Disruption of paraoxonase 3 impairs proliferation and antioxidant defenses in human A549 cells and causes embryonic lethality in mice

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Abstract.

We had previously shown that paraoxonase 3 (PON3), a putative circulating antioxidant, was systemically up-regulated in late gestation rat, sheep and human fetuses. Our overarching hypothesis is that preterm human infants are delivered with low levels of PON3 and that this contributes to a state of oxidative stress. We sought to determine whether absence of Pon3 was associated with reduced neonatal viability in mice and studied the offspring from crosses between Pon3\(^{+/c}\) mice. The number of Pon3\(^{-/-}\) animals at E10.5 and E17.5 was significantly lower than the expected 25% (9.3% and 7.9% respectively, \(P<0.001\)). On the first day of post-natal life, this was further reduced (2.4%, significantly less than the proportion in fetal life \(P=0.04\)). Pon3\(^{+/c}\) animals had lower body and placental weights than wild type littermates at E17.5, an effect which was independent of the parent of origin of the mutant allele. We then studied the effect of PON3 knock down in a human cell line, A549. Stable knock down of PON3 using short hairpin RNA reduced cell proliferation in 21% oxygen. We then studied the effect of transient knock down of PON3 using short interfering RNA (siRNA) in the same cell line in low (2%) or ambient (21%) oxygen. Knock down of PON3 using siRNA reduced total antioxidant capacity in 21% (\(P=0.008\)) but not 2% oxygen. We conclude that absence of Pon3 in mice resulted in increased rates of early fetal and neonatal death. Knock down of PON3 in human cells reduced cell proliferation and total antioxidant capacity.
Introduction.

Preterm birth is associated with a huge burden of morbidity, mortality and economic consequences. Hence, ameliorating the consequences of preterm birth has very significant potential benefits for the public health. Following birth there is a rapid rise in oxygen levels in all mammalian species. The rise in PO$_2$ following birth is a key trigger for postnatal adaptation of the cardiovascular system, such as promoting closure of the ductus arteriosus (15) and increasing pulmonary blood flow (8). However, rapid increases in PO$_2$ lead to the increased generation of reactive oxygen species (ROS). The fetus up-regulates expression of several anti-oxidant enzymes in late gestation in preparation for the sudden rise in pulmonary and arterial oxygen tension following birth. Some of the complications of preterm birth are due to exposure to ROS prior to the completion of these preparative changes, leading to a state of oxidative stress (3; 7). This has led to evaluation of exogenous anti-oxidants in the treatment of preterm infants, but none of these trials have demonstrated any improvement in clinical outcome (2; 16; 18).

We conducted a series of experiments with the aim of identifying endogenous antioxidants involved in fetal preparation for birth (1). Using whole genome array in preterm and term fetal rats, we found that paraoxonase-3 (Pon3) was one of only four transcripts up-regulated >20-fold in both the lung and intestine in late gestation and it was the only one of the four that had been identified as a putative antioxidant. We quantified this transcript in biological replicates and confirmed massive up-regulation. We then studied fetal sheep and found that PON3 mRNA levels also increased in the lung and intestine in late gestation. We also obtained evidence that its expression was regulated
by glucocorticoids in the fetal sheep (1). Finally, we obtained human umbilical cord blood and found that serum PON3 levels were greater than 6-fold higher at term (P<0.0001) than preterm (<30 weeks).

We concluded from these analyses that up-regulation of PON3 may be a systemic preparative process for birth which is observed in the rat, sheep and human fetus. The last common ancestor of these three species lived ~97 million years ago (9). The conservation of this preparative change in fetal life suggests that it has some fundamental role in the perinatal period. We speculated that low levels of PON3 in human preterm infants may lead to oxidative stress and, therefore, that supplementation of infants with exogenous synthetic PON3 may improve outcome. However, there is currently no direct evidence that low levels of PON3 are harmful. There are two other paraoxonase enzymes, PON1 and PON2. All three PONs hydrolyze a range of lactones and hydroxyl acids and have some common substrates (5). Hence, it is plausible that PON3 is redundant due to the presence of PON1 and PON2 and that its absence would be without biological effect. The aim of the present study was to determine the effect of eliminating or reducing PON3 in mice and in a human cell line.
Methods

Genetically modified animals

All experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 under an appropriate Home Office License and with approval from the local ethical review committee. Sperm from mice that were heterozygous for a knock out Pon3 allele were purchased from the Texas A&M Institute for Genomic Medicine. In the knock out allele, the first two coding exons of Pon3 are replaced by a β-geo (lacz/neo) cassette. This results in deletion of paraoxonase domain (Interpro ID: IPR008364) located in these exons and also the incorporation of a polyadenylation signal that prevents transcription further downstream. Correct gene targeting was confirmed by Southern blot using 5’ and 3’ probes outside the homology arms by the Texas A&M Institute for Genomic Medicine. To determine the effect of Pon3 loss on fetal development, embryos were collected at embryonic day (E) 10.5 and E17.5 from heterozygous timed matings, where day of plug = E0.5. To determine the effect of Pon3 loss in neonatal mice, pups were sacrificed on the morning following delivery (P1, delivery occurs during the night). Genomic DNA was isolated by phenol chloroform extraction and ethanol precipitation and used as template for genotyping by PCR with a Pon3 specific reverse primer (5’-AAAAGCCATCTAGACGCTGG-3’) and forward primers either directed against Pon3 or the inserted neomycin cassette (5’-TTCTGTAGGCACAGTGTTAC-3’ and 5’-GCAGCGCATCGCTTCTATC-3’ respectively). PCR with Taq polymerase (Bioline, London, UK) conditions consisted of denaturation at 95°C for 5 minutes and 40 cycles of 95°C for 30 seconds, 61°C for 30 seconds and 72°C for 1 minute followed by a final extension of 72°C for 10 minutes in the presence of 3mM MgCl₂ and 0.5mM primers.
Detection of Pon3 mRNA in mouse tissue

Total RNA was extracted from E17.5 mouse embryonic lungs using Trizol (Invitrogen, Paisley, UK) according to the manufacturers instructions, DNAse treated (Promega, Southampton, UK) and reverse transcribed to cDNA with Superscript II (Invitrogen) with random hexamer primers. cDNA was used as template for PCR with Pon3 and beta-actin specific primers. A 362bp fragment relating to mouse Pon3 was amplified with primers against the 3´ end of Pon3 mRNA (5´-GATCTTGACCCTCACTGGA-3´ and 5´-TTTGTAGATAAGCCCAGGG-3´) with PCR cycling conditions of 95ºC for 5 minutes followed by 40 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 1 minute followed by a final extension of 72ºC for 10 minutes. A 527bp beta-actin PCR product was amplified using beta-actin specific forward and reverse primers (5´-CTACAATGAGCTGCGTGTGG-3´ and 5´-GCTCTTCTTCCAGCCTTCCT-3´). PCR cycling conditions included an initial denaturation of 95ºC for 5 minutes followed by 35 cycles of 95ºC for 30 seconds, 48ºC for 30 seconds and extension at 72ºC for 1 minute followed by a final extension at 72ºC for 10 minutes.

Studies of human cell lines

The human lung adenocarcinoma cell line, A549, was employed to study the effects of PON3 knock down. Cells were maintained in DMEM supplemented with 10% FCS, 2mM glutamine and antibiotics (penicillin [50 U/ml] and streptomycin [50 μg/ml]). Permanent knock-down of PON3 was achieved using SureSilencing short-hairpin RNA (shRNA) plasmids purchased from SA Biosciences (Frederick, MD, USA) and all experiments were conducted in the presence of 21% oxygen. We employed four different short hairpins targeting PON3 and a non-targeting shRNA. To generate A549 cell lines stably expressing shRNAs, 2 x 10^5 cells per well were plated on 24-well plates in medium with
no antibiotics. Cells were transfected 24 hours later using 1μg supercoiled plasmid, 2 μL Lipofectamine 2000 and 150 μL Optimem. The next day cells were passaged onto 3.5 cm dishes and transfected cells were selected with G418 (400 μg/ml) for 21 days. The resultant colonies were trypsinized to generate a polyclonal population of cells expressing the shRNA plasmids. Cell proliferation was assessed by Thiazolyl Blue Tetrazolium Bromide (MTT) assay as previously described (13). 10,000 cells per well were plated onto 96 well plates (n=16 for each cell line). Cells were grown in G418 (400 μg/ml) containing medium for 72 hours. Twenty microliters of MTT (5 mg/ml solution) was added to each well and the cells were incubated at 37ºC for 1 hour. Medium was removed and 100 μL DMSO added. Optical density was determined at 550 nm and 690 nm.

Transient knock down of PON3 was achieved using short interfering RNAs (siRNAs). 1.5x10^5 A549 cells were plated onto each well of a 6-well plate (35 mm diameter) in media without antibiotics and cultured for 72 hours at 37ºC in an atmosphere of 2% O₂ and 5% CO₂ in a BioSpherix Xvivo controlled atmosphere tissue culture system (BioSpherix Ltd., Lacona, NY, USA). Cells were then transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In brief, 100 pmol siRNA (against Pon3 and non-targeting siRNA, On-Target Plus, pool of 4) (Dharmacon, Lafayette, USA), was used along with 5μL Lipofectamine 2000 and 500μL Opti-Mem Reduced Serum Medium (Invitrogen) per well. Eight transfections were done both with PON3 siRNA and with non-targeting siRNA. All biological replicates were transfected individually. Mock transfection was performed with Lipofectamine 2000 and OptiMem with ddH₂O. Transfection was performed overnight in 2% O₂. After 24 hours, cells were passaged 1:6 using reagents equilibrated at 2% oxygen and plated onto 6-well plates in medium with antibiotics. Each six-well plate contained three wells from one of the
transfections with siPON3 and three wells from one of transfections with non-targeting siRNA. 48 hours after the transfection, plates were placed in incubators with low (2%) or ambient (21%) oxygen. After a further 24 hours (i.e. 72 hours after the start of the transfection) cells were harvested using trypsin (for RNA analysis) or with a cell scraper for antioxidant activity assay. Cells for RT-PCR analysis were resuspended in 350µL RA1 buffer supplemented with beta-mercapto-ethanol (Nucleospin RNA II, Macherey-Nagel, Düren, Germany), frozen immediately in dry ice and stored at -80ºC. Scraped cells for antioxidant activity assay were stored at -80ºC. All analyses were of transfected and control cells processed in parallel over the same period of 72 hours.

Quantitative real-time RT-PCR of A549 cells

Total RNA was purified from cells using Nucleospin RNA II kit (Macherey-Nagel) with on-column DNAse treatment. Total RNA was reverse transcribed to cDNA using superscript II (Invitrogen) with random hexamer primers and used as template for quantitative RT-PCR. PCR was performed with an ABI Prism 7900HT system (Applied Biosystems, Warrington, UK) with Fast QPCR mix (Applied Biosystems) and primers and probes for PON3, GAPDH and 18S rRNA. These primers and probes were pre-designed and pre-optimized (Applied Biosystems: Hs00412993_m1, Hs02758991_g1 and Hs99999901_s1 respectively). All samples were assayed in triplicate. PON3 transcript levels were quantified using normalization to the geometric mean of GAPDH and 18S rRNA (19).

Western blotting

Samples (mouse lung, liver or A549 cells) were homogenized in RIPA buffer (50mM Tris HCl pH7.4, 150mM NaCl, 2mM EDTA, 1% nonident P40 and 0.1% SDS) with protease inhibitors (Roche). 50µg of protein per lane was reduced by addition of Nupage Sample
Reducing agent (Invitrogen), heat-denatured and loaded on a 10% Novex Bis-Tris Gel (Invitrogen). After electrophoresis, proteins were transferred to a PVDF membrane (Invitrogen), blocked with 5% skimmed milk in TBS-tween-20 (0.05%) for 1 hour and subsequently probed with a goat polyclonal anti-human PON3 antibody (Lifespan Biosciences) at 0.2\( \mu \)g/ml on a shaker at 4°C overnight. Antibody binding was detected by horseradish peroxidase-conjugated rabbit anti-goat secondary antibody (Dako). ECL substrate (Amersham Biosciences) was then used to visualize binding. Membranes were stripped with Restore™ Western stripping buffer (Pierce) for 15 minutes at room temperature and re-probed with a rabbit polyclonal antibody to human GAPDH (Abcam) at 0.2\( \mu \)g/ml to confirm equal loading of protein.

Analysis of total antioxidant capacity

Total antioxidant capacity was measured using a commercial antioxidant assay kit (Cayman chemicals, Cambridge Bioscience, Cambridge, UK). This assay estimates the sum of all antioxidant activity in a sample by quantifying its ability to prevent oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] by metmyoglobin, relative to Trolox (a water-soluble tocopherol analogue). Briefly, cell pellets were resuspended in lysis buffer (5mM potassium phosphate pH 7.4, 0.9% NaCl, 0.1% glucose) and centrifuged. The supernatant was analyzed for antioxidant capacity using 10\( \mu \)L of sample, 10\( \mu \)L metmyoglobin and 150\( \mu \)L chromogen, added to the well of a 96 well plate and all samples were assayed in duplicate. 40\( \mu \)L H\(_2\)O\(_2\) was added to each well and the plate incubated at room temperature for 5 minutes. Absorbance was read at 750 nm. Antioxidant capacity was expressed in Trolox equivalents using a standard curve generated by serial dilution of a standard Trolox solution.
Continuous data were described using mean and 95% confidence intervals. Comparison of continuous data was performed using Student’s t test, paired or unpaired as appropriate. Where continuous data were not normally distributed, non-parametric tests were employed. Proportions were compared using the chi squared test and binomial confidence intervals. Statistical significance was assumed at P<0.05.
Results

Pon3 knockout mice showed no Pon3 mRNA in liver or lung at E17.5 by RT-PCR whereas it was clearly present in heterozygous and wild type littermates (Figure 1A). Uniformity of cDNA synthesis was established by RT-PCR for the housekeeping gene beta-actin. Western blotting also showed no Pon3 protein in either lung or liver from knock out mice but confirmed the presence of Pon3 protein in wild type and heterozygous littermates of the expected size (40 kilodaltons). The membrane was stripped and re-probed for GAPDH, which confirmed equal loading of protein (data not shown). When the offspring of het/het matings were studied there were 4 null mutants out of 43 offspring from 8 litters (9.3%, 95% CI = 2.6 to 22.1%) at E10.5 and 5 null mutants out of 63 offspring from 7 litters (7.9%, 95% CI 2.6-17.6) at E17.5: the proportions were significantly less than the expected 25% at both gestational ages (P<0.001). On the first day of post-natal life (P1), there were 3 null mutants out of 127 offspring from 23 litters (2.4%, 95% CI 0.5 to 6.7%) which was significantly less (P=0.04) than the proportion observed in fetal life. It is likely that dead neonates were ingested by the mothers. After excluding null mutants, 25 out of 39 fetuses at E10.5 were heterozygous (64.1%, 95% CI 47.2-78.8), 33 out of 58 fetuses at E17.5 were heterozygous (56.9%, 95% CI 43.2-69.8) and 81 out of 124 neonates at P1 were heterozygous (65.3%, 95% CI 56.3-73.6). In all cases the 95% confidence intervals included the 66.7% heterozygotes predicted by Mendelian ratio (having excluded null mutants).

The effect of Pon3 heterozygosity on fetal growth was studied by mating 6 heterozygous animals with 6 wild type animals. The placental and body weight of Pon3 heterozygous offspring was expressed as a percentage of the mean body or placental weight of their
The body weight of 25 heterozygous fetuses at E17.5 was 94.1% (95% CI 91.0-97.1, P<0.001) of their wild type littermates (n = 24). The reduction in body weight was similar whether the Pon3 mutant allele was inherited from the mother (96.0%, 95% CI 93.1-98.9, 3 litters of 22 pups, 12 heterozygous) or father (92.3%, 95% CI 86.8-97.8, 3 litters of 27 pups, 13 heterozygous). The placental weight was 85.7% of the mean of the wild type littermates, which was significantly reduced (95% CI 81.2-90.2, P<0.001). The placental weight was slightly smaller when the mutant allele was paternally inherited (81.6%, 95% CI 76.2-87.1 verses 86.7%, 95% CI 81.8-93.3 for maternal inheritance) but this difference was not statistically significant (P>0.05).

We next conducted a series of experiments using a human cell line, A549, derived from human lung adenocarcinoma. Using two different shRNAs we permanently reduced PON3 expression in these cells. Compared with cells expressing a non-targeting shRNA, PON3 transcript levels were reduced by an average of 43% and 55% resulting in a 62% and 55%, reduction, respectively, in PON3 protein level. Both knock-down cell lines demonstrated significantly reduced cell proliferation in 21% oxygen using MTT assay compared with control cells expressing the non-targeting shRNA (61.7% and 58.2% expression, 95% CI 52.4-71.0 and 51.7-64.6 respectively, n=16 for each group, p<0.001). We next studied transient knock down of PON3 using siRNA in 2% oxygen and, after 24 hours, cells were either exposed to 21% oxygen for 48 hours or were maintained in 2% oxygen. At the end of this (72 hours after transfection), total antioxidant capacity and PON3 transcript levels were compared. PON3 transcript levels were reduced significantly in both 2% and 21% oxygen (average reduction 64% and 68%, respectively). PON3 knock down had no effect on total antioxidant capacity in 2% oxygen but was associated with significantly reduced total antioxidant capacity in 21% oxygen (P = 0.0078, Figure 2).
Discussion.

The key finding of the current paper is that absence of PON3 had adverse effects on both mice and in a human cell line. When studying the offspring of matings of animals which were heterozygous for null mutation of the Pon3 gene, we found that the proportion of homozygous null mutants was well below the expected Mendelian ratio at E10.5. There was no further significant decline in the proportion of null mutants between E10.5 and E17.5. However, there was a further significant reduction in the proportion of null mutants on neonatal day 1. On the basis of our previous studies, we had hypothesized that up-regulation of PON3 was an important preparative process for birth and that absence of PON3 may be harmful following birth. The increased rate of neonatal loss is clearly supportive of that hypothesis. However, there was no direct evidence, to our knowledge, that PON3 protected cells from the effects of increased levels of oxygen. In order to address this, we studied a human cell line (A549). First, we demonstrated that stable knock down of PON3 in ambient oxygen was associated with reduced cell proliferation. However, such an effect could be through multiple mechanisms, other than through oxidative stress. Hence, we next sought to determine the effects of absence of PON3 (using siRNA knock down) on total antioxidant capacity in low (2%) and ambient (21%) oxygen. We found that PON3 knock down had no effect on total antioxidant capacity in low oxygen, but resulted in reduced total antioxidant capacity in ambient oxygen. We conclude that Pon3 has a non-redundant biological role both in early murine development and adaptation to extra-uterine life. Moreover, we provide direct evidence that PON3 acts as an antioxidant in the presence of atmospheric oxygen levels in human cells.
The motivation for studying the Pon3 knock out was to determine whether absence of Pon3 was associated with increased neonatal mortality. However, when comparing the number of null mutants in fetal life, we found that the proportion was well below the predicted 25% Mendelian ratio. This was the case at both E10.5 and E17.5 and there was no difference in the number of reabsorbed fetuses at these time points. These observations suggest that there was loss of Pon3 null mutants in the first half of pregnancy and, therefore, that Pon3 must have an important non-redundant role in early development. Further studies will be required to determine the mechanism of these early losses. Previous studies have demonstrated that Pon3 mRNA is present in pre-implantation embryos (see GEO data series GDS578 from Hamatani et al, 2004 (6)) and that Pon3 is expressed in early embryonic life (10). It is possible that increased rates of early loss may be explained by absence of the anti-oxidant effect of PON3. However, in addition to their role as antioxidants, paraoxonases are capable of metabolizing derivatives of important regulatory hormones, have anti-atherogenic and anti-inflammatory properties, can detoxify or degrade xenobiotics and have the potential to alter host/microbe interaction (12). Further studies will be required to establish the effect of Pon3 on early embryonic and placental development.

A previous study reported retained lactonase activity in lysates of tracheal epithelial cells isolated from Pon3 knock-out mice (17). These authors did not comment on any effect of Pon3 null mutation on viability. However, the authors analyzed Pon3 mRNA levels in relation to the Pon3 genotype and found that the apparent null mutants had Pon3 mRNA levels which were 10% of the wild type animals (17). It is also possible that these animals may have increased translation of mRNA to protein resulting in >10% of normal PON3 levels. Using the commercially derived animals, we found that there was no evidence for
Pon3 mRNA or protein in the knock outs and we believe that the greater efficacy of this
knock out may explain the high rates of loss observed in the present study. Moreover, we
had discussions with a second commercial supplier who had an identical knock out to the
one employed in the present study and they commented that the number of null mutants
was severely reduced compared with the expected Mendelian ratio.

An analysis of a number of genes on mouse chromosome 6 demonstrated that Pon3
expression in the placenta and embryo was maternally biased at E10 and that the degree
of maternal bias was greater in the placenta. However, these authors commented that as
the C57Bl6 allele was always expressed more strongly, the maternal/paternal ratios
observed “are not a good indicator of imprinting in this case” (10). If PON3 was strongly
imprinted, we would have expected a reduction in the normal 2:1 ratio of heterozygotes to
wild types following matings of pairs of heterozygous animals. Given the normal ratio of
heterozygotes, we conclude that the gene is not expressed exclusively from the maternal
or paternal allele in the cell or tissue type which determines the early lethality of null
mutants. However, we observed that animals which were heterozygous for the Pon3 null
allele exhibited a phenotype, namely, the body weight and placental weight were
approximately 5-10% lower than wild type littermates at E17.5 and we considered that
this may be due to imprinting. Hence, we studied body and placental weight in mating of
heterozygous mothers and wild-type fathers and wild-type mothers and heterozygous
fathers. Body weight and placental weight were similar among the heterozygotes
irrespective of the parent of origin of the mutant allele, which is not supportive of
imprinting.
Previous evidence that PON3 acts as an antioxidant was somewhat indirect. It had been shown that PON3, but not PON2, protected low density lipoprotein from copper induced oxidation (4). Moreover, a mouse model of oxidative stress had suggested an antioxidant role for PON3. Mice given carbon tetrachloride demonstrate hepatotoxicity due to the conversion of carbon tetrachloride to chloride radicals in the liver (11). Mice expressing circulating recombinant human PON3 demonstrated evidence of reduced liver damage and reduced depletion of other hepatic antioxidant enzymes compared with controls. However, these previous findings used model systems and did not directly address the effect of oxygen. Using A549 cells, which are proposed as a model for type II pneumocytes, (14) we found that knocking down endogenous PON3 had no effect on total antioxidant capacity in the presence of 2% oxygen. However, in the presence of 21% oxygen, PON3 knock down was associated with reduced total antioxidant capacity. The fact that this was only observed in the presence of elevated oxygen tension suggests that the presence of endogenous PON3 prevented the consumption of other antioxidants (such as reduced glutathione), as was observed in the mouse liver exposed to carbon tetrachloride (11). This is the first direct evidence, to our knowledge, that PON3 protects against the effect of increasing partial pressure of oxygen in human cells. Moreover, stable knock down of PON3 reduced proliferation in the same cell line in ambient oxygen levels.

In conclusion, we had observed that up-regulation of PON3 in late fetal life was conserved between the rat, sheep and human. We hypothesized that this was an important preparative process to reduce oxidative stress following the exposure of the infant to the elevated oxygen levels of extra-uterine life. We have now found that Pon3\(^{-/-}\) mice exhibited increased early embryonic and neonatal mortality and PON3 knock down
in a human cell line reduced cell proliferation and total antioxidant capacity in ambient oxygen levels. These data add further support to the proposal that low levels of PON3 may contribute to morbidity and mortality in preterm human infants and that administration of synthetic PON3 may have potential as a means of reducing oxidative stress in neonatal intensive care.

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Disclosures.

GB, SLK & DL have nothing to declare. DSCJ & GCSS are named inventors on a patent application filed by Cambridge Enterprise relating to the use of paraoxonase 3 as a therapeutic product.
Reference List


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Figures captions

Figure 1. A. Pon3 mRNA in relation to Pon3 genotype in offspring of matings of Pon3 heterozygotes. WT denotes wild type, HET denotes heterozygote, KO denotes knock out, Lu denotes lung and Li denotes liver. All bands were of the expected size. B. Pon3 protein expression in E17.5 wildtype (WT), heterozygote (HET) and knockout (KO) mouse lung and liver. The estimated molecular weight of the Pon3 protein was ~40 kilodaltons.

Figure 2. The effect of transient \textit{PON3} knock down in A549 cells on total antioxidant capacity in the presence of A. 2\% or B. 21\% oxygen. In controls, Lipofectamine 2000 was replaced with ddH\textsubscript{2}O. Transfection was performed in 2\% oxygen. 48 hours after transfection, cells were divided between incubators with 2\% or 21\% oxygen. 72 hours after the start of the transfection cells were harvested. Total antioxidant activity of the lysate was quantified by its ability to prevent oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] by metmyoglobin and this was quantified as equivalence to Trolox, a water-soluble tocopherol analogue. \textit{P} by Wilcoxon signed rank test (all groups n=8).
Figure 1.

A

![Pon3 and Beta-actin gel images](image)

B

![Lung and Liver gel images](image)
Figure 2.

A. 2% oxygen

B. 21% oxygen
Figure 1.

A

B
Figure 2.

A. 2% oxygen

B. 21% oxygen

Trolox-equivalents, mM

p = NS

p = 0.0078