Chronic gestational hypoxia accelerates ovarian aging and lowers ovarian reserve in next-generation adult rats

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List of non-standard abbreviations

Alox 12 - Arachidonate 12-lipoxygenase

Alox 15 - Arachidonate 15-lipoxygenase

Chk 1-6 – Checkpoint kinase 1-6

Cuznsod – Copper/zinc superoxide dismutase

Ecsod – Extracellular superoxide dismutase

Gpx1 - Glutathione peroxidase 1

Gp91phox - NADPH oxidase 1

Hif1a - Hypoxia-inducible factor 1-alpha

Hmox1 - Heme Oxygenase 1

Neil1 - Endonuclease VIII-like 1

Nfkb - Nuclear factor kappa-B DNA binding subunit

Nrf2 - Nuclear respiratory factor 2

Nthl1 - Nth Like DNA Glycosylase 1

Nox2 - NADPH oxidase 2

Ogg1 - 8-Oxoguanine DNA Glycosylase

P53 – tumour protein 53

P21 – cyclin-dependent kinase inhibitor 1

P16ink - Cyclin-dependent kinase inhibitor 2A

P22phox - human neutrophil cytochrome b light chain

P47phox - neutrophil cytosolic factor 1

P67phox – neutrophil cytosolic factor 2

Pot1 – Protection of telomeres protein 1

Ppia - Cyclophilin A

Xo – xanthine oxidase

Xrcc1 - X-Ray Repair Cross Complementing 1

Abstract:

Chronic fetal hypoxia is a common complications observed in human pregnancy, impacting pregnancies across global contexts. Exposure to chronic intrauterine hypoxia has major short and long-term consequences for offspring health. However, the impact of chronic gestational hypoxia on female reproductive system development is unknown. We aimed to understand the impact of exposure to chronic fetal hypoxia on the developing female reproductive system. Wistar rat dams underwent normoxia (21%) or hypoxia (13%) during pregnancy. Postnatally, all female offspring were maintained in normoxic conditions into early adulthood. Females rats exposed to chronic gestational hypoxia (13%) during their intrauterine development had decreased ovarian primordial follicular reserve compared to controls ($p<0.05$). Adult females who had been exposed to chronic fetal hypoxia had significantly reduced somatic ovarian telomere length ($p<0.05$), and reduced ovarian protein expression of KU70, a critical component of the DNA-PK repair complex ($p<0.01$). Gene expression of NOX2-mediated oxidative stress markers was increased ($p<0.05$). Exposure to chronic hypoxia during fetal development leads to accelerated ageing of the somatic ovary and decreased ovarian reserve in adulthood. Ovarian ageing is highly sensitive to gestational hypoxia, with implications for future fertility in next-generation offspring of high-risk pregnancies.

Key words: ovary, follicles, reproductive ageing, fetal hypoxia, developmental programming

Introduction

Chronic gestational hypoxia is a common feature of a number of suboptimal intrauterine environments, including placental insufficiency, preeclampsia, maternal smoking and pregnancy at high altitude(1, 2) Exposure to chronic hypoxia during gestation adversely influenced fetal and placental development, and is associated with adverse pregnancy outcomes (2-6). The short term adverse effects of chronic gestational hypoxia include increased risks of late miscarriage, fetal growth restriction, and low birth weight (3-8). Chronic gestational hypoxia also has long-term effects on the physiology of exposed offspring, termed developmental programming. The effects of gestational hypoxia are best characterized in the cardiovascular system, where the impact of low oxygen tension on the developing heart and vasculature has been extensively studied in animal models (1, 3). The consequences of a suboptimal fetal environment on long term reproductive health is an under explored area in the field of developmental programming but an area of huge importance given that the reproductive system is the mediator of information across generations. In particular, the impact of chronic hypoxia on the development of the female reproductive system is unknown.

The developing female reproductive system is particularly vulnerable to the impact of a suboptimal intrauterine environment because of the specific developmental windows during which ovarian reserve is established. Ovarian reserve refers to the total finite number of primordial follicles remaining in both ovaries at any point in life, and is the key determinant of fertility potential in the female (4). Disruptions to the fetal environment during the crucial phase of ovarian follicular endowment result in a decreased ovarian reserve in adult reproductive life (5-9). *In vitro* evidence suggests that the ovarian follicle is particularly sensitive to oxygen tension. Oocyte development within follicles in the adult ovary is markedly influenced by the oxygen content of the follicular fluid (10), with hypoxic follicles containing a higher percentage of oocytes with derangements of chromosomal organization. Therefore, there is a strong rationale to hypothesise that follicular dynamics in the developing follicles in the ovary *in utero* may be highly influenced by exposure to chronic gestational hypoxia. In this study, we investigated whether ovarian reserve in the young adult female is influenced by exposure to chronic hypoxia during gestation and determined underlying mechanisms.

Materials and Methods

All animal experiments were approved by the University of Cambridge Animal Welfare and Ethical Review Board. All animal experiments were conducted in accordance with the British Animals (Scientific Procedures) Act (1986) and were compliant with EU Directive 2010/63/EU.

Study design

Wistar rat dams at 10-12 weeks of age (Charles River Ltd., Margate, UK) were housed in individually ventilated cages (21% oxygen, 70-80 air changes/hour) under standard conditions. All animals were fed a standard laboratory chow diet (20% protein) and fed *ad libitum* with free access to water. After initial acclimatization (10 days) they were mated with fertile male Wistar rats (n=14), and pregnancy confirmed through the observation of a vaginal plug. The day of the plug was designated day 0 of pregnancy (full term 21-22 days). Upon confirmation of pregnancy, dams were weighed and housed individually. On day 6 of pregnancy, dams were randomly divided into two groups; control (21%) and hypoxic (13%) pregnancy (n=8 per group). Pregnant rats assigned to the hypoxia group were placed inside a chamber, which combined a PVC isolator with a nitrogen generator, as previously described (11, 12). Hypoxic pregnancies were maintained at a constant inspired fraction of oxygen of 13% from day 6 to 20 of gestation. This model of hypoxic pregnancy does not decrease maternal food intake (11). All dams delivered under normoxic conditions. There were no complete pregnancy losses in either group during the study. The respective litter sizes were 12.3±1.0 pups in the normoxia group compared to 9.3±1.2 pups in the hypoxia group ($p<0.05$). Gestational length averaged 20±1 days in both normoxic and hypoxic groups. Normoxia (21%) was maintained for all animals during lactation, weaning and thereafter. Following determination of birth weight, litters were culled to 4 males and 4 females to standardise nutritional access and maternal care during suckling (11, 12). All pups were suckled by their own mothers. At four months of age, adult female pups underwent euthansia. At *post mortem*, the reproductive tract tissues were harvested and weighed fresh, immediately after dissection. One ovary from each animal was snap-frozen in liquid nitrogen and the other fixed in formalin/paraldehyde. The fixed ovaries were sectioned and subjected to haematoxylin and eosin (H&E). An equal distribution of estrous cycle stages in each group was confirmed using the serial sections of whole H&E stained ovary prepared for primordial follicle counting. However, the study was not powered for comparisons between estrous

cycle stages and thus parameters were selected to be non-varying with cycle stage. Sample analysis was performed using project codes to blind the investigators to the experimental groups. The adequacy of the sample size was determined via a power calculation based on the effect sizes for ovarian primordial follicle counts in Wistar rats reported in our previous studies (6, 13) using an alpha level of 0.05 to give power of 0.8.

Primordial follicle counts

Primordial follicle counts were performed as described previously (6, 13). Fixed ovaries were processed for microscopy and the entire ovary sectioned at 8μm. Every 9^{th} section was stained with H&E for morphometric analysis (72μm between analysed sections). Only follicles with a visible oocyte nucleus were counted, in order to avoid repeat counts of the same follicle (14). Primordial follicles were identified morphologically by the presence of a single layer of flattened granulosa cells surrounding the oocyte (15) (Supplementary figure 1). The total volume of each ovary was derived (section areas x section thickness x number of sections) and the follicle count normalized to ovarian mass, as follicles/mm^3 of ovarian tissue.

Telomere length analysis

High-molecular weight DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quantity and purity was determined using a Nanodrop spectrophotometer (Nanodrop Technologies). Agarose gels were run to ensure all DNA samples were of high-molecular weight. DNA (1.2μg) was digested with *Hinf*I and *Rsa*I restriction enzymes for 2h at 37^{o}C. The restricted samples were quenched with 5x SDS loading buffer (Roche Diagnostics, Mannheim, Germany) and loaded onto agarose gels containing SYBR safe stain (Invitrogen, Paisley, Scotland, UK). After pulsed field gel electrophoresis (PFGE), the gels were checked for non-specific degradation of an undigested DNA control and complete digestion of the enzyme-restricted DNA by visualizing the stained gels under UV light (Syngene, Cambridge, UK). The separated DNA fragments were transferred to nylon membrane (Roche Diagnostics, Mannheim, Germany) by Southern blotting, and telomeric repeat length was determined using a commercial method of chemiluminescent detection as described previously (16). Molecular weight markers on each gel were a mid-range pulsed-field gel marker (New England Biolabs, Ipswich, MA, USA) and dioxygenin (DIG; low range) molecular-weight marker (Roche Diagnostics, Mannheim, Germany). Standard digested genomic samples of DNA from a 4-month control animal were

also included on each gel to verify digestion efficiency. Telomere signals were analyzed using Adobe Photoshop (Adobe Systems Inc. San Jose, CA, USA) and Alpha-Ease software (Alpha Innotech, San Leandro, CA, USA). Telomere length was measured as described previously (16).

Gene expression analysis

An initial panel of 32 candidate genes was developed to test which molecular pathways might be altered in the somatic ovary following exposure to chronic gestational hypoxia. These genes were chosen based on (i) previous work on the effects of developmental programming on ovarian, para-ovarian adipose tissue, and oviductal gene expression (5, 17, 18), (ii) knowledge of programming mechanisms in other organ systems in the same gestational hypoxia rat model (11, 19, 20), and (iii) relevant literature review. RNA was extracted from snap-frozen ovaries using a miRNeasy mini kit (Qiagen, Hilden, Germany) following manufacturers' instructions, with the addition of a DNaseI digestion step to ensure no genomic DNA contamination. RNA quantification was performed using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). For the RT-PCR process, RNA (1 μg) was reverse transcribed to cDNA using oligo-dT primers and M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA). Gene expression was determined using custom designed primers which were designed within one exon (Table 1; Sigma, Poole, Dorset, UK) and SYBR Green reagents (Applied Biosystems, Warrington, UK) as previously described (21). Quantification of gene expression was performed using a Step One Plus RT-PCR machine (Applied Biosystems, Warrington, UK). All cDNA samples were run against a gDNA standard curve of known concentrations using a commercially-available rat genomic DNA standard (Sigma, Poole, Dorset, UK) in order to express gene expression as average copy number. Equal efficiency of the reverse transcription of RNA from all groups was confirmed through quantification of expression of the house-keeping gene *ppia*, the expression of which did not differ between groups.

Protein quantification

Protein was extracted from whole tissue lysates of snap-frozen ovaries, as described previously (18, 22). To ensure equal protein loading, protein assays were performed on all samples to ensure that each sample was diluted to the same protein concentration (1mg). Protein (20μg) was loaded onto 10%, 12% or 15% polyacrylamide gels, dependent upon the molecular weight of the protein to be measured. The samples were electrophoresed and

transferred to polyvinylidene fluoride membranes. Detection steps used the following primary antibodies; OGG1 (Novus Biologicals; cat no: NB100-106,1:1000), NTH1 (Proteintech; cat no: 11154-1-AP, 1:1000), HIF1α (Abcam: cat no: Ab51608, 1:1000), Catalase (Abcam, Cambridge, UK, cat. no.: Ab1877-10, 1:10000), MnSOD (Upstate, Watford, UK; cat. no.: 06-984), CuZnSOD (ProteinTech, Cambridge, UK; cat. no.: 10269-1-AP, 1:1000), $GP91^{phox}$ (ProteinTech, Cambridge, UK; cat no: 19013-1-AP), $P67^{phox}$ (ProteinTech, Cambridge, UK; cat. no: 15551-1-AP, 1:1000), XO (Santa-Cruz, Wimbledon, Middlesex, UK; cat. no: SC-20991, 1:200), P53 (R & D Systems; cat no: MAB1355, 1:1000), $P16^{INK}$ (Abcam, Cambridge, UK; cat no: Ab189034, 1:1000), KU70 (ProteinTech, Cambridge, UK, cat no: 10723-1-AP, 1:1000), KU80 (Novus, cat no: NB100-508, 1:1000). Anti-rabbit secondary antibodies (Cell Signaling Technology, Danvers, MA, USA, 1:2000) were utilised for all primary antibodies except P53, which required an anti-mouse secondary antibody (Cell Signaling Technology (Danvers, MA, USA), 1:2000) (Supplementary figure 2). Equal protein loading was confirmed by staining electrophoresed gels with Coomassie Blue (Bio-Rad, Hemel Hempstead, Herts, UK) to visualize total protein. This methodology was selected to avoid the use of house-keeping proteins that may be vulnerable to expression differences following developmental hypoxia-exposure (23, 24). To ensure that the chemiluminescent signal changed in a linear manner, the ratio between loading controls (100% and 50% pooled sample) was confirmed for each detected protein.

Statistical Analysis

Maternal hypoxia effects were compared between groups using 2-tailed Student's T tests. In order to correct for multiple hypothesis testing of gene expression levels, p values were transformed to q values to take account of the false discovery rates using the p.adjust function in R stats package (R Foundation for Statistical Computing, Vienna, Austria). This adjustment was designed for this study in order to take account of the specific number of genes that were tested within the initial screen (32) and therefore to ensure that the p values were optimally transformed. Data are represented as means ± SEM. Where p values are reported, an alpha level <0.05 was considered statistically significant. All data analysis was conducted using the R statistical software package version 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria). Only ovaries of one female offspring per litter were used for analysis to account for within litter variation. Therefore, in all cases, n refers to the number of litters, and n=8 was used for all groups.

Results

There was no significant difference in the body weight of female rats at 4 months of age exposed to gestational hypoxia compared to those that experienced normoxia, however there was a trend towards a slightly lower body weight in the hypoxia group ($p=0.06$; Table 2). Ovarian weight was not significantly different between the groups, whether expressed as absolute organ weight or normalized to body weight (Table 2).

Primordial follicle counts per cubic millimeter of ovarian tissue at 4 months of age were significantly lower in the gestational hypoxia than in the gestational normoxia group ($p<0.01$; Figure 1). Absolute follicle counts were also lower in the gestational hypoxia group than the normoxic group (131.0±12.4 *v.*183.7±20.6 follicles per ovary, $p<0.05$).

At 4 months of age, there were significantly fewer very long (145-48.5kB, $p<0.05$) and long (48.5-8.6kB, $p<0.05$) telomeres in the somatic ovarian tissue of gestational hypoxia-exposed animals compared to the normoxic group (Figure 2). Conversely, there was a higher proportion of very short telomeres in the hypoxia exposed group animals (1.1-4.2kB, $p<0.05$), strongly suggesting that telomere length maintenance is impaired in the somatic ovary following the developmental challenge of hypoxia *in utero*.

One possible mechanism of accelerated telomere shortening is impaired recognition of DNA damage. Accordingly, we measured the gene expression levels of a range of DNA-damage sensing and repair proteins in the hypoxia-exposed animals. Expression of *Ogg1* ($p<0.05$) and *Neil1* ($p<0.05$) was elevated in the hypoxia-exposed group compared to the normoxic group (Figure 3), which is in keeping with an increased burden of DNA damage in the hypoxia-exposed group. There was no difference in gene expression of either *Nthl1* or *Xrcc1* in either group (Figure 3). At the protein level, NTH1 was increased in the hypoxia-exposed group compared to the controls ($p<0.01$), but there was no difference in the protein level of OGG1 (Table 3).

Gene expression levels of the key functional subunit components of DNA-PK, *Ku70 and Ku80*, primarily responsible for repairing double-stranded DNA breaks and hence playing a role in maintaining telomere length, did not vary significantly between the hypoxia and normoxia-exposed groups (Figure 4A). However, at the protein level, there was a highly significant reduction in KU70 in the animals exposed to gestational hypoxia ($p<0.001$), with

no difference between groups in KU80 levels (Figure 4B). Inability to repair double-stranded DNA breaks, despite adequate detection, is consistent with the accelerated telomere shortening observed in the somatic ovarian tissue of the group exposed to chronic gestational hypoxia. There were no differences between the hypoxic and normoxic groups in the gene expression of any other DNA damage-sensing or protection mechanisms that were assayed (*Pot1, Chk1, Chk2, Cdk4, Cdk6*; Table 4).

Gene expression levels of *p53* were significantly higher in the somatic ovarian tissue of gestational hypoxia-exposed animals, than in normoxic controls ($p<0.01$; Table 4). There were trends towards a similar increase in levels of *p21* and *p16ink*, but these were not significant after correction for multiple hypothesis testing (Table 4). At the protein level there was a significant increase in both P53 ($p<0.001$) and $P16^{ink}$ ($p<0.05$) in the hypoxia-exposed group compared to the normoxia-exposed group (Figure 5).

There was a significantly higher gene expression of *Hif1α* in the somatic ovary following exposure to chronic gestational hypoxia than in the normoxic control group ($p<0.05$, Table 4), however there was no difference in expression levels of *Nfkβ* (Table 4). Various oxidative stress markers were included in the initial gene expression screen (Table 3). There was a specific up-regulation of oxidative stress markers $Gp91^{phox}$ ($p<0.05$) and $P22^{phox}$ ($p<0.05$) in the hypoxia-exposed group at the gene expression level, but other markers (*Xo,* $P47^{phox}$, $P67^{phox}$, *Nrf2, Hmox1, Gpx1*) were not significantly different between experimental groups (Table 4). At the protein level, there was an increase in $GP91^{phox}$ expression in the hypoxia-exposed group but this did not reach statistical significance ($p=0.08$; Table 4). There was no difference between groups at the protein level in expression of XO, $P67^{phox}$, or HIF1α (Table 3).

In keeping with increased levels of oxidative stress in the somatic ovary in the hypoxia-exposed group, there was also a significantly higher gene expression of the cytoplasmic anti-oxidant *CuZnsod* ($p<0.001$, Table 4). There was no difference in the gene expression levels of several other anti-oxidants included in the initial screen (*Mnsod, Ecsod, Catalase;* Table 4) between the gestational hypoxia and normoxia-exposed groups. There were no differences in anti-oxidant protein expression between the hypoxia- and normoxia-exposed groups, except for Catalase, which was decreased in the hypoxia exposed group ($p<0.05$; Table 3). Gene expression levels of markers of lipid peroxidation included in the initial screen (*Alox12,*

316 *Alox15;* Table 4) were unchanged between the gestational hypoxia and normoxia-exposed
317 groups.
318
319 **Discussion**
320 Hypoxia during fetal life is the final common pathway of a number of important pregnancy
321 complications (1-3). Chronic gestational hypoxia may arise from maternal hypoxaemia, for
322 example during pregnancy at high altitude (25), or from maternal smoking (26) or from
323 insufficiency of utero-placental blood flow (27). A hypoxic intrauterine environment may
324 also result from failure of conversion of the spiral arteries during early placental development
325 (28). Failure to adapt the uterine blood flow to the demands of pregnancy also promotes
326 chronic fetal hypoxia which is associated with pregnancy complications including pregnancy
327 loss, fetal growth restriction, and increased risk of pre-eclampsia (29, 30), Taken together, the
328 various aetiologies leading to chronic gestational hypoxia affect a large number of human
329 pregnancies globally (3), including 389 million people who live at altitudes greater than
330 1500m and at least 70 million who live above 2500m (31, 32). The immediate adverse
331 impacts of chronic gestational hypoxia, including the high risk of fetal loss (33) and low birth
332 weight (34) are well established. Many aspects of longer-term health in survivors of a
333 hypoxic intrauterine environment, including adverse cardiovascular (11) and metabolic (12)
334 impacts have also been characterized. However, our study provides a conceptual advance
335 presenting important new evidence of a significant impact on reproductive potential through
336 accelerated cellular ageing in adult female offspring of hypoxic pregnancy.
337
338 We show that chronic gestational hypoxia leads to decreased ovarian reserve in female
339 offspring in early adulthood. Ovarian reserve is a key determinant of female fecundity and
340 hence a reduction in the number of primordial follicles available in early adulthood is highly
341 likely to be associated with an early decline in fertility (35). Fecundity in later life relates to
342 both oocyte quality and quantity, however there is, as yet, no well-established method of
343 reliably predicting oocyte quality (36), hence reliance on occyte quantity. Our results suggest
344 that accelerated ageing in the somatic ovary in response to early-life hypoxia may be the
345 result of a post-transcriptional reduction in expression of a component of the DNA-PK
346 complex, which in turn prevents telomere maintenance and leaves ovarian follicular cells
347 vulnerable to accumulating age-associated damage. It is thus highly plausible that accelerated
348 reproductive ageing is a key mechanism by which ovarian reserve in the next generation
349 female offspring is reduced following exposure to chronic gestational hypoxia.

Understanding the developmental basis of accelerated reproductive ageing is particularly important in light of global trends towards increasing maternal age.

Physiological early embryonic and fetal development proceeds in a low oxygen tension environment, with high levels of antioxidants, in order to protect the conceptus from potential oxidative damage during organogenesis (37, 38). However, after the establishment of the placental circulation, at the end of the first trimester in human pregnancy, oxygen tension rises dramatically (39). Failure of the oxygen tension to rise sufficiently during this developmental phase, whether as a result of limited utero-placental flow (40) or, as in our model and at altitude, low ambient oxygen levels, results in an increase in placental oxidative stress (41, 42). This early accumulation of oxidative stress has important consequences for the development of the fetal heart (43) and potentially other organ systems (3, 43, 44). Of particular note, endowment of the ovarian follicular reserve occurs concomitantly with the physiological rise in oxygen tension at around 12 weeks in human pregnancy (39). In the fetal ovary at this stage, the primordial germ cells have completed migration to the genital ridge and enter meiosis, irrevocably setting the maximum potential number of oocytes and commencing the oxygen-sensitive process of follicular development (45). Hence, there is rationale to consider whether the decreased ovarian reserve that we observe in adulthood may be a consequence of failure to experience the expected increase in oxygen tension during this crucial period of early development.

In keeping with the findings of this study, previous work has demonstrated a similar phenotype of early renal ageing in response to developmental hypoxia (46). However, previous work exploring the developmental response to hypoxia in the developing cardiovascular system does not show a direct accelerated ageing effect (47). Thus, the long-term impacts of developmental hypoxia on the female reproductive tract are likely to represent a tissue-specific effect, rather than a ubiquitous response to a developmental stressor.

Numerous studies in human populations (35, 48) have established the link between reduced ovarian reserve and female reproductive potential. Evidence from >15,000 healthy women across cultural contexts in Latin America suggests that high altitude (>2000m, hypobaric hypoxia) is associated with earlier age at menopause (49). Smaller studies from Peru and Nepal also suggest a shorter reproductive lifespan in high altitude populations (50, 51). At a

population level, observational studies in humans (35, 52) suggest that ovarian reserve reflects age at menopause, which is the best available proxy in women for the point at which unassisted conception becomes highly unlikely. Hence, our finding may translate into an important functional deficit in fertility, particularly in the older mother, following exposure to a suboptimal intrauterine environment. This is particular relevant in many populations where age at first pregnancy is progressively increasing. A key advantage of the model used in this study (13% oxygen) is that it closely reflects the oxygenation during human pregnancy at altitude. At altitudes of 3000-3500m above sea level, maternal arterial oxygen tension can fall to around 60% of the value at sea level (95mmHg at sea level v. 50mmHg (25)). The severity of the hypoxia used in our study is approximately equivalent to women experiencing pregnancy in the city of La Paz in Bolivia (3600m - 4150m), where ~40,000 women give birth annually (53). When considering high altitude populations, it is important to consider population mobility and thus the impact not only of prenatal hypoxia, but also the postnatal environment on ovarian reserve into adulthood. This is an important area for future study.

As immediate survival of high-risk pregnancies improves (54, 55), it becomes increasingly important to understand the multitude of ways in which the health of survivors of adverse intrauterine environments may be affected in the longer term (56-58). Our study provides important novel evidence that fertility issues may also be among these programmed complications. Advances in assisted reproductive technologies mean that fertility problems are now often amenable to treatment, but this is much more likely to be successful if high-risk groups can be identified early in reproductive life (59). The finding that chronic fetal hypoxia results in decreased ovarian reserve in adulthood is therefore an important conceptual advance in understanding which future potential mothers are at high risk of experiencing fertility problems in later life. Moreover, our results provide mechanistic insight into how hypoxia-induced low ovarian reserve is associated with a specific defect in DNA repair and telomere maintenance in the somatic ovarian tissue. Insight into such molecular pathways is the first step towards developing effective interventions to protect ovarian reserve in the female offspring of high-risk pregnancy.

Author contributions:

C. Aiken, D. Giussani and S. Ozanne designed the research. J. Tarry-Adkins, T. Ashmore

A. Spiroski, A. Nuzzo, T. Ashmore, A. Rolfo, M. Sutherland, and E. Camm performed the research. C. Aiken analysed the data. C. Aiken, D. Giussani and S. Ozanne drafted the paper. All authors edited and approved the paper.

References:

1. Giussani, D. A. (2016) The fetal brain sparing response to hypoxia: physiological mechanisms. J Physiol 594, 1215-1230
2. Ducsay, C. A., Goyal, R., Pearce, W. J., Wilson, S., Hu, X. Q., and Zhang, L. (2018) Gestational Hypoxia and Developmental Plasticity. Physiol Rev 98, 1241-1334
3. Giussani, D. A., and Davidge, S. T. (2013) Developmental programming of cardiovascular disease by prenatal hypoxia. J Dev Orig Health Dis 4, 328-337
4. Richardson, S. J., and Nelson, J. F. (1990) Follicular depletion during the menopausal transition. Ann N Y Acad Sci 592, 13-20; discussion 44-51
5. Aiken, C. E., Tarry-Adkins, J. L., Penfold, N. C., Dearden, L., and Ozanne, S. E. (2016) Decreased ovarian reserve, dysregulation of mitochondrial biogenesis, and increased lipid peroxidation in female mouse offspring exposed to an obesogenic maternal diet. FASEB J 30, 1548-1556
6. Aiken, C. E., Tarry-Adkins, J. L., and Ozanne, S. E. (2013) Suboptimal nutrition in utero causes DNA damage and accelerated aging of the female reproductive tract. FASEB J 27, 3959-3965
7. Ho, S. M., Cheong, A., Adgent, M. A., Veevers, J., Suen, A. A., Tam, N. N. C., Leung, Y. K., Jefferson, W. N., and Williams, C. J. (2017) Environmental factors, epigenetics, and developmental origin of reproductive disorders. Reprod Toxicol 68, 85-104
8. Chan, K. A., Bernal, A. B., Vickers, M. H., Gohir, W., Petrik, J. J., and Sloboda, D. M. (2015) Early life exposure to undernutrition induces ER stress, apoptosis, and reduced vascularization in ovaries of adult rat offspring. Biol Reprod 92, 110
9. Winship, A. L., Gazzard, S. E., Cullen McEwen, L. A., Bertram, J. F., and Hutt, K. J. (2018) Maternal low protein diet programmes low ovarian reserve in offspring. Reproduction
10. Van Blerkom, J., Antczak, M., and Schrader, R. (1997) The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum Reprod 12, 1047-1055
11. Giussani, D. A., Camm, E. J., Niu, Y., Richter, H. G., Blanco, C. E., Gottschalk, R., Blake, E. Z., Horder, K. A., Thakor, A. S., Hansell, J. A., Kane, A. D., Wooding, F. B., Cross, C. M., and Herrera, E. A. (2012) Developmental programming of cardiovascular dysfunction by prenatal hypoxia and oxidative stress. PLoS One 7, e31017
12. Camm, E. J., Martin-Gronert, M. S., Wright, N. L., Hansell, J. A., Ozanne, S. E., and Giussani, D. A. (2011) Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring. FASEB J 25, 420-427
13. Aiken, C. E., and Ozanne, S. E. (2014) Transgenerational developmental programming. Hum Reprod Update 20, 63-75
14. Bernal, A. B., Vickers, M. H., Hampton, M. B., Poynton, R. A., and Sloboda, D. M. (2010) Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. PLoS One 5, e15558

15. Picut, C. A., Remick, A. K., Asakawa, M. G., Simons, M. L., and Parker, G. A. (2014) Histologic features of prepubertal and pubertal reproductive development in female Sprague-Dawley rats. Toxicol Pathol 42, 403-413
16. Tarry-Adkins, J. L., Ozanne, S. E., Norden, A., Cherif, H., and Hales, C. N. (2006) Lower antioxidant capacity and elevated p53 and p21 may be a link between gender disparity in renal telomere shortening, albuminuria, and longevity. Am J Physiol Renal Physiol 290, F509-516
17. Aiken, C. E., Tarry-Adkins, J. L., and Ozanne, S. E. (2015) Transgenerational Developmental Programming of Ovarian Reserve. Sci Rep 5, 16175
18. Tarry-Adkins, J. L., Aiken, C. E., Ashmore, T. J., and Ozanne, S. E. (2018) Insulin-signalling dysregulation and inflammation is programmed trans-generationally in a female rat model of poor maternal nutrition. Sci Rep 8, 4014
19. Camm, E. J., Hansell, J. A., Kane, A. D., Herrera, E. A., Lewis, C., Wong, S., Morrell, N. W., and Giussani, D. A. (2010) Partial contributions of developmental hypoxia and undernutrition to prenatal alterations in somatic growth and cardiovascular structure and function. Am J Obstet Gynecol 203, 495 e424-434
20. Herrera, E. A., Camm, E. J., Cross, C. M., Mullender, J. L., Wooding, F. B., and Giussani, D. A. (2012) Morphological and functional alterations in the aorta of the chronically hypoxic fetal rat. J Vasc Res 49, 50-58
21. Tarry-Adkins, J. L., Chen, J. H., Smith, N. S., Jones, R. H., Cherif, H., and Ozanne, S. E. (2009) Poor maternal nutrition followed by accelerated postnatal growth leads to telomere shortening and increased markers of cell senescence in rat islets. FASEB J 23, 1521-1528
22. Tarry-Adkins, J. L., Fernandez-Twinn, D. S., Madsen, R., Chen, J. H., Carpenter, A., Hargreaves, I. P., McConnell, J. M., and Ozanne, S. E. (2015) Coenzyme Q10 Prevents Insulin Signaling Dysregulation and Inflammation Prior to Development of Insulin Resistance in Male Offspring of a Rat Model of Poor Maternal Nutrition and Accelerated Postnatal Growth. Endocrinology 156, 3528-3537
23. Yamaji, R., Fujita, K., Takahashi, S., Yoneda, H., Nagao, K., Masuda, W., Naito, M., Tsuruo, T., Miyatake, K., Inui, H., and Nakano, Y. (2003) Hypoxia up-regulates glyceraldehyde-3-phosphate dehydrogenase in mouse brain capillary endothelial cells: involvement of Na+/Ca2+ exchanger. Biochim Biophys Acta 1593, 269-276
24. Staudacher, J. J., Naarmann-de Vries, I. S., Ujvari, S. J., Klinger, B., Kasim, M., Benko, E., Ostareck-Lederer, A., Ostareck, D. H., Bondke Persson, A., Lorenzen, S., Meier, J. C., Bluthgen, N., Persson, P. B., Henrion-Caude, A., Mrowka, R., and Fahling, M. (2015) Hypoxia-induced gene expression results from selective mRNA partitioning to the endoplasmic reticulum. Nucleic Acids Res 43, 3219-3236
25. Postigo, L., Heredia, G., Illsley, N. P., Torricos, T., Dolan, C., Echalar, L., Tellez, W., Maldonado, I., Brimacombe, M., Balanza, E., Vargas, E., and Zamudio, S. (2009) Where the O2 goes to: preservation of human fetal oxygen delivery and consumption at high altitude. J Physiol 587, 693-708
26. Longo, L. D. (1976) Carbon monoxide: effects on oxygenation of the fetus in utero. Science 194, 523-525
27. Kuzmina, I. Y., Hubina-Vakulik, G. I., and Burton, G. J. (2005) Placental morphometry and Doppler flow velocimetry in cases of chronic human fetal hypoxia. Eur J Obstet Gynecol Reprod Biol 120, 139-145
28. Parks, W. T. (2017) Manifestations of Hypoxia in the Second and Third Trimester Placenta. Birth Defects Res 109, 1345-1357

29. Lyall, F., Robson, S. C., and Bulmer, J. N. (2013) Spiral artery remodeling and trophoblast invasion in preeclampsia and fetal growth restriction: relationship to clinical outcome. Hypertension 62, 1046-1054
30. Ball, E., Bulmer, J. N., Ayis, S., Lyall, F., and Robson, S. C. (2006) Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. J Pathol 208, 535-542
31. Cohen, J. E., and Small, C. (1998) Hypsographic demography: the distribution of human population by altitude. Proc Natl Acad Sci U S A 95, 14009-14014
32. Moore, L. G., Charles, S. M., and Julian, C. G. (2011) Humans at high altitude: hypoxia and fetal growth. Respir Physiol Neurobiol 178, 181-190
33. Keyes, L. E., Armaza, J. F., Niermeyer, S., Vargas, E., Young, D. A., and Moore, L. G. (2003) Intrauterine growth restriction, preeclampsia, and intrauterine mortality at high altitude in Bolivia. Pediatr Res 54, 20-25
34. Giussani, D. A., Phillips, P. S., Anstee, S., and Barker, D. J. (2001) Effects of altitude versus economic status on birth weight and body shape at birth. Pediatr Res 49, 490-494
35. Depmann, M., Faddy, M. J., van der Schouw, Y. T., Peeters, P. H., Broer, S. L., Kelsey, T. W., Nelson, S. M., and Broekmans, F. J. (2015) The Relationship Between Variation in Size of the Primordial Follicle Pool and Age at Natural Menopause. J Clin Endocrinol Metab 100, E845-851
36. Hoshino, Y. (2018) Updating the markers for oocyte quality evaluation: intracellular temperature as a new index. Reprod Med Biol 17, 434-441
37. Jauniaux, E., Gulbis, B., and Burton, G. J. (2003) Physiological implications of the materno-fetal oxygen gradient in human early pregnancy. Reprod Biomed Online 7, 250-253
38. Jauniaux, E., Gulbis, B., and Burton, G. J. (2003) The human first trimester gestational sac limits rather than facilitates oxygen transfer to the foetus--a review. Placenta 24 Suppl A, S86-93
39. Jauniaux, E., Watson, A. L., Hempstock, J., Bao, Y. P., Skepper, J. N., and Burton, G. J. (2000) Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol 157, 2111-2122
40. Wang, Y., and Walsh, S. W. (1998) Placental mitochondria as a source of oxidative stress in pre-eclampsia. Placenta 19, 581-586
41. Tissot van Patot, M. C., Murray, A. J., Beckey, V., Cindrova-Davies, T., Johns, J., Zwerdlinger, L., Jauniaux, E., Burton, G. J., and Serkova, N. J. (2010) Human placental metabolic adaptation to chronic hypoxia, high altitude: hypoxic preconditioning. Am J Physiol Regul Integr Comp Physiol 298, R166-172
42. Richter, H. G., Camm, E. J., Modi, B. N., Naeem, F., Cross, C. M., Cindrova-Davies, T., Spasic-Boskovic, O., Dunster, C., Mudway, I. S., Kelly, F. J., Burton, G. J., Poston, L., and Giussani, D. A. (2012) Ascorbate prevents placental oxidative stress and enhances birth weight in hypoxic pregnancy in rats. J Physiol 590, 1377-1387
43. Burton, G. J., and Jauniaux, E. (2018) Development of the Human Placenta and Fetal Heart: Synergic or Independent? Front Physiol 9, 373
44. Zhang, L. (2005) Prenatal hypoxia and cardiac programming. J Soc Gynecol Investig 12, 2-13
45. Gondos, B., Westergaard, L., and Byskov, A. G. (1986) Initiation of oogenesis in the human fetal ovary: ultrastructural and squash preparation study. Am J Obstet Gynecol 155, 189-195

46. Gonzalez-Rodriguez, P., Jr., Tong, W., Xue, Q., Li, Y., Hu, S., and Zhang, L. (2013) Fetal hypoxia results in programming of aberrant angiotensin ii receptor expression patterns and kidney development. Int J Med Sci 10, 532-538
47. Allison, B. J., Kaandorp, J. J., Kane, A. D., Camm, E. J., Lusby, C., Cross, C. M., Nevin-Dolan, R., Thakor, A. S., Derks, J. B., Tarry-Adkins, J. L., Ozanne, S. E., and Giussani, D. A. (2016) Divergence of mechanistic pathways mediating cardiovascular aging and developmental programming of cardiovascular disease. FASEB J 30, 1968-1975
48. Pelosi, E., Simonsick, E., Forabosco, A., Garcia-Ortiz, J. E., and Schlessinger, D. (2015) Dynamics of the ovarian reserve and impact of genetic and epidemiological factors on age of menopause. Biol Reprod 92, 130
49. Castelo-Branco, C., Blumel, J. E., Chedraui, P., Calle, A., Bocanera, R., Depiano, E., Figueroa-Casas, P., Gonzalez, C., Martino, M., Royer, M., Zuniga, C., Dulon, A., Espinoza, M. T., Futchner, C., Mostajo, D., Soto, E., Albernaz, M. A., Aravena, H., Busquets, M., Campodonico, I., Germain, A., Alba, A., Baron, G., Gomez, G., Monterrosa, A., Onatra, W., Broutin, G., Manzano, B., Gabriela, A., Hidalgo, L., Leon, P., Orbea, M., Sanchez, H., Vallejo, S., Vallecillo, G., Hernandez-Bueno, J., Motta, E., Andrade, R., Tserotas, K., Gonzalez, M. C., Benitez, Z., Calle, E., Danckers, L., Del Castillo, A., Izaguirre, H., Ojeda, E., Rojas, J., Bencosme, A., Lima, S., Motta, E., and Figueroa-Casas, P. (2006) Age at menopause in Latin America. Menopause 13, 706-712
50. Gonzales, G. F., and Villena, A. (1996) Body mass index and age at menarche in Peruvian children living at high altitude and at sea level. Hum Biol 68, 265-275
51. Beall, C. M. (1983) Ages at menopause and menarche in a high-altitude Himalayan population. Ann Hum Biol 10, 365-370
52. Wallace, W. H., and Kelsey, T. W. (2010) Human ovarian reserve from conception to the menopause. PLoS One 5, e8772
53. Roost, M., Altamirano, V. C., Liljestrand, J., and Essen, B. (2009) Priorities in emergency obstetric care in Bolivia--maternal mortality and near-miss morbidity in metropolitan La Paz. BJOG 116, 1210-1217
54. Santhakumaran, S., Statnikov, Y., Gray, D., Battersby, C., Ashby, D., Modi, N., and Medicines for Neonates Investigator, G. (2018) Survival of very preterm infants admitted to neonatal care in England 2008-2014: time trends and regional variation. Arch Dis Child Fetal Neonatal Ed 103, F208-F215
55. Ganzevoort, W., Mensing Van Charante, N., Thilaganathan, B., Prefumo, F., Arabin, B., Bilardo, C. M., Brezinka, C., Derks, J. B., Diemert, A., Duvekot, J. J., Ferrazzi, E., Frusca, T., Hecher, K., Marlow, N., Martinelli, P., Ostermayer, E., Papageorghiou, A. T., Schlembach, D., Schneider, K. T. M., Todros, T., Valcamonico, A., Visser, G. H. A., Van Wassenaer-Leemhuis, A., Lees, C. C., Wolf, H., and Group, T. (2017) How to monitor pregnancies complicated by fetal growth restriction and delivery before 32 weeks: post-hoc analysis of TRUFFLE study. Ultrasound Obstet Gynecol 49, 769-777
56. Lawn, J. E., Blencowe, H., Oza, S., You, D., Lee, A. C., Waiswa, P., Lalli, M., Bhutta, Z., Barros, A. J., Christian, P., Mathers, C., Cousens, S. N., and Lancet Every Newborn Study, G. (2014) Every Newborn: progress, priorities, and potential beyond survival. Lancet 384, 189-205
57. Salam, R. A., Das, J. K., and Bhutta, Z. A. (2014) Impact of intrauterine growth restriction on long-term health. Curr Opin Clin Nutr Metab Care 17, 249-254

58. Van Wassenaer-Leemhuis, A. G., Marlow, N., Lees, C., Wolf, H., and investigators, T. (2017) The association of neonatal morbidity with long-term neurological outcome in infants who were growth restricted and preterm at birth: secondary analyses from TRUFFLE (Trial of Randomized Umbilical and Fetal Flow in Europe). BJOG 124, 1072-1078
59. Alviggi, C., Humaidan, P., Howles, C. M., Tredway, D., and Hillier, S. G. (2009) Biological versus chronological ovarian age: implications for assisted reproductive technology. Reprod Biol Endocrinol 7, 101

Table and figure legends:

Figure 1: Primordial follicular reserve in adult female rats exposed to gestational hypoxia compared to normoxia. Box plots: median ± upper and lower quartiles, whiskers: maximum and minimum values. Open bars: normoxia (21% oxygen) during gestation, grey bars: hypoxia (13% oxygen) during gestation. Primordial follicle count is shown normalized to mm^3 of ovarian tissue. **p<0.01.

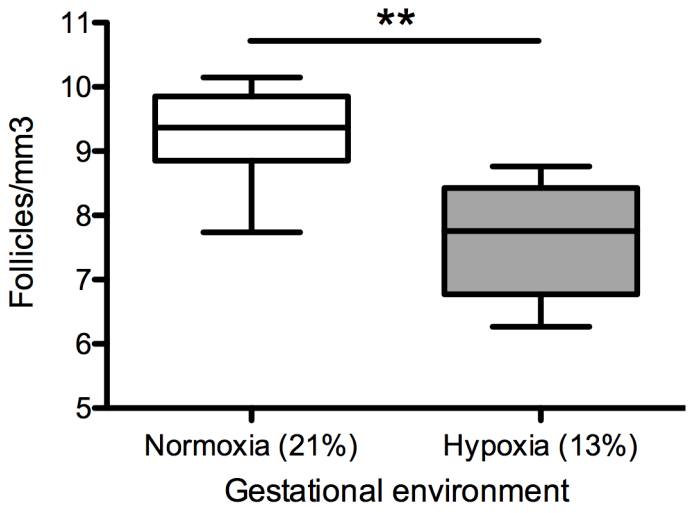
Figure 2 Ovarian telomere length in adult female rats exposed to gestational hypoxia compared to normoxia. Data shown as mean ± SEM. Open bars: normoxia (21% oxygen) during gestation, grey bars: hypoxia (13% oxygen) during gestation. *p<0.05.

Figure 3 Effect of gestational hypoxia compared to normoxia on DNA damage sensing gene expression in the ovary of adult female rats. Data shown as mean ± SEM. Open bars: normoxia (21% oxygen) during gestation, grey bars: hypoxia (13% oxygen) during gestation. *p<0.05.

Figure 4 Effect of gestational hypoxia compared to normoxia on expression of components of the DNA-activated protein kinase (DNA-PK) in the ovary. Data shown as mean ± SEM. Open bars normoxia (21% oxygen) during gestation, grey bars: hypoxia (13% oxygen) during gestation. A) Gene expression B) Protein expression. Protein expression is represented as the percentage of the 4 month normoxia group (assigned baseline value of 100%).. ***p<0.001.

Figure 5 Effect of gestational hypoxia compared to normoxia on expression of cellular senescence proteins in the ovary. Data shown as mean ± SEM. Data are represented as the percentage of the 4 month normoxia group (assigned baseline value of 100%). Open bars:

639 normoxia (21% oxygen) during gestation, grey bars: hypoxia (13% oxygen) during gestation.
640 A) P53 B) $P16^{ink}$. ***p<0.001, *p<0.05.
641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9
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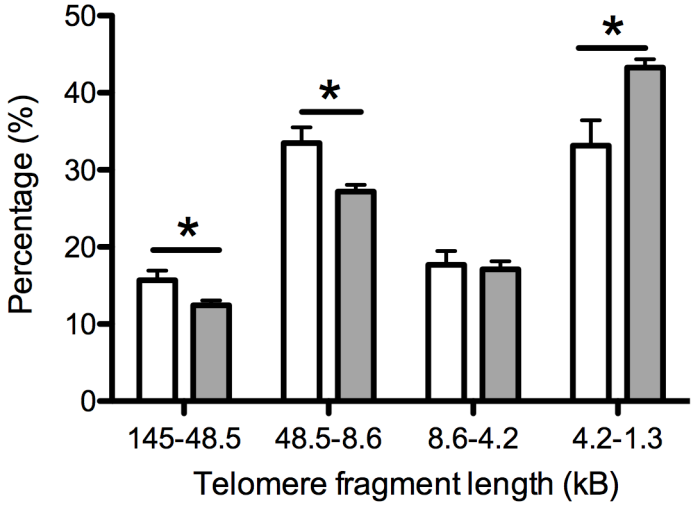
5
Follicles/mm3
Normoxia (21%)
Hypoxia (13%)
Gestational environment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Percentage (%)
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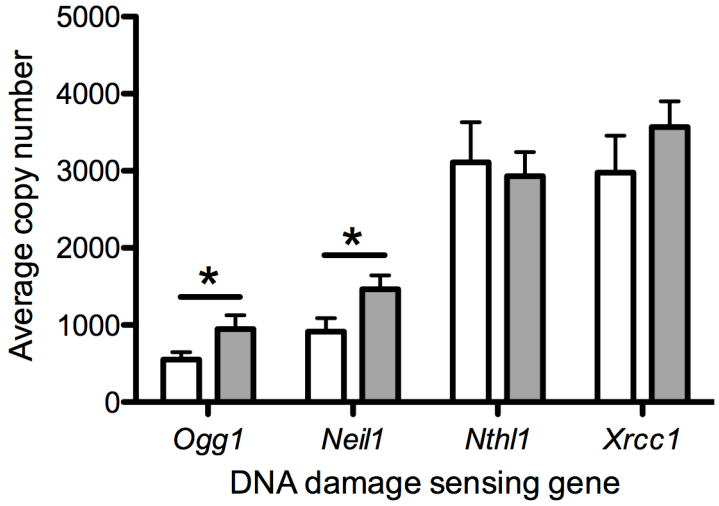
8.6-4.2
4.2-1.3
Telomere fragment length (kB)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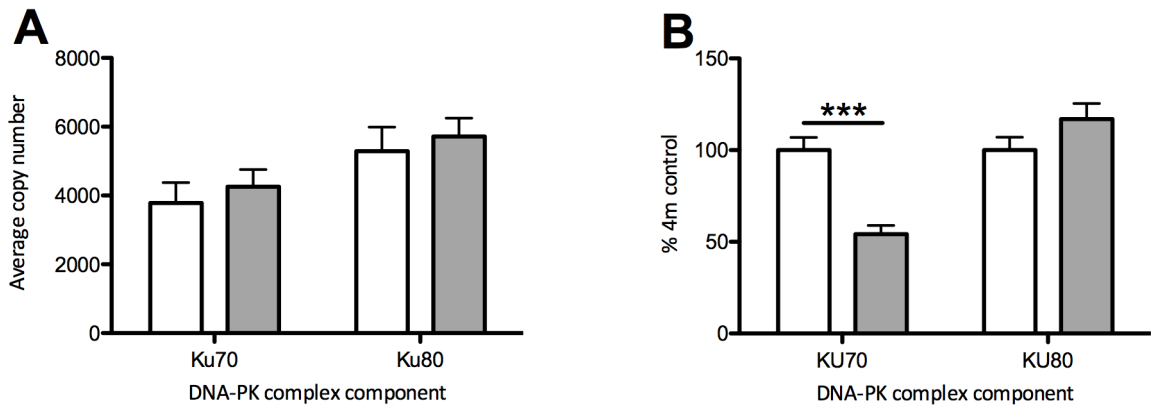
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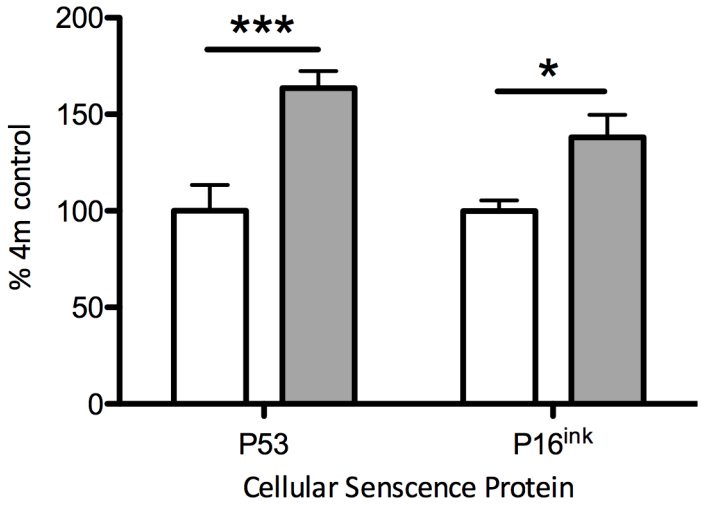
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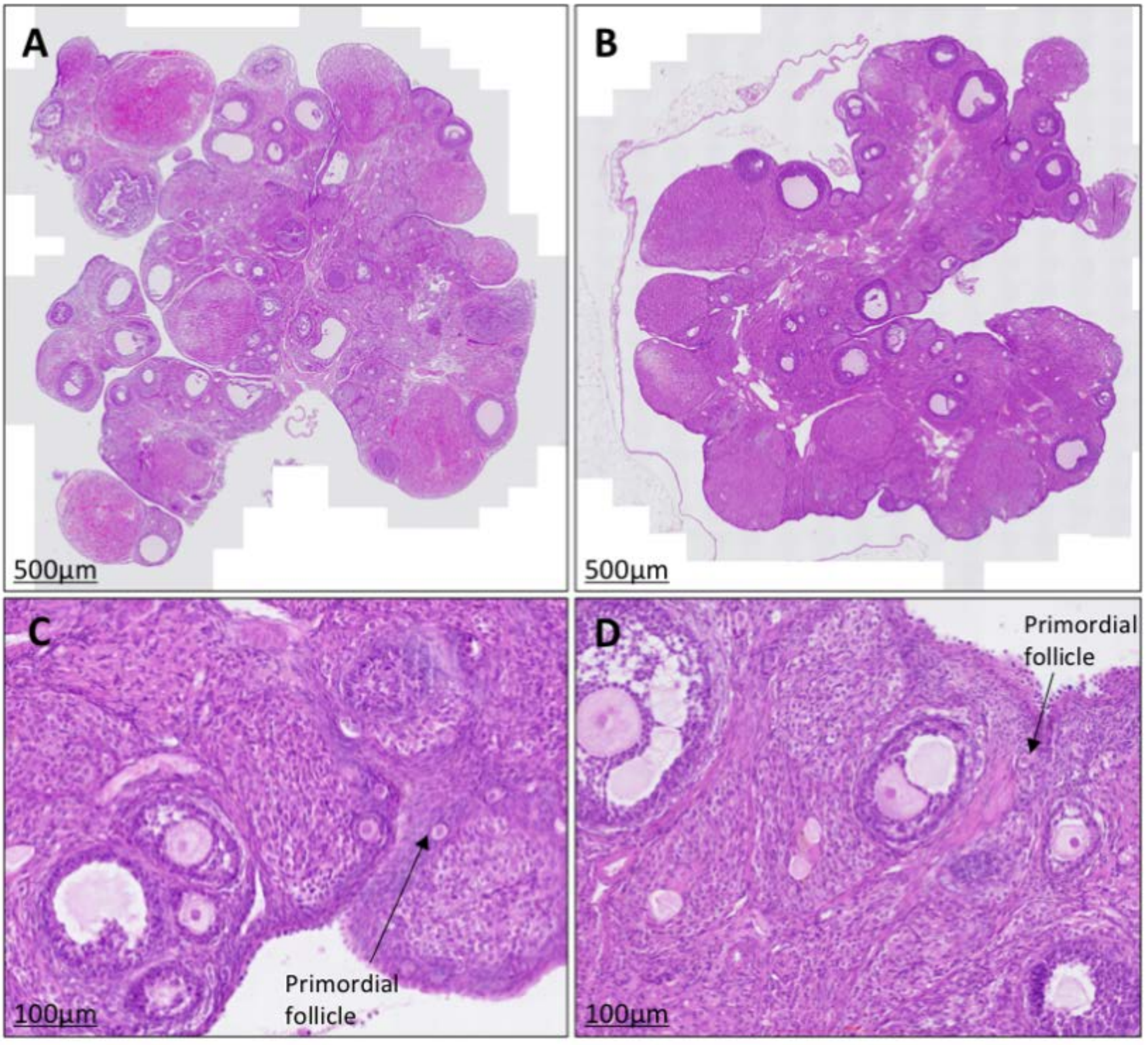
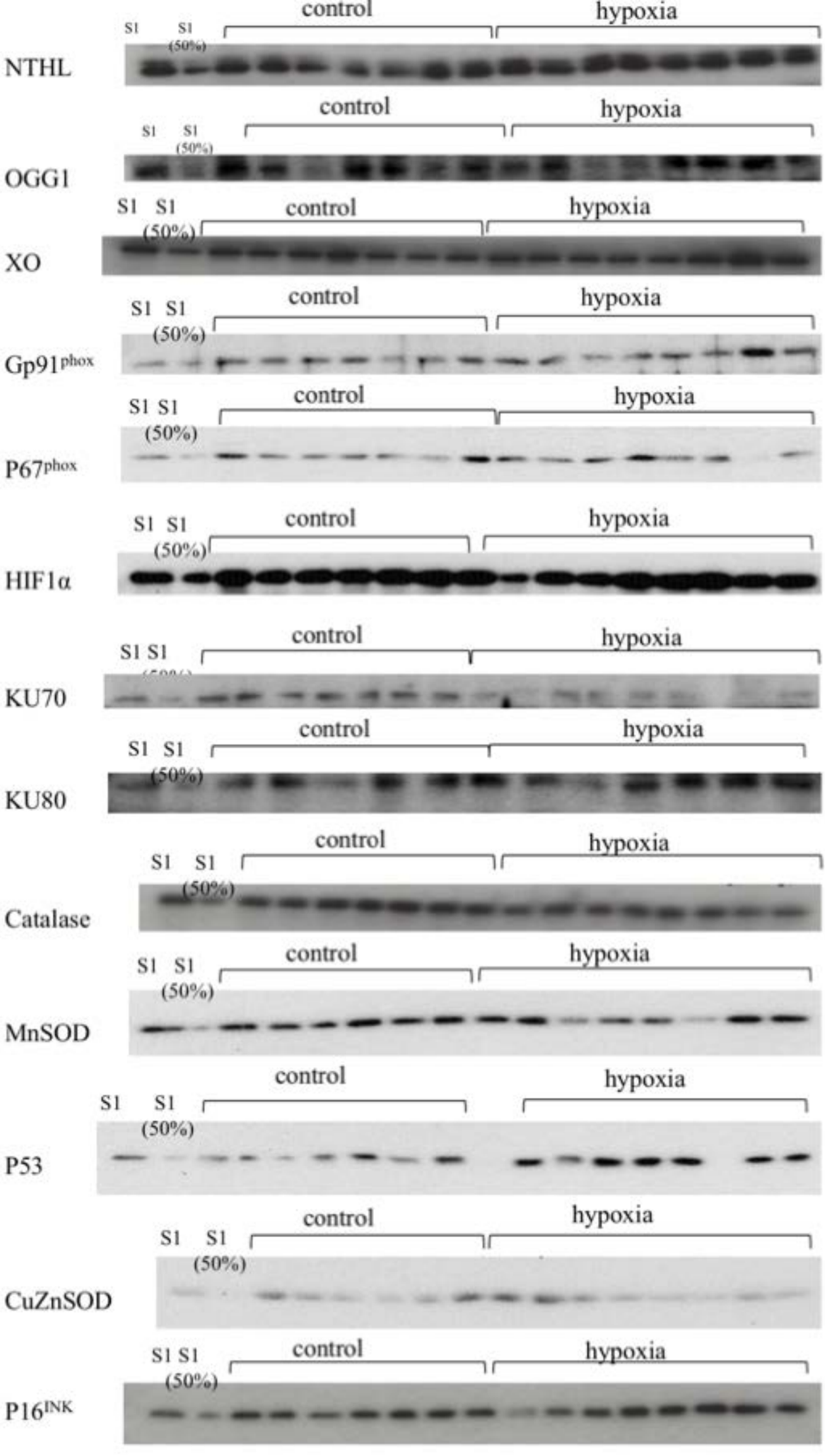


Supplementary figure 1: Representative images of ovarian tissue stained with H&E A) Normoxia, whole ovary section B) Hypoxia, whole ovary section C) Normoxia, representative area with primordial follicle highlighted D) Hypoxia, representative area with primordial follicle highlighted



Supplementary figure 2: Western blot images for all reported proteins