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Prenatal expression of thioredoxin reductase 1 (TRXR1) and microsomal glutathione transferase 1 (MGST1) in humans

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ABSTRACT

Thioredoxin reductase 1 (TRXR1) and microsomal glutathione transferase 1 (MGST1) are important redox and detoxifying enzymes in adult life. The aim of this study was to investigate the expression of these enzymes during fetal life. In addition, the role of gene methylation was studied since this might play an important role in the on-and-off switch of gene expression between fetal and adult life.

To this end, the expression of the TRXR1-encoding gene *TXNRD1* and the MGST1-encoding gene *MGST1* was studied in fetal tissues. The mean mRNA expression of *TXNRD1* in fetal livers were seven times higher compared to the mean expression in adult livers ($p < 0.001$). Of the six studied splice variants of *TXNRD1*, four had a significantly higher expression in the fetal livers as compared to adult livers. The mean expression of MGST1 was twofold higher in adult compared to fetal liver tissue ($p = 0.01$). For *MGST1* the alternative first exon 1B was the predominant splice variant in both fetal and adult liver samples. The highest mRNA expression of both *TXNRD1* and *MGST1* was found in fetal adrenals, whereas expression was lower in fetal liver, lungs and kidneys. There was a significant correlation between the hepatic expression of *TXNRD1* and *MGST1*. Treatment with the demethylating agent 5-AZA resulted in decreased levels of *TXNRD1* in human liver HepG2 cells but did not affect the expression of *MGST1*.

In conclusion, the expression of *TXNRD1* is higher in fetuses than in adults and might be of importance during fetal life. Hepatic *TXNRD1* and *MGST1* are co-expressed in both fetuses and adults suggesting common regulatory mechanisms.

1. Introduction

Thioredoxin reductase 1 (TRXR1) and microsomal glutathione transferase 1 (MGST1) are important enzymes for redox control and detoxification during adult life. Animal studies have shown that TRXR1 is important also in fetal life [1,2] but it has never been investigated in human fetal tissue. The aim of this study was to investigate the expression of TRXR1 and MGST1 in human fetal tissues and compare with the expression in adult liver.

The mammalian thioredoxin system consists of thioredoxin, thioredoxin reductase (TRXR) and NADPH [3]. This system is involved in many cellular processes including redox control of transcription factors, reduction of peroxides, regulation of apoptosis and synthesis of deoxyribonucleotides [4]. Mammalian TRXRs are selenocysteine-containing oxidoreductase flavoproteins with remarkable broad substrate specificity [4]. Three different human genes that encode TRXR enzymes have been identified; the classical cytosolic TRXR1, the mitochondrial TRXR2 and the mitochondrial TRXR3 (also known as TGR) mainly expressed in the testis [4].

The TRXR1 encoding gene *TXNRD1* is widely expressed and is up-regulated by various stimuli such IL-1B, lipopolysaccharide, epidermal growth factors and hydrogen peroxide [4], whereas for example statins have been shown to inhibit the transcription of *TXNRD1* [5]. However, the mechanisms of these transcriptional regulations remain unclear. In fact the transcriptional regulation of *TXNRD1* is complex and involves several mechanisms such as alternative splicing and different transcriptional start sites [6]. In humans, 21 different mRNA forms have been identified, all differing in the 5′-end [7]. A putative CpG island in the *TXNRD1* promoter

Abbreviations: TRXR1, thioredoxin reductase 1; MGST1, microsomal glutathione transferase 1; 5-AZA, 5-AZA-2-deoxyxytidine

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(−18 to +90) has been identified [8], but the methylation grade of the CpG sites has not been studied.

TRXR1 has been shown to be essential for embryonic development. *Txnrd*$^{-/-}$ mice resulted in early embryonic death between days 9.5 and 10.5, with a defect in cell proliferation and severe growth retardation [1]. Jurado et al. noted that *Txnrd1* expression peaked around week 10 in mice. Interestingly the *Txnrd1* level was 6-fold higher early in the mouse embryo compared to adult organs [2]. Even though it is known that TRXR1 is essential in the embryonic stage of mice, little is known about the ontogeny of *TXNRD1* expression in humans.

MGST1 is an enzyme that exerts both glutathione transferase activity as well as reduction of various hydroperoxides, being important in the protection against oxidative stress [9]. The expression of the MGST1 encoding gene *MGST1* is abundant in most tissues, with the highest gene expression levels found in liver and pancreas [10]. Several alternative first noncoding exons have been identified, whereas one is predominantly expressed in the liver [10,11]. The ontogeny expression of *MGST1* in humans has never been studied, but it is well known that cytosolic GSTs are functional in various human fetal tissues [12–14].

The specific aims of this study were to measure and compare the gene expression of *TXNRD1* and *MGST1* in adult and fetal livers, and to study the intra-individual gene expression profile of *TXNRD1* and *MGST1* in fetal livers, lungs, adrenals and kidneys. We also chose to co-investigate the gene expression and ontogenetic of *TXNRD1* and *MGST1* to investigate whether common regulatory mechanisms were involved. Moreover the effect of 5-AZA-2-deoxyxytidine (5-AZA), a demethylating agent, on *TXNRD1* and *MGST1* gene expression was studied in HepG2 cells since methylation plays an important role in the on-and-off switch of gene expression between fetal and adult life.

2. Materials and methods

2.1. Fetal and adult liver samples

Human adult liver specimens from 20 subjects were collected from a human donor liver bank established at the Division of Clinical Pharmacology, Karolinska University Hospital, as previously described [15]. Human fetal specimens from liver ($N = 60$), adrenal ($N = 46$), kidney ($N = 43$) and lung ($N = 37$) from 60 fetuses were obtained at legal abortions which were performed for sociomedical reasons at the Karolinska university hospital between year 2000 and 2003. The fetal tissues were excised and immediately frozen in liquid nitrogen and stored at −70 within 2 h. The study was approved by the regional Ethics committee in Stockholm and by the National Board of Health and Welfare.

The gestational ages were determined by crown-rump length and ranged from 5 to 12 weeks (median age 10.2). The maternal age ranged from 18 to 43 years (median 29 years). None of the women reported any chronic or acute disease, regular drug use, or drug abuse. Smoking was reported in 22 women (37%), non smoking in 21 (35%), whereas for 17 there were no reports.

2.2. RNA preparations and real time PCR

Total RNA from 5 to 30 mg of fetal tissue samples and approximately 200 mg of adult liver tissues was prepared using Allprep DNA/RNA and RNAeasy kit (Qiagen), respectively. RNA concentration and quality were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE) and all the RNA samples displayed a 260/280 ratio between 1.6 and 2.1.

RNA (0.5 μg) was reverse transcribed into cDNA with hexamer primer using Superscript III (Invitrogen) according to the manufacturer's protocol and diluted ten times.

The relative mRNA level of *TXNRD1* in liver tissues was determined by real-time PCR using the 7500 Fast System (Applied Biosystems). 18S (# 4310893E Applied Biosystems) was chosen as endogenous housekeeping control gene. For total *TXNRD1* and *MGST1* analysis reaction mix Hs00182418_ml or Hs00220393_m1 (Applied Biosystems) and 2× Taqman reaction mix (Applied Biosystems) were used. For analysis of different TRXR1 and MGST1 isoforms the reactions contained 2× SYBR green reaction mix (Applied Biosystems, Foster City, CA) and 450 mmol of α1/2, α6, α7/8, α10/11, α13, γ2–4 primers for TRXR1 splice variants (sequences described in [16]), and 1A, 1B, 1C, 1D exon specific MGST1 primers (sequences described in [11]). All the reactions included 1 μl cDNA template in a total volume of 15 μl. Thermal cycling conditions included activation at 95 °C (10 min) followed by 40 cycles each of denaturation at 95 °C (15 s) and annealing/elongation at 60 °C (1 min). Each reaction was performed in duplicates and no-template controls were included in each experiment. For the comparison between the mRNA expression in adult and fetal liver samples, an adult liver sample was employed as a calibrator and the delta CT-formula was used as described [17]. For intra-individual comparison between fetal tissues, a fetal liver sample was used as a calibrator. When different splice variants were compared, the TRXR1 α1/2 and MGST1 1B were chosen as calibrators, respectively.

2.3. Cell culture

Human liver cancer HepG2 cells were cultured in MEM supplemented with 5% FCS, 1% penicillin/streptomycin, 1% L-glutamine and maintained in humidified atmosphere at 37 °C and 5% CO_2. No additional selenium source above that present in 5% FCS was added and the cells were thereby selenium starved but not selenium deficient [18]. Prior to 5-AZA exposure the HepG2 cells were split and plated in 12-well plates and pre-incubated for 2 days. 5-AZA were diluted in DMSO and added to the cells for 24 h at final concentration 5 mM. The non-treated controls were incubated with vehicle only. The experiments were performed in four independent experiments. The cells were harvested with Trizol (Invitrogen, UK) and RNA was extracted according the protocol and kept at −80 °C.

2.4. Statistical analysis

To investigate if there was any significant difference in relative hepatic expression between two groups (adult–fetal, smoking–non-smoking, 5-AZA-control) the nonparametric Mann–Whitney test was used since the measurements were few and could not be proven to be normally distributed. When looking for significant difference in splice variants and in tissue-specific (livers, kidneys, adrenals, lungs) expression of TXNRD1 and MGST1 the nonparametric Kruskal–Wallis test was performed followed by Dunn's multiple comparison post-test for identification of which groups differed from the other. The results are presented as mean ± SEM and in box and whisker plots, i.e. the bottom and top of the box are the first and third quartiles, and the band inside the box is the median. The ends of the whiskers represent the 5–95th percentile. The correlation analyses were performed by the Spearman rank method. All statistical tests were performed using GraphPad Prism v. 5.00 and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. The mRNA expression of total TXNRD1 and MGST1 in adult and fetal liver

In order to compare the *TXNRD1* mRNA expression between adult and fetal liver samples we used primers that bind to exon 5 and exon 6. *TXNRD1* was detectable in 59 of 60 fetal and 17 of 20 adult liver samples. Significantly higher levels of *TXNRD1* mRNA transcripts were observed in the fetal liver samples as compared to the adult liver samples. The mean *TXNRD1* was 7 times higher in fetal liver compared to adult liver (mean 12.8 ± 0.82 vs mean 1.82 ± 0.48; $p < 0.0001$) as shown in Fig. 1. In the adults specimens a larger inter-individual variation in *TXNRD1* mRNA was found (90-fold) compared to the fetal specimens in which a 30-fold variation was found.

MGST1 was detectable in 59 (98%) and 18 (90%) of the fetus and adult samples investigated. There was a 2-fold higher expression of MGST1 in adults as compared to fetal expression ($p = 0.01$) (Fig. 1). A large (30-fold) inter-individual variation of *MGST1* gene expressions were noted both in adults and fetuses.

3.2. The mRNA expression profile of six TRXR1 splice variants in fetuses

Several isoforms of TRXR1 have been identified. Here we have studied the abundance of six splice variants previously described [16]. Of these, four splice variants (α1/2, α6, α7/8, and α10/11) were significantly higher in the fetal livers as compared to adult livers. The mean relative mRNA levels (±SEM) in fetal compared to adult livers were 21 ± 1 and 2.4 ± 0.66 for α1/2, 37 ± 2.4 and 1.4 ± 0.36 for α6, 15 ± 0.86 and 0.79 ± 0.10 for α7/8, 22 ± 1.6 and 1.0 ± 0.1 for α10/11 respectively.

All the six splice variants were frequently abundant in the fetal samples. α1/2, α6, α7/8, α10/11, α13 and γ2–4, were detected in 98%, 97%, 92%, 97%, 93% and 97% of the samples, respectively. In the adult samples however, occurrence of the splice variants was only observed in 65%, 40%, 90%, 20%, 60% and 25% of the samples analyzed.

In fetal liver samples α7/8 was the most abundant transcript, three times higher than the wildtype α1/2 ($p < 0.001$), whereas the isoforms α13 and γ2–4 were found at lowest levels (Fig. 2). In the adult liver samples α13 was found at the highest levels, but with a large inter-subject variation (200-fold). Isoform α6 was present at the lowest levels compared to the other isoforms (Fig. 2).

3.3. The mRNA expression profile of MGST1 splice variants

MGST1 1B and MGST1 1D were found in 93% and 88% of the fetal samples and in 70% and 60% of the adult liver samples investigated. Both in the fetal and adult liver samples the alternative first exon 1B was the predominant variant, the mean expression

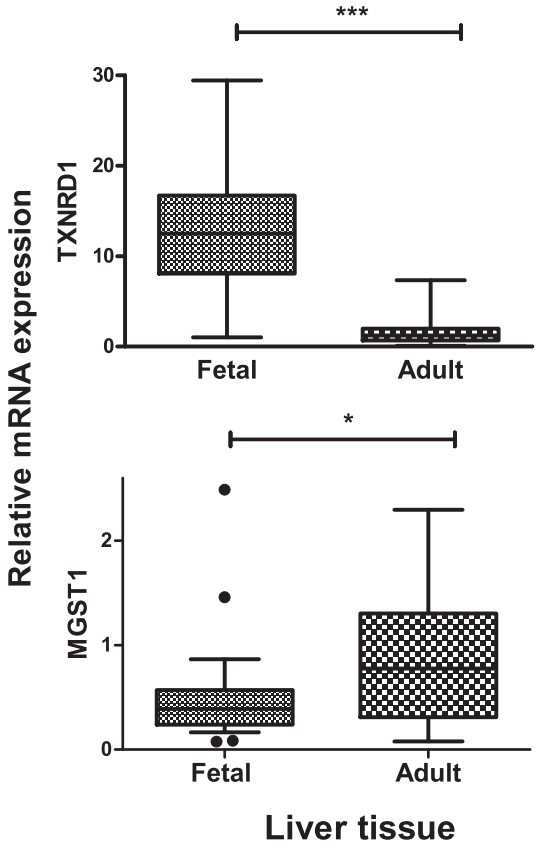

Fig. 1. Relative mRNA expression of *TXNRD1* and *MGST1* mRNA in 59 fetal and 17 adult liver tissue samples. There was a 7-fold higher expression of *TXNRD1* in fetal than adult liver tissue ($p < 0.001$). In contrast, there was a 2-fold higher expression of *MGST1* in adult compared to fetal liver tissue ($p = 0.01$). Statistical analyses were performed using Mann–Whitney test.

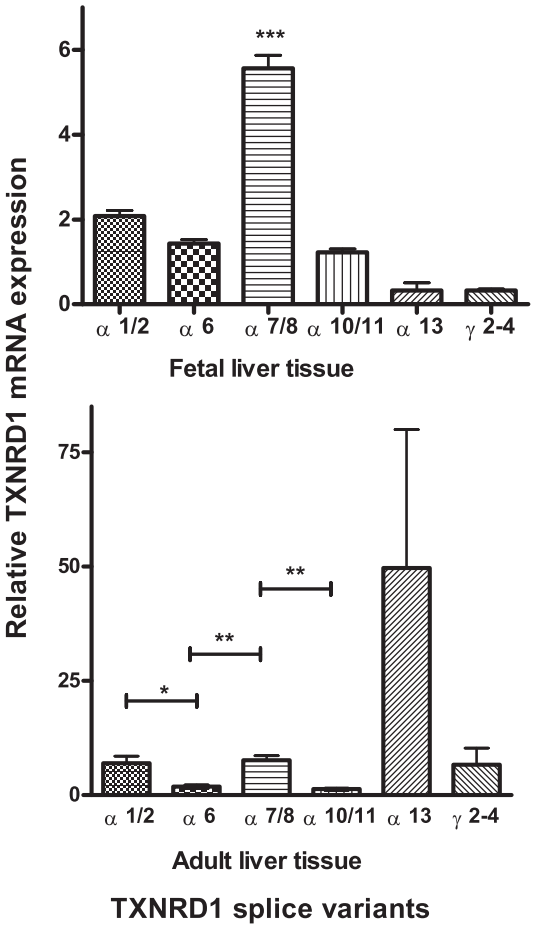

Fig. 2. Relative mRNA expression of 6 different splice variants of *TXNRD1*. In fetal liver samples ($N = 59$) α7/8 was the most abundant transcript, and significantly higher than the other variants ($p < 0.001$). In the adult liver samples ($N = 17$) α13 was found at high levels in some but not in all samples, i.e. there was a large inter-subject variation. The average expressions of α7/8 and α1/2 were significantly higher compared to α6 and α10/11. Statistical analysis were performed using Kruskal–Wallis test followed by Dunn's multiple comparison post-test for identification of which groups differed from the other.

being 400 and 100-fold higher in the fetuses and adults, respectively. There was no difference in the expression of *MGST1* 1B transcript between adults (mean 1.84 ± 0.58) and fetuses (mean 1.59 ± 0.24), whereas *MGST1* transcript including the alternative exon 1D was 5-fold higher in adults (mean 4.92 ± 1.7) as compared to fetuses (mean 1.06 ± 0.13; $p = 0.0006$). The other putative alternative exons 1 (1A and 1C) were not identified in any sample, further supporting that these exons are non-expressed.

3.4. Expression of TRXR1 and MGST1 in different fetal tissue

TXNRD1 and *MGST1* transcripts were abundant in all adrenals, kidney and lung samples analyzed. There was no correlation between the extra-hepatic expressions of these genes. The highest mRNA expression of *TXNRD1* was found in the adrenals whereas the liver, lungs and kidney had lower and about equal expression. For *MGST1* also the adrenals had the highest expression, followed by the liver whereas the lowest expression was found in lungs and kidneys (Fig. 3).

3.5. Correlation between hepatic TXNRD1 and MGST1 mRNA expression

There was a significant correlation between hepatic *TXNRD1* and *MGST1* mRNA levels in both the fetuses ($r = 0.49$, $p < 0.0001$) and adult samples ($r = 0.60$, $p = 0.01$) (Fig. 4).

There was no correlation between the fetal gestational age and the mRNA expression of *TXNRD1* or *MGST1* in any specimens studied. Moreover, there was no apparent effect of maternal smoking on the gene expression of *TXNRD1* and *MGST1* in fetal liver (data not shown).

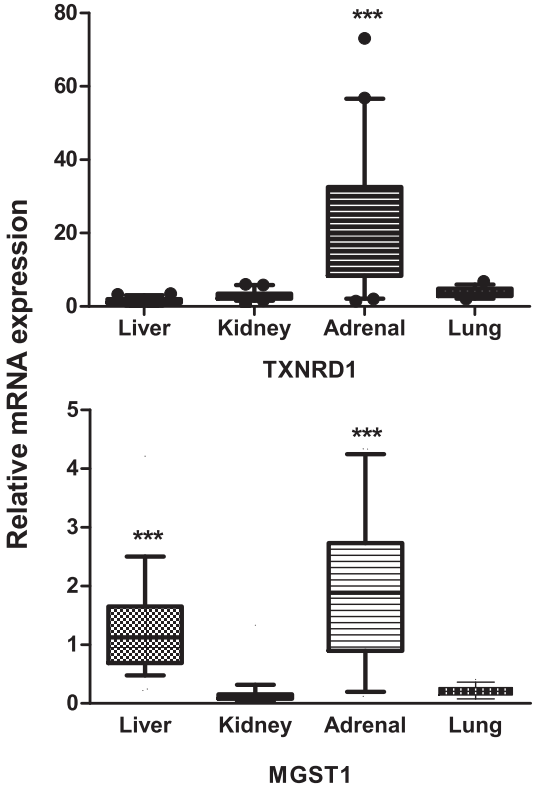

Fig. 3. Relative mRNA expression of *TXNRD1* and *MGST1* in different fetal tissues; liver ($N = 59$), adrenal ($N = 46$), kidney ($N = 43$) and lung ($N = 37$). *TXNRD1* had a significantly higher expression in adrenal tissue compared to the other tissues ($p < 0.001$). *MGST1* had a significantly higher expression in adrenal and liver tissue compared to kidney and lung tissue ($p < 0.001$). Statistical analysis were performed using Kruskal–Wallis test followed by Dunn's multiple comparison post-test for identification of which groups differed from the other.

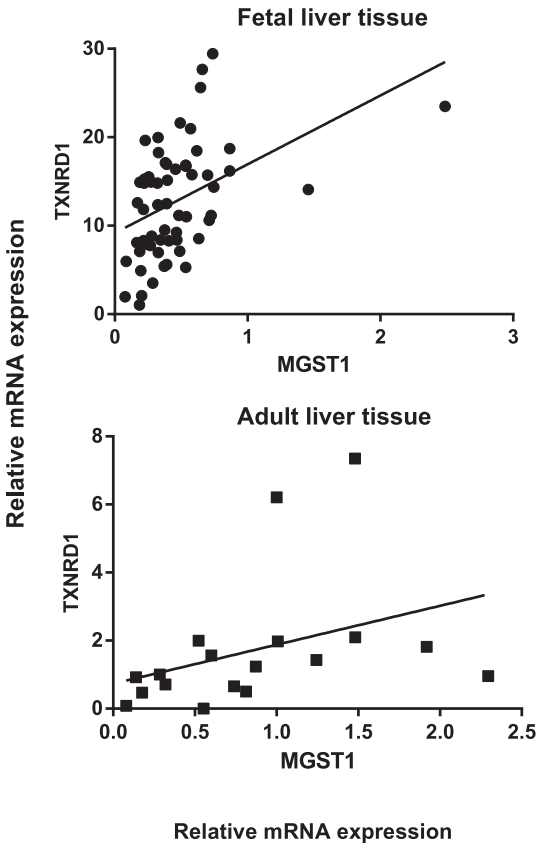

Fig. 4. Correlation analyses between the relative mRNA expression of *TXNRD1* and *MGST1* in fetal ($N = 59$) and adult ($N = 17$) liver tissue were performed using Spearman rank method. There was a significant correlation between hepatic *TXNRD1* and *MGST1* mRNA levels in both fetal ($r = 0.49$, $p < 0.0001$) and adult liver tissue ($r = 0.60$, $p = 0.01$).

3.6. Methylation in HepG2 cells

HepG2 cells were treated with 5 mM 5-AZA and analyzed for the gene expression of *TXNRD1* and *MGST1* and were performed in four independent experiments. The mRNA expression of *TXNRD1* was inhibited in every experiment and in average by 34% by the exposure of 5-AZA in the HepG2 cells ($p = 0.03$), whereas 5-AZA did not affect the expression of *MGST1* (data not shown).

4. Discussion

This is the first time the expression profile of both *MGST1* and *TXNRD1* has been studied in human fetal samples. Both genes were highly expressed in the first trimester. *TXNRD1* mRNA was found at 7-fold higher levels in fetal than in adult livers. This is in agreement with previous studies performed in mice where embryos expressed higher levels of *Txnrd1* compared to adults [2]. Our results, i.e. that *TXNRD1* is highly abundant and consistently found in human fetuses indicates that TRXR1 is an important enzyme during human embryonic fetal development.

In contrast to *TXNRD1*, but in agreement with several other phase II drug metabolizing enzymes, *MGST1* transcripts seems to be more abundant in adults than in fetuses. This is in line with previous result showing that *Mgst1* mRNA expression was detected prenatally (2 days before birth) and increased with age in newborn mice [19]. Moreover, the GST activity has been shown to be higher in cytosolic liver human samples obtained from adults as

compared to samples from the first trimester [20] as was the case for microsomal GST activity in the rat [21].

Here we show for the first time that mRNAs specific for *TXNRD1* and *MGST1* are correlated in the liver, both in fetuses and adults. The reason for this correlation is not known, but the genes share some common transcriptional regulatory mechanisms. Both genes comprises functional Sp1 sites in their proximal promoter regions [8,19]. Both *MGST1* and *TXNRD1* genes also display elements for Oct-4, a transcription factor known to drive the gene expression in early embryogenesis [22]. So this may be a putative factor for high correlation observed in the first trimester. Future studies are warranted to further understand how these genes are co-regulated in different settings.

We did not note any correlation between gestational age and expression level. However, all our samples were collected during the first trimester. It is possible that the expression profile of *TXNRD1* and/or *MGST1* is altered during fetal development. Unfortunately, sex was not determined at the collection of fetus specimens.

As in the case of *TXNRD1* and *MGST1*, the mRNA expression profile during the development is often different than in adults. This developmental switch may be driven by different transcriptional mechanisms such as alternative splicing. Here we noted different distribution of the *TXNRD1* isoforms between adults and fetuses, indicating that there may be some switches in the splicing events during ontogeny. Most splice variants investigated here were found at higher levels in the fetal liver samples. α7/8 was the predominant isoform during first trimester. The same splicing profile has been reported in adult malignant mesothelioma cells, where α1/2 and α7/8 were the major transcripts, whereas α6 was found at the lowest levels [16]. For *MGST1*, our results indicate that the alternative first exon 1B is preferred in both fetuses and adults. This is in agreement with previous findings [10,11]. This first exon is the exon that gives rise to the predominant mature mRNA transcripts in human tissues [10].

In addition to *TXNRD1* and *MGST1* gene expression in livers, we performed studies in fetal samples obtained from adrenals, kidneys and lungs. The expression of *TXNRD1* was most pronounced in the adrenal tissue suggesting an important role for the enzyme in this tissue during fetal life. To our knowledge a comparison of the expression of TRXR1 in different tissues in adults has not been done. *MGST1* was most abundant in adrenals and livers during the first trimester. In adults *MGST1* transcripts have been found at highest levels in livers and pancreas [10].

Epigenetic events such as DNA methylation of CpG sites has been suggested to play a role in the developmental switch in gene expression of several genes [22,23]. Methylation of CpG islands may result in inhibition of transcriptional rate or in some cases activation. Even though a CpG island was identified *in silico* in the proximal promoter of *TXNRD1*, no one has investigated the methylation grade of the *TXNRD1* promoter *in vivo*. Our 5-AZA results indicate that methylation of *TXNRD1* may be a transcriptional determinant in liver cells. We showed that de-methylation leads to decrease in *TXNRD1* expression in HepG2 cells. Even though up-regulation is more common, several genes have been shown to be repressed by 5-AZA exposure in HepG2 cells [24]. It is possible that de-methylation increases access of repressor binding sites. Alternatively, the availability of trans-active factors may decrease. It would be of interest to further assess the CpG island in *TXNRD1* and study whether or not the methylation grade differs throughout development.

MGST1 expression was not affected by 5-AZA indicating that DNA methylation does not play an important role in *MGST* gene expression, at least not in HepG2 cells. This is consistent with an *in silico* analysis using UCSC Genome Bioinformatics (www.http://genome-euro.ucsc.edu/index.html), which did not identify any CpG islands in the *MGST1* gene.

Many proteins that are highly expressed during fetal life are often overexpressed also in malignant cells. Such proteins can be used as biomarkers for different types of cancer, for example alfa-fetoprotein that is a biomarker for liver cancer. TRXR1 is overexpressed in many cancer cells and plays an important role during carcinogenesis [25–27]. This is in accordance with the results presented here showing high expression of TRXR1 during fetal life. Indeed, it has been discussed whether this enzyme could be used as a biomarker for different cancers [25,28].

In conclusion we have found that the expression of *TXNRD1* is higher in the first trimester than in adult life and might be of importance during fetal life, whereas *MGST1* was more abundant in adults. Interestingly, hepatic *TXNRD1* and *MGST1* are co-expressed in the fetuses as well as in adults suggesting common mechanism for regulation. In addition, DNA methylation may be important in the regulation of the transcriptional activity of *TXNRD1* but is probably of minor importance for *MGST1*.

Author contributions

LBB, MJ, RM, AR and LE conceived and designed the study. LBB, MJ and LE acquired and analysed the data. LBB, MJ, RM, AR and LE interpreted the data and wrote the paper.

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