 copies of GAPDH mRNA). This expression kinetics indicates that at the peak of GPx4 expression during murine embryogenesis, almost 500 copies of GPx4 mRNA species were present per 1000 copies of GAPDH mRNA. Such values may be considered high level expression. Interestingly, expression of the n-GPx4 (Fig. 2B, inset) and m-GPx4 suddenly drops down at late gestation.
GPx4 Expression in Murine Embryogenesis

between E14.5 and E17.5) and remains low until birth. In contrast, the expression levels of the c-GPx4 isoform remain unchanged. Taken together, these data indicate profound regulatory events in GPx4 expression during late embryogenesis.

Localization of phGPx Isoforms in Murine Embryos—To investigate the intraembryonic distribution of GPx4 expression, we performed in situ hybridization using isoform-specific probes for the nuclear (n-GPx4) and the mitochondrial (m-GPx4) isoenzyme (Figs. 3 and 4). In addition, a probe (m+c+n-GPx4) was employed to test total GPx4 expression (Scheme 1). At early embryonic development (Fig. 3; early bud gastrula E6.5 to late primitive streak embryos E7.5), a similar expression pattern of total GPx4 (m+c+n-GPx4) and m-GPx4 was observed (Fig. 3, A, C, D, and F). The most intense hybridization signals were seen in the ectoplacental cone, the extraembryonic ectoderm, the exocoelomic cavity, and the embryonic epiblast. In this developmental stage, it was impossible to decide whether the m- or the c-GPx4 were dominant at these sites. Using the n-GPx4-specific probe, the hybridization signals were somewhat less intense, suggesting low level expression of this isoform at early developmental stages. At E6.5, n-GPx4 (Fig. 3B) was mainly localized in the yolk sac endoderm, the exocoelomic cavity, and embryonic endoderm (amnion). At a later stage (E7.5), a similar expression pattern was observed, but the signals became even less intense (Fig. 3E). These data suggest that m-GPx4 and c-GPx4 (represented by the probe m+c+n-GPx4) may be of functional relevance during early gastrulation, mainly in ectoplacental and ectodermal tissues. In contrast, the nuclear isoform appears to be involved mainly in endodermal development. In addition, we found strong GPx4 expression (mitochondrial and/or cytosolic isoforms) in the primitive streak (arrows in Fig. 3, D and F) but not in embryonic mesoderm, which prompted us to conclude a function of m- and/or c-GPx4 in early embryonic lineage development.

At late embryonic development (Fig. 4; early somite E8.0 to midsomite stages E10.0), total GPx4 expression (indicated by m-GPx4 and m+c+n-GPx4 probes) continues in extraembryonic ectoderm, but now we also detected hybridization signals in the developing embryos (Fig. 4, A, C, D, and F). Expression of the mitochondrial isoform (m-GPx4) was particularly intense in the head fold region of E8.0 embryos (Fig. 4A) but also throughout the rostral to caudal neural tube at E8.5 (Fig. 4D). A similar hybridization pattern was observed when the m+c+n-probe was used (Fig. 4, C and F). Because of the cross-hybridization properties of the latter probe, it was not possible to distinguish which of the two isoforms (c-GPx4 or m-GPx4) contributed more or less to the total signal intensity. The signals caused by the n-GPx4 probe in the endodermal layers were rather weak (Fig. 4, B and E). Expression of m- and/or c-GPx4 in the differentiating neuroepithelium of the rostral neural tube was extended to the forebrain, midbrain, and hindbrain at later developmental stages E9.5 and E10.0 (Fig. 4, G, I, J, and L). This stage-dependent expression pattern suggested a role of these isoforms in embryonic brain development. The intensity of the hybridization signals obtained with the probe for the n-GPx4 was rather weak in the developing head fold region and in the tail (Fig. 4, H and K).

Expression of Different GPx4 Isoforms during Embryonic Development of Various Organ Systems—Abundant expression of GPx4 isoforms in the developing neuroepithelium (Fig. 4) prompted us to follow the kinetics of GPx4 expression during embryonic cerebral development in more detail. From Fig. 5, it...
can be seen that cerebral expression of the GPx4 isoforms is characterized by a unique profile (Fig. 5, top). The mRNA concentration for the cytosolic isoform does not undergo major alterations during perinatal brain development (E10.5–N3). In fact, it remains constant at a level of about 100 mRNA copied per 10³ copies of GAPDH mRNA. In contrast, the m-GPx4 was expressed at higher levels until E13.5 but then dropped down to lower levels during E14.5–E17.5. Toward birth, expression levels recover, reaching copy numbers of up to 200 per 10³ GAPDH copies. A similar transient drop was observed for n-GPx4 during these developmental stages. In contrast, such regulatory kinetics were not observed for other organs, such as the heart (Fig. 5, bottom). Here, we observed constant expression levels of the different isoforms at all stages of embryonic development.

**Targeted Knockdown of GPx4 Expression during in Vitro Embryogenesis**—For more detailed information on the biological function of the GPx4 isoforms during murine embryogenesis, we established an *in vitro* embryogenesis model and silenced expression of different GPx4 isoforms using the siRNA technology. Because of the interesting expression kinetics of m-GPx4 and n-GPx4 during early embryogenesis and because no c-GPx4-specific siRNA probes can be prepared, we focused our attention on knocking down expression of these two GPx4 isoforms (Fig. 6). Injecting the sham control m-GPx4 siRNA into the amniotic cavity did not lead to any silencing of m-GPx4 expression as indicated by *in situ* hybridization (Fig. 6C). Moreover, we did not observe major deviations from normal embryo development, especially in the brain (Fig. 6, A and B). In contrast, injection of m-GPx4 knockdown constructs strongly impaired m-GPx expression in the differentiating neuroepithelium of the rostral neural tube underlying rhombomeres 5 and 6 (arrow in Fig. 6F). Furthermore, we observed that the siRNA constructs induced minor microencephaly (Fig. 6D) and abnormal hindbrain development in lower rhombomeres (arrow in Fig. 6E). In fact, we found that these rhombomeres extended abnormally underneath the developing otic vesicle with interference of normal expression of m-GPx4 in the developing hindbrain (arrow in Fig. 6F). These data suggested that m-GPx4 may play an important role in segmentation of rhombomeres 5 and 6 during hindbrain development.

Specific n-GPx4 siRNA constructs, which selectively silenced expression of this isoform, were also used for targeted knockdown studies (Fig. 7). Injecting the sham control constructs, we did not observe major deviation from normal embryo development (Fig. 7A). However, we observed growth depression (Fig. 7D) and abnormal formation of the atrial structure (Fig. 7, B and E (arrow)) following injection of n-GPx4 knockdown constructs. Expression of n-GPx4 in the left atrium of siRNA-treated embryos (arrow in Fig. 7F) was absent when compared with sham control embryos (Fig. 7C). These data implicate n-GPx4 in normal embryo development and also suggest a role of this GPx4 isoform in development of the left atrium. Taken together, one may conclude that GPx4 isoforms appear to be involved in embryonic development of brain and heart.

**Knockdown of m-GPx4 Expression Induces Apoptosis in Developing Hindbrain**—Since m-GPx4 has been suggested as an antiapoptotic protein (26, 28), we tested whether knockdown of m-GPx4 expression might induce apoptosis in the developing hindbrain. When staining cross-sections of primitive hindbrain prepared from embryos treated with m-GPx4 siRNA (Fig. 8), we observed severe underdevelopment of rhombomeres 5/6 (compare A and B). In fact, the neuroepithelium between the two rhombomeres was much smaller in size, and m-GPx4 expression was strongly reduced although not completely abolished. These data are consistent with our results shown in Fig. 6. Using the TUNEL technique, which stains for apoptotic cells, we detected a high degree of programmed cell death in the abnormal hindbrain regions (dark brown nuclei in Fig. 8B). In contrast, corresponding sections of control embryos did not show any signs of apoptosis (Fig. 8A). These data suggest that m-GPx4 may be important for hindbrain development via regulation of cell survival.
DISCUSSION

Expression regulation of the GPx4 gene is rather complex and involves activation of several transcriptional regulatory elements (21–24) as well as post-transcriptional mechanisms (22, 24). In mammalian cells, several GPx4 transcripts have been described (10, 25), and some of them contain the m-AUG (Scheme 1B) as well as the complete mitochondrial insertion sequence. These mRNA species encode for the mitochondrial GPx4. Other GPx4 mRNA species do not contain the m-AUG, and those transcripts are translated to the cytosolic isoform. There is, however, the possibility that during translation of an m-GPx4 mRNA, the m-AUG start codon may be functionally silenced by protein binding. In such cases, protein synthesis will start at the c-AUG, leading to c-GPx4 protein. Thus, in theory, c-GPx4 protein might be synthesized from m-GPx4 mRNA, but for the time being there is no experimental evidence for such translational silencing of the m-AUG start codon in vivo.

GPx4 was originally discovered as glutathione peroxidase capable of reducing lipid hydroperoxides even if they are incorporated in complex lipid protein assemblies (2). However, in the past few years, more specific functions have been suggested for GPx4 (26–28). So far, three different gene products have been found to originate from the GPx4 gene, and each of these isoforms exhibits a specific cellular and subcellular distribution pattern. Here we report that during development of murine embryos (Fig. 2), expression of m-GPx4 parallels that of the nuclear isoform, suggesting identical mechanisms of expression regulation. A similar co-expression was observed during brain development, where the two isoforms were down-regulated in parallel at midgestation (Fig. 5). From these kinetic similarities, one might conclude that transcriptional regulation of the two isoforms might involve joint regulatory mechanisms (23). On the other hand, m- and c-GPx4 mRNA, which on the protein level form an identical protein with differential subcellular localization, exhibit different expression kinetics (Figs. 2 and 5). In fact, the cytosolic isoform is expressed at constant levels, whereas m-GPx4 shows a maturation-dependent regulation. These findings are rather surprising, transcription of these two isoforms appears to involve the same cis-regulatory elements (23, 24). If this is the case, post-transcriptional elements of expression regulation (isoform-specific splicing mechanisms, differential mRNA stability) may be responsible for the observed isoform-specific differences.

The specific biological roles of the different GPx4 isoforms are still a matter of discussion, and several molecular functions have been suggested (1, 5, 12). To explore their functions in more detail, GPx4-deficient mice were created (7, 8). Unfortunately, homozygous GPx4−/− mice turned out to be not viable and die in utero at midgestation. In contrast, selective knock-out of n-GPx4 did not reveal major deficits (18), and thus m- and/or c-GPx4 appear to be essential for embryo development. To test this hypothesis, we first monitored the time course of expression of the three GPx4 isoforms during murine embryogenesis and found that all three isoforms are expressed on the mRNA level. For c-GPx4, we measured constant steady state mRNA concentrations of about 150 copies per 10^3 copies of GAPDH throughout the entire time window. During early embryogenesis, m-GPx4 mRNA was present at similar concentrations, whereas the nuclear isoform was only expressed at a low level. Interestingly, the steady state concentrations of m-GPx4 and n-GPx4 mRNAs were strongly reduced at late gestation, and these kinetics might be related to the antiapoptotic activity of the enzymes (see below).

The cytosolic GPx4 is considered to be a part of the antioxi-
dative defense system of mammalian cells (1), and thus, constant high level expression of this enzyme may be required during murine embryogenesis to protect the developing embryo from oxidative damage. It is well known that early rodent embryos are sensitive to oxidative stress, but at later stages, they actually require more oxygen for proper organogenesis. The selective drop in the steady state concentrations of both, m- and n-GPx4 mRNAs toward the end of the gestation period suggests a more specific function of these isoforms when the embryo approaches delivery. The mitochondrial isoform has been characterized as an antiapoptotic gene product (26). Its overexpression in various cell lines suppresses markers of programmed cell death, such as release of cytochrome c, DNA fragmentation, and activation of caspases (28). We found that silencing m-GPx4 expression induces apoptosis in the developing hindbrain, which was not observed in controls (Fig. 8). These data suggest that retardation of brain development induced by silencing m-GPx4 expression may be related to programmed cell death.

It has been reported in the literature that expression of Bcl-2, another antiapoptotic protein, is also down-regulated during late murine embryogenesis, and there are further similarities in expression regulation of these two proteins (29). Like GPx4, Bcl-2 can be detected during early murine embryogenesis in many tissues derived from all three germ layers. However, its expression becomes restricted with maturation (29). Although we did not measure Bcl-2 expression under our experimental conditions, the apparent parallelism between Bcl-2 and GPx4 expression suggests a maturation-dependent down-regulation of the antiapoptotic capacity of murine embryos in the perinatal period. It is well known that regular perinatal lung development involves massive apoptosis (30), and parallel down-regulation of Bcl-2 and m-GPx4 may contribute to trigger these perinatal alterations.

Excessive apoptosis is certainly detrimental for developing organs. On the other hand, a certain degree of programmed cell death is required for embryonic development of the central nervous system (31–33), and knock-out studies of members of the apoptotic cascade cause embryonic lethality and/or neuronal hyperplasia (34). Antiapoptotic Bcl-2 is present in embryonic and adult central nervous system, and its expression peaks at stages of massive neuronal development. When neurogenesis is complete, bcl-2 concentrations decline to very low levels (35). In contrast, bcl-2 expression remains high in the adult peripheral nervous system (35).

In the present study, we showed that GPx4 protein is expressed in cerebral neurons adult mice. Moreover, we observed a remarkable down-regulation of m- and n-GPx4 mRNA during brain development at midgestation (E14.5–17.5), and given the anti-
apoptotic properties of m-GPx4, one may predict that this stage of embryonic brain development might involve strong neuronal differentiation. Neuron production increases exponentially during mouse brain development, starting from midgestation at E14 to completion of neurogenesis at E17 (36). However, the newly produced neurons undergo apoptosis unless the cells are stimulated by growth factors secreted by their target cells (33). This mechanism of responding to survival signals was suggested to match the number of neurons for the number of target cells that need to be innervated. Thus, excessive neurons, which do not find target cells for innervation, may undergo apoptosis (37).

The expression kinetics of m- and n-GPx4 during embryogenesis implicate these isoforms in brain development and suggest that intrauterine lethality of GPx4−/− mice may be related to irregularities in the development of the central nervous system. To provide more direct evidence for this hypothesis, we carried out targeted knockdown experiments of different GPx4 isoforms employing the siRNA technology. For this purpose, we cultured murine embryos (E7.5) in vitro for 3 days in the presence and absence of siRNA constructs and checked expression silencing by in situ hybridization. We found that targeted knockdown of m-GPx4 expression led to microencephaly and abnormal hindbrain development, suggesting a pivotal role of this isoform in brain development. On the other hand, selective knockdown of n-GPx4 did not lead to disturbance of brain development, but it rather induced growth depression of atrium structure. Thus, improper heart development might also contribute to embryonic lethality of GPx4-deficient mice. Interestingly, n-GPx4 knock-out mice are fully viable and do not exhibit major functional deficits (18). Unfortunately, embryonic development of the cardiovascular system has not been studied in these animals. Thus, it may well be that the observed atrial growth retardation will be overcome at later developmental stages so that the mature animals will not show major cardiovascular malfunction.

Taken together, the data presented here indicate that all GPx4 isoforms are expressed during murine embryogenesis. There are isoform-specific differences in the expression kinetics, which implicate m-GPx4 in embryonic development of the central nervous system. Functional silencing of GPx4 expression by the siRNA technology confirmed this conclusion but also revealed a function of the n-GPx4 during embryonic heart development.

REFERENCES

1. Imai, H., and Nakagawa, Y. (2003) Free Radic. Biol. Med. 34, 145–169
2. Ursini, F., and Bindoli, A. (1987) Chem. Phys. Lipids 44, 255–276
3. Thomas, J. P., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) J. Biol. Chem. 265, 454–461
4. Sattler, W., Maiorino, M., and Stocker, R. (1994) Arch. Biochem. Biophys. 309, 214–221
5. Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J. S., and Flohe, L. (1999) Science 285, 1393–1396
6. Roveri, A., Flohe, L., Maiorino, M., and Ursini, F. (2002) Methods Enzymol.