Testicular Dysgenesis Syndrome-like morphology and gene expression, and activation of Hypoxia Inducible Factor 1 Alpha in juvenile lamb testes following developmental exposure to low-level environmental chemical mixture.

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Abstract

Current declines in male reproductive health may, in part, be driven by anthropogenic environmental chemical exposure. Using the biosolid treated pasture (BTP) sheep model, this study examined the effects of gestational exposure to a translationally relevant mixture of environmental chemicals. At 8 weeks of age, ram lambs gestationally exposed to BTP were lighter than control, and their testes contained fewer germ cells and had a greater proportion of Sertoli-cell-only seminiferous tubules. Effects of biosolid exposure on the testicular transcriptome correlated with human testicular dysgenesis syndrome (TDS) patient data. Common differentially expressed genes indicated changes in apoptotic and mTOR signalling, which concurs with previous gene expression data from neonatal BTP lambs. Gene expression data and immunohistochemistry indicates increased HIF1α activation and nuclear localisation in BTP exposed animals, known to disrupt testosterone synthesis. Together, these results provide a potential mechanism for the pathogenesis of this phenotype, and TDS in humans.

30 Keywords

31 Developmental toxicity. Reproductive toxicity. Environmental chemicals. Testicular dysgenesis
32 syndrome. Hypoxia Inducible Factor 1 Alpha.

33 Abbreviations

Biosolid treated pasture	BTP
Bisphenol A	BPA
Blood-testes barrier	BTB
Differential gene expression	DGE
Differentially expressed genes	DEGs
Endocrine disrupting chemicals	EDCs
Environmental chemicals	ECs
False discovery rate	FDR
Gene ontology	GO
Gonadotropin-releasing hormone	GnRH
Human chorionic gonadotropin	hCG
Hypothalamic–pituitary–testicular	HPT
Hypoxia Inducible Factor 1 Alpha	HIF1 α
Hypoxia-responsive elements	HREs
Luteotropic hormone	LH
No observable adverse effect level	NOAEL
Pharmaceuticals and personal care products	PPCPs
Polybrominated diphenyl ethers	PBDEs
Polychlorinated biphenyls	PCBs
Polycyclic aromatic hydrocarbons	PAHs
Sertoli-cell-only	SCO
Testicular dysgenesis syndrome	TDS
Tolerable daily intake	TDI
Von Hippel-Lindau tumour suppressor protein	VHL

34

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37 1. Introduction

38 Male reproductive health has been reported to be in decline for the past 80 years ¹, with reduced
 39 semen quality and fecundity, and increased incidence of testicular dysgenesis syndrome (TDS;
 40 encompassing cryptorchidism, hypospadias, hypogonadism, infertility, and testicular germ cell
 41 cancer) ². While precise mechanisms behind the pathogenesis of TDS are still to be fully elucidated, a
 42 link has been made to foetal Sertoli and Leydig cell dysfunction ^{3,4}. While many contributory factors
 43 to the decline in male fertility have been identified, including improper nutrition, sedentary lifestyle,
 44 and stress, much attention has focused on the role of environmental chemicals (ECs), in particular
 45 endocrine disrupting chemicals (EDCs) ⁵⁻⁷. A variety of ECs and families of ECs are known to be able
 46 to adversely affect testicular development, for example, gestational exposure to phthalates can
 47 negatively affect germ cell development, sperm motility, and testosterone production ^{8,9}. In addition
 48 to such studies which have examined the effects of individual ECs, several studies have used
 49 component-based methodologies to examine the effects of low dose mixtures of ECs. When
 50 presented in this form, it is noteworthy that adverse effects of EC exposure have been reported even
 51 when individual chemicals were present at doses at or below their respective tolerable daily intake
 52 (TDI) values ^{10,11}. This exposure scenario is of note as it more realistically reflects human EC
 53 exposure, which is characterised as chronic, extremely complex, and very low-level.

54 The human EC exposome is represented in our wastewater. The solids from wastewater treatment,
 55 biosolids, are extensively used as an agricultural fertiliser and reflect the human EC exposome in
 56 terms of complexity and concentration ¹²⁻¹⁶. ECs can be measured in tissue samples collected from
 57 sheep pastured on biosolid treated pasture (BTP) ^{12,17-20}, as well as the organs of their offspring
 58 ^{12,19,20}. These ECs include alkylated phenols, dioxin-like compounds, flame retardants such as
 59 polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), pharmaceuticals and
 60 personal care products (PPCPs), plasticising agents such as phthalates and bisphenol A (BPA),
 61 polycyclic aromatic hydrocarbons (PAHs), and metabolites thereof ¹³⁻¹⁵. Experiments using the BTP
 62 sheep model have previously shown that *in utero* EC exposure can cause a multitude of effects in
 63 offspring. These include altered behaviour, differences in bone composition, disruption to cellular
 64 and hormonal processes, and changes in liver function, as well as effects on gonadal development in
 65 males and females ^{17,18,21-30}. With specific regards to male gonadal development, gestational
 66 exposure to BTPs has been associated with reduced testicular weights and fewer gonocytes, Leydig
 67 cells, and Sertoli cells in 110-day-old fetuses ²³. Neonatal (1-day-old) lambs also display fewer
 68 gonocytes as well as an increased incidence of Sertoli-cell-only (SCO) seminiferous tubules ²⁴. This
 69 study indicated that there may be two phenotypic responses to EC exposure within the male lambs,

one more susceptible to disruption and another resistant. This observation mirrors findings in 9-month-old offspring where a subset of animals was identified that had reduced germ cell numbers and an increased incidence of SCO seminiferous tubules¹⁷. Puberty in male sheep begins at approximately 8 weeks of age, therefore in this study the morphology and transcriptome of gestationally BTP exposed juvenile (8-week-old) ram lamb testes were examined to provide insights into the mechanisms underlying observed adverse morphological and functional outcomes.

2. Results

2.1. Anatomical and histopathological analyses

Mean morphological indices are presented in Table 1. Mean body weight was significantly ($p = 0.010$) lower in biosolid compared to control lambs. Mean pituitary weight was significantly ($p = 0.042$) lower in biosolid (0.435 ± 0.054 g) compared to control (0.489 ± 0.060 g) lambs, but when corrected for body weight there was a trend ($p = 0.054$) for pituitary/BWT to be higher in biosolid lambs. Mean adrenal weight was not affected by treatment but when expressed relative to body weight was significantly ($p = 0.038$) higher in biosolid compared to control lambs.

Representative immunohistochemical images from control and biosolid testes are shown in Figure 1(A and B). The mean relative germ cell : Sertoli cell ratio (Figure 1C) was significantly ($p = 0.009$) lower in biosolid lambs (0.67 ± 0.22) compared to controls (1 ± 0.29). The mean percentage of SCO seminiferous tubules (Figure 1D) was significantly ($p = 0.007$) higher in biosolid lambs (12.56 ± 11.49 %) compared to control (2.27 ± 1.81 %). The mean minimum Feret's diameter of seminiferous tubules (Figure 1E) was significantly ($p = 0.021$) smaller in biosolid (77.51 ± 16.51 μm) than in control (94.89 ± 13.36 μm) lambs.

2.2. Gene expression and gene ontology analyses

DGE analysis identified 1382 DEGs between the biosolid and control groups (726 with higher expression, 656 with lower). 99 DEGs had an FDR ≤ 0.1 (60 with higher expression, 39 with lower). A z-score heatmap of these genes is shown in Figure 2 (Differential expression data presented in Supplementary Data 2). GO analysis of the 99 DEGs indicated 6 GO terms as significantly ($p < 0.05$) enriched (Supplementary Data 3). Within these identified GO terms, expression levels of 2 genes showed significant ($p < 0.05$) correlation with the geometric mean of germ cell markers, both from different GO term groups (Supplementary Data 4). These represent 12.5% and 50% of genes within respective GO terms (Nucleic acid binding and Protein ADP-ribosylation, respectively), and 2.94% of total genes identified.

2.3. BaseSpace correlations

A significant ($p < 0.0001$) positive correlation was identified between the biosolid DEG data and 7 published DEG data sets from 3 separate human testes gene expression studies (Supplementary Data 5). A gene list was created by identification of DEGs in common across each dataset per study and then combining between studies. This list comprised of 520 genes and was submitted to DAVID for GO and pathway analysis. 9 KEGG pathways and 25 GO terms were identified as significantly ($p < 0.05$) enriched (Table 2). Within these pathways and terms, 6 genes showed significant ($p < 0.05$) correlation with the geometric mean of germ cell markers, each from different GO term groups (Error! Reference source not found. Supplementary Data 6). This represented between 9% and 33% of genes within respective GO terms, and 1.3% of total genes identified.

2.4. Quantitative PCR

Of the 13 genes quantified by qPCR, 4 had significantly ($p < 0.05$) higher expression in biosolid animals than in control (Figure 3): *FASN* ($p = 0.045$), *HK1* ($p = 0.026$), *PDPK* ($p = 0.042$), and *VEGFA* ($p = 0.037$).

2.5. Hypoxia Inducible Factor 1 Alpha analysis

Representative images from control and biosolid testes showing immunofluorescent staining for HIF1 α and the nuclear marker DAPI are shown in Figure 4A, with the quantified overlap of HIF1 α and DAPI signal (Manders' overlap coefficient) shown in Figure 4B. In Leydig cells, a significantly ($p = 0.0043$) greater proportion of HIF1 α was seen in the nucleus of biosolid (0.403 ± 0.106) compared to control (0.251 ± 0.097) animals (Figure 4B).

Western blots for HIF1 α and α -Tubulin are shown in Figure 4C with the mean relative abundance of HIF1 α in the testes of control and biosolid animals shown in Figure 4D. There was significantly ($p = 0.0164$) less HIF1 α detected in the testes of biosolid (0.710 ± 0.219) than control (1.00 ± 0.291) animals.

3. Discussion

The present study demonstrates the ability for gestational exposure to a low dose, extremely complex chemical mixtures to adversely affect prepubertal gonadal development. Similarities between the morphological and DGE patterns seen in biosolid exposed lambs and TDS patients suggest that the changes observed prior to puberty in this model may predispose animals to TDS and demonstrates the model's utility to investigate the pathogenesis of TDS. Of specific note, biosolid

exposure was associated with differential expression of genes related to apoptotic processes and mTOR signalling, and increased expression of a group of mTOR regulated genes which are all transcriptionally controlled by a common nuclear factor: HIF1 α . The activation of HIF1 α was also found to be altered in biosolid exposed animals visually, specifically where it was more localised to the nuclei of Leydig cells in biosolid exposed animals than in control. As HIF1 α has previously been shown to inhibit STAR transcription and testosterone synthesis³¹, this may be a mechanism through which gestational EC exposure may generate the altered testicular phenotype in biosolid exposed males, and may result in reduced fertility in affected animals.

There is increasing evidence of synergistic actions between different chemical components within low dose chemical mixtures producing adverse effects on the development of male gonads. Male offspring gestationally exposed to low doses of some simple chemical mixtures (containing ≤ 10 chemical components) have greater rates of feminised phenotypes, genital malformations, pathological testicular morphology, and impaired spermatogenesis^{11,32–35}. These studies exemplify the ability for even relatively simple chemical mixtures to elicit adverse effects in developing male gonads at low doses. In this study pregnant ewes were exposed to realistic levels and numbers of ECs as to be of human relevance. The present study showed anatomical differences between control and biosolid offspring, including lower bodyweights and pituitary weights in biosolid lambs. These results resemble anatomical differences seen in late gestation fetuses²³ and neonatal lambs²⁴, respectively, following similar exposure. The present study also showed fewer germ cells, with a concurrent increase in the occurrence of Sertoli-cell-only seminiferous tubules, in biosolid exposed lamb's testes compared to controls, which phenotypically resembles mixed testicular atrophy – a hallmark of TDS^{2,36}. These results also resemble morphological differences seen in the testes of late gestation fetuses²³, neonatal lambs²⁴, and a subset of adult offspring¹⁷, following similar exposure.

DGE analysis of the testicular transcriptome yielded many DEGs which may be involved in the pathogenesis of the observed phenotype. Noteworthy DEGs are a group involved in maintaining the blood-testes barrier (BTB) and cell polarity (*PRICKLE*, *CTNNA2*, and *DLG1*)^{37–39} which had significantly lower expression in the testes of biosolid animals than in controls. BTB and cell polarity maintenance are crucial for germ cell stem cell survival and differentiation. Also noteworthy are a group of DEGs essential for spermatogenesis (*CIB1*, *GBF1*, *CFAP44*, and *PLK4*)^{40–43}, again with significantly lower expression in the testes of biosolid animals than in controls. This was also reflected in the GO term “Spermatid Development” being identified as significantly enriched within these DEGs. Comparison of the DEG data from this study with DEG data from human TDS patients allowed the identification

of GO terms and KEGG pathways that were affected in both data sets, including two mTOR and two apoptotic entries. mTOR is known to be a crucial component of proper testicular development and spermatogenesis⁴⁴ and was previously indicated in transcriptomic analysis of testes from neonatal sheep gestationally exposed to BTP²⁴. Additionally, a range of chemicals have been shown to disrupt mTOR signalling pathways, causing testicular autophagy in pubescent rodents^{45–47}. A selection of genes were investigated which are known to have expression increased following mTOR activation⁴⁸. Of the four genes found to have higher expression in the testes of biosolid animals compared to controls, three (*VEGFA*, *HK1*, and *PDPK1*) are transcribed *via* Hypoxia Inducible Factor 1 Alpha (HIF1 α) activation and contribute to the angiogenic and metabolic adaptive responses to hypoxia.

HIF1 α is an important factor in embryonic development which is expressed from early embryonic stages, continuing in germ cells and other tissues into adulthood⁴⁹. In the classical scenario HIF1 α responds to physiological oxygen status. Under normoxic conditions HIF1 α is bound by the von Hippel-Lindau tumour suppressor protein (VHL) and degraded by ubiquitin-mediated proteolysis⁵⁰. Under hypoxic conditions the mechanisms for VHL association are suppressed and HIF1 α will translocate to the nucleus to bind with HIF1 β , forming the heterodimeric HIF-1 transcription factor, which binds to hypoxia-responsive elements (HREs) of target genes, increasing transcription⁵¹. Separately to the classical scenario, HIF1 α activation can occur independently of oxygen status by biochemical pathways (e.g. mTOR)⁵² or small molecules and reactive oxygen species^{53,54}, with subsequent nuclear localisation, HIF-1 formation, and increased target gene transcription. Interestingly, contrary to other tissues, HIF1 α can be stable under normoxic conditions in the testes. As HIF1 α is expressed in germ cells throughout the life cycle, a role in germ cell development and maturation has been hypothesised^{49,55}, although precise details are yet to be discovered. However, it is Leydig cells which express the highest levels of HIF1 α in the testes⁵⁶. The primary function of Leydig cells is testosterone production, which is tightly regulated in a closed-loop feedback mechanism by the hypothalamic-pituitary-testicular (HPT) axis. In steroid biosynthesis, the main rate-limiting step is the STAR mediated transport of cholesterol from the outer to inner mitochondrial membrane⁵⁷. HIF-1 has been shown to repress STAR transcription in Leydig cells by way of binding site blocking, and thus reduce testosterone synthesis³¹. As greater nuclear localisation of HIF1 α was observed in Leydig cells of biosolid animals compared to controls, further indicating HIF1 α activation, this could provide a mechanistic reasoning to lower testosterone levels previously seen in neonatal biosolid lambs²⁴. However, hypoxia has also been reported to increase testosterone secretion by Leydig cells *in vitro*⁵⁸, which is potentially HIF1 α mediated⁵⁹. It is also interesting that while nuclear localisation of HIF1 α was greater within Leydig cells of biosolid animals, total testicular amounts of HIF1 α were lower. However, this may be attributable to the

198 changes in cellularity and the loss of germ cell HIF1 α , and the magnitude of changes in localisation
199 (160% of control) also outweighs the magnitude of changes in protein levels (71% of control).

200 Foetal development is an extremely complex and dynamic period, which increases vulnerability to
201 xenobiotic induced toxicity. This is the first study to show similarity of DGE between low level EC
202 exposure and human TDS patients. Additionally, this is the first study to link HIF1 α activation to low
203 level EC exposure. These findings add to the body of evidence to suggest that exposure to real-world
204 levels of environmental chemical mixtures during pregnancy may be having an adverse effect on
205 male offspring reproductive health, contributing to the decline in sperm quality and fecundity in
206 humans.

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4. Online Methods

4.1. *Ethics statement*

All animals were maintained under normal husbandry conditions at the University of Glasgow Cochno Farm and Research Centre. The research programme was approved by the University of Glasgow School of Veterinary Medicine Research Ethics Committee. All procedures were conducted in accordance with the Home Office Animal (Scientific Procedures) Act (A(SP)A), 1986 regulations.

4.2. *Experimental animals*

EasyCare ewes were maintained on pastures fertilised with either biosolids, at conventional rates (4 tonnes/ha, twice per annum (April/September); biosolid), or with inorganic fertiliser at a rate which supplied equivalent levels of nitrogen (225 kg N/ha per annum; control) for one month prior to being mated and for the duration of pregnancy. Ewes were maintained indoor for the last two week of pregnancy and fed forage supplemented with concentrates as per normal husbandry practice, biosolids ewes received forage harvested from Biosolid treated pasture. Mating was by artificial insemination with semen from 4 rams which had only been maintained on control pasture. After parturition all ewes and lambs were maintained on control pastures, therefore all EC exposure was maternal i.e., through placental or lactational transfer. A subset of the male offspring from ewes exposed to conventionally fertilised pastures (n=11, control), and biosolid treated pastures (BTP) (n=11, biosolid), were weighed before euthanised by intravenous barbiturate overdose (140 mg/kg Dolethal, Vetroquinol, UK) for tissue collection.

4.3. *Tissue collection*

At post-mortem, gonads, thyroids, adrenals, and pituitaries were removed and weighed. Epididymides were removed from the testes and weighed. Testes weights were determined by subtraction of epididymides weights from gonadal weights. Two transverse slices were taken from the centre of the left testis, fixed overnight in 10% neutral buffered formalin (NBF, Thermo Scientific – 16499713), then transferred to 70% ethanol (VWR – 20821.330) prior to processing and embedding in paraffin wax for histology (Excelsior AS, Thermo Scientific). A 5mm transverse slice was taken from the centre of the right testis and frozen on dry ice prior to storage at -80°C until RNA extraction.

4.4. *Immuno-histochemistry*

Formalin-fixed paraffin embedded testicular tissue was sectioned (5µm) using a microtome (Leica Biosystems, model RM2125RT). Immuno-histochemistry was used to identify germ cells. One section per animal was mounted on a glass slide, dewaxed, and processed for antigen retrieval (autoclave for 21 minutes while immersed in citrate buffer 10mM, pH 6). Slides were washed in TBS and taken through peroxidase, avidin, and biotin blocking solutions (15 minutes each with TBS washes in between). Non-specific binding was blocked by incubation for 30mins with 20% goat serum in TBS before incubation with the primary antibody (rabbit anti-DDX4 polyclonal antibodies; Abcam – ab13840) diluted 1:1000 in antibody diluent (Agilent DAKO – S2022) overnight at 4°C. Sections were then washed in TBS + 1% Tween20 before being incubated with a biotinylated secondary antibody (goat anti-rabbit biotinylated polyclonal antibodies; Agilent DAKO – E0432) diluted 1:200 in antibody diluent for 30 minutes. Following incubation in secondary antibody, sections were treated with Vectrastain ABC-HRP system (Vector Laboratories – PK4000) for 60 minutes before being washed in TBS + 1% Tween20 and stained using DAB for 30 seconds. Slides were then washed in TBS, and counter-stained with haematoxylin and cover-slipped. Four representative images of the lobuli testis were captured (Leica DM4000B microscope with a Leica DC480 digital camera at 40x magnification using Leica Qwin software) from separate areas (top, bottom, left, and right) of each tissue section for each animal. Using ImageJ (version 1.53a), individual tubules which were entirely captured within images were manually selected (n = 2145 Control, 3673 Biosolid). DDX4 positive and negative cells were counted by automated macro (pre-validated on a subset of data – Supplementary Data 1). Mean germ cell to total cell populations, per tubule, were calculated relative to the mean control, as well as the proportion of seminiferous tubules without germ cells (Sertoli-cell-only; SCO). Separately, tubule selections were filtered for circularity using the equation $4\pi(Area/Perimeter)^2$ with a threshold of ≥ 0.9 (n = 587 Control, 1326 Biosolid) and minimum Feret's diameters measured.

Fluorescent immuno-histochemistry was used to localise HIF1α. One section per animal was mounted on a glass slide, dewaxed, and microwaved at medium heat in 1 mM EDTA, pH 8.0, for 10 minutes. Sections were probed overnight at 4°C using mouse anti-HIF1α antibodies (Invitrogen – MA1-16504) diluted 1:250 in 5% BSA / TBS-T. Slides were washed with TBS and probed for 1 hour at room temperature using goat anti-mouse antibodies (Abcam – ab150113) diluted 1:1000 in 5% BSA / TBS-T. Sections were counter stained with DAPI (Abcam – ab104139) and cover-slipped. Four representative images of the lobuli testis were captured (Leica DM4000B microscope with a Leica DC480 digital camera at 160x magnification using Leica Qwin software) from separate areas (top, bottom, left, and right). Using ImageJ, areas out-with seminiferous tubules were manually selected and co-localisation analysis performed using the JACoP plugin⁶⁰, which provided Maders' overlap coefficients for the proportion of HIF1α staining that overlapped DAPI staining.

4.5. RNA extraction, cDNA library preparation, sequencing, and data analysis

Transcriptome analysis was performed as previously described²⁴. Briefly, RNA was extracted, purified, reverse transcribed, and ligated to individual DNA barcodes for multiplexing. As the barcoding kit only contains twelve individual barcodes, samples were split into two groupings of eleven (mixed control and biosolids) for sequencing. Barcoded cDNA samples within a grouping were pooled and sequenced using a MinION Nanopore sequencer (Oxford Nanopore). Data were processed and filtered for quality prior to alignment to the reference transcriptome (constructed from NCBI's Oar_v4.0 reference genome and annotation files) and counted. Differential gene expression (DGE) analysis was performed on gene counts by EdgeR. Differentially expressed genes (DEGs) were called using a p-value threshold of 0.05, log₂ fold change threshold of <-1 or >1, and false discovery rate (FDR) threshold of 0.1. Gene ontology (GO) analysis was performed using DEG lists in DAVID (version 6.8).

4.6. BaseSpace Analysis

Illumina's BaseSpace correlation Engine enables the comparison and correlation of DEG datasets through a combination of ranked-based enrichment statistics, meta-analyses, and biomedical ontologies⁶¹. BaseSpace correlation Engine employs a rank-based, nonparametric analysis strategy driven by a Running Fisher's test algorithm which performs the rank-based directional enrichment process. This enrichment process utilises a Fisher's exact test to calculate four p-values: two p-values for the genes which are positively correlated between the datasets (genes that are either up or down-regulated in both datasets) and two for the negatively correlated genes (genes that are up-regulated in dataset 1 and down-regulated in dataset 2, or vice versa). The overall correlation p-value was calculated by converting the four p-values to -log₁₀ p-values and subtracting the sum of the negative correlation p-values from the sum of the positive correlation p-values. A p-value threshold for significance of 0.0001 was used. To enable cross-platform and cross-species comparisons BaseSpace correlation engine software uses a database compiled of commonly used gene identifiers and reference identifiers along with ortholog information to standardise mapping across platforms and species.

Expression data for DEGs with $p \leq 0.05$ were analysed for correlation to published data by Illumina's BaseSpace software. Positively correlating DEGs were filtered for those common across each dataset per study. These gene lists were then combined and submitted to DAVID for pathway and GO analyses.

4.7. Effect of changes to cellularity

To assess the effect of changes in testicular cellular composition, DEGs with pathways or GO terms identified as enriched were tested for correlation using generalised linear models, on gene counts against the geometric mean of gene counts for germ cell specific biomarkers, and p values corrected for false discovery. Germ cell specific biomarkers used were CD9, CD14, THY1, NOTCH1, GFRA1, CDH1, and UCHL1.

4.8. Quantitative qPCR

RNA was extracted from approximately 30mg of frozen testes using RNeasy Mini Kit (Qiagen – 74104). Genomic DNA was degraded and cDNA synthesised using QuantiTect Reverse Transcription Kit (Qiagen – 205311). qPCR was performed using qPCR Brilliant II SYBR Master Mix (Agilent – 600828) on a Stratagene 3000. Primer details can be found in Supplementary Data 7. Raw fluorescent data were regressed by PCR Miner⁶² to produce primer efficiencies and Ct values which were used in $\Delta\Delta C_t$ analysis to produce Log₂ Fold Change values.

4.9. Western blots

Approximately 15 mg of frozen tissue samples were homogenisation using a 1 mL tapered PTFE tissue homogeniser into 19x volume of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0)) containing protease inhibitors (Merck – 11697498001) and phosphatase inhibitors (Merck – 4906845001). Samples were centrifuged at 12,000 xg for 20 minutes at 4 °C. Following protein determination (Thermo Scientific – 23227), samples were made to a final concentration of 2 µg/µL using LDS Sample Buffer (Invitrogen – NP0007) and Reducing Agent (Invitrogen – NP0004) and heated to 95 °C for 10 minutes. 10 µL of reduced, denatured samples (20 µg protein) were loaded into wells of 4 to 12%, Bis-Tris, 1.0 mm acrylamide gels (Invitrogen – WG1403BOX) using 5 µL of protein reference standard (BioRad – 1610375EDU) in the first and last wells. Gels were run under constant voltage using MOPS SDS Running Buffer (Invitrogen – NP0001) with added antioxidant (Invitrogen – NP0005) and transferred to nitrocellulose membranes (Invitrogen – IB23001 NC) using an iBlot2 (Invitrogen – IB21001). Membranes were blocked using Intercept TBS Blocking Buffer (Licor – 927-60001), washed with TBS-T and TBS, and probed with overnight with primary antibody diluted 1:1000 in 5% BSA/TBS at 4 °C. The next morning membranes were washed with TBS-T and TBS before incubation in secondary antibody diluted 1:10,000 in 5% BSA/TBS at room temperature for one hour. Membranes were then washed in TBS-T, TBS, and finally MilliQ water before imaging on an Odyssey DLx Imager (Licor – 9142). Primary antibodies used were mouse-anti-HIF1α monoclonal (Invitrogen – MA1-16504) and rabbit-anti-α-tubulin polyclonal (Invitrogen – PA1-38814), with donkey anti-mouse (Invitrogen – SA5-10172) and

336 donkey anti-rabbit (Invitrogen – SA5-10044) fluorophore conjugated secondary antibodies.
 337 Fluorescent intensities were quantified using Licor Image Studio Software (version 5.2.5). HIF1 α
 338 signal intensities were normalised to α -Tubulin and expressed as relative to the average of control
 339 values.

340 4.10. Statistical analysis

341 All calculations and statistical analyses were performed in R (version 4.1.1) using base functionality.
 342 Unless otherwise stated, data were fitted to generalised linear models with gamma distribution and
 343 groups compared using one-way ANOVA. Plots were created using the R package ggplot2 (version
 344 3.3.5). Data are presented as mean $\pm$ SD.
 345

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498 Tables

Morphological indices	Control (n=11)			Biosolid (n=11)			p-value
<i>Body Weight (BWT) (kg)</i>	<i>25.227</i>	<i>±</i>	<i>3.629</i>	<i>21.000</i>	<i>±</i>	<i>3.369</i>	<i>0.010</i>
Gonads (g)	43.993	±	12.538	35.270	±	12.296	0.115
Testes (g)	27.690	±	8.926	21.191	±	7.700	0.089
Epididymides (g)	16.269	±	4.899	14.079	±	5.197	0.334
Adrenals (g)	1.914	±	0.374	2.148	±	0.457	0.203
Thyroids (g)	2.038	±	0.648	1.748	±	0.439	0.233
<i>Pituitary (g)</i>	<i>0.489</i>	<i>±</i>	<i>0.060</i>	<i>0.435</i>	<i>±</i>	<i>0.054</i>	<i>0.042</i>
Gonads / BWT (g/kg)	1.756	±	0.459	1.680	±	0.598	0.743
Testes / BWT (g/kg)	1.111	±	0.327	1.003	±	0.340	0.469
Epididymides / BWT (g/kg)	0.653	±	0.180	0.677	±	0.277	0.818
<i>Adrenals / BWT (g/kg)</i>	<i>0.079</i>	<i>±</i>	<i>0.026</i>	<i>0.105</i>	<i>±</i>	<i>0.029</i>	<i>0.038</i>
Thyroids / BWT (g/kg)	0.082	±	0.025	0.085	±	0.026	0.751
<i>Pituitary / BWT (g/kg)</i>	<i>0.019</i>	<i>±</i>	<i>0.002</i>	<i>0.021</i>	<i>±</i>	<i>0.019</i>	<i>0.054</i>

Table 1. Anatomical metrics for control and biosolid exposed juvenile ram lambs. Data is presented as mean ± SD for animal body weights, absolute organ weights, and relative organ to body weights. All p-values derived from generalised linear models of biosolid animals compared with controls. Bold italics indicates p<0.05.

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Category	Term	Fold Enrichment	p-value
GO: Cellular Component	Cytosol	2.00	0.000030
GO: Molecular Function	Zinc ion binding	1.75	0.000140
GO: Cellular Component	Nucleolus	2.04	0.000285
GO: Cellular Component	Intracellular ribonucleoprotein complex	6.03	0.000926
GO: Molecular Function	mRNA 3'-UTR binding	7.51	0.000999
KEGG	Cell cycle	3.52	0.001055
GO: Cellular Component	Early endosome	3.16	0.002503
GO: Molecular Function	Hydrolase activity	3.98	0.003734
KEGG	Purine metabolism	2.76	0.004030
GO: Biological Process	Apoptotic process	3.88	0.004223
KEGG	Metabolic pathways	1.45	0.007357
GO: Biological Process	mTOR signalling	8.96	0.008819
KEGG	Lysine degradation	4.51	0.010057
GO: Molecular Function	GTPase activator activity	2.61	0.014330
KEGG	ErbB signalling pathway	3.33	0.018098
GO: Biological Process	Ras protein signal transduction	4.70	0.020505
GO: Molecular Function	DNA-directed DNA polymerase activity	6.59	0.021242
GO: Biological Process	Positive regulation of gene silencing by miRNA	12.48	0.021909
GO: Cellular Component	mTORC2 complex	11.95	0.024152
GO: Molecular Function	DNA-dependent ATPase activity	5.96	0.027828
GO: Biological Process	Mitochondrial fragmentation involved in apoptotic process	10.92	0.028559
GO: Biological Process	Protein localization to microtubule	10.92	0.028559
KEGG	Pyrimidine metabolism	2.98	0.029146
GO: Biological Process	Platelet-derived growth factor receptor signalling pathway	5.82	0.029464
GO: Molecular Function	RNA polymerase II core promoter proximal region sequence-specific DNA binding	1.92	0.030620
GO: Cellular Component	Nucleus	1.25	0.032457
GO: Biological Process	Retrograde transport, endosome to Golgi	4.04	0.033621
KEGG	Phosphatidylinositol signalling system	2.86	0.034587
GO: Biological Process	Peptidyl-tyrosine autophosphorylation	9.71	0.035899
GO: Molecular Function	ATP binding	1.32	0.036382
KEGG	Biosynthesis of antibiotics	2.07	0.039467
GO: Biological Process	Negative regulation of viral transcription	8.74	0.043875
KEGG	Base excision repair	5.01	0.044115
GO: Biological Process	Mitochondrion organization	4.85	0.047341

502 Table 2. GO terms and KEGG pathways identified as potentially enriched by DAVID within the list of common DEG between
503 the biosolid data and at least one correlating study identified by BaseSpace.

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506 Figures

Figure 1. Histopathological findings in juvenile control and biosolid lamb testes. Representative images of H&E and DDX4-DAB stained tissue sections from 8-week-old control (A) and biosolid (B) lambs, taken at 40x magnification. Arrows indicate Sertoli-cell-only (SCO) seminiferous tubules. Relative proportions of germ cells to Sertoli cells (C) and percent of tubules which were SCO (D) ($p = 0.009$ and $p = 0.007$ respectively). Seminiferous tubule minimum Feret's diameters ($p=0.021$) (E). Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

Figure 2. Heat map of DEGs plotted against z-score. Genes are ordered from top to bottom by increasing p-value.

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Figure 3. Log₂ fold change of testicular gene expression between biosolid and control animals. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

Figure 4. HIF1 α in testes. Immunofluorescent staining of testes sections (A). Images to the right are of selections seen in images to the left. Blue staining is of DAPI and green of HIF1 α . Co-localisation analysis (B) performed on regions outside of seminiferous tubules shows significantly ($p = 0.0043$) more nuclear localisation of HIF1 α in biosolid animals than control. Manders' Overlap Coefficients of the proportion of green signal overlapping with blue signal were determined by ImageJ. Western blots for HIF1 α and α -Tubulin (C and D) show significantly ($p = 0.0164$) less HIF1 α in biosolid animals than control. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

514 Supplementary Data 1

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 516 Supplementary Data 1. Comparative plots, and Ratio and Delta Bland-Altman plots for DAB (A, B, and C) and haematoxylin
 517 (D, E, and F) stained cells. Dots represent counts for individual seminiferous tubules. Blue lines represent theoretical
 518 complete agreement and red lines represent regressed lines of best fit (solid lines) and 95% confidence intervals (dotted
 519 lines).

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521 Supplementary Data 2

Gene	logFC	logCPM	F	PValue	FDR
SIPA1L1	4.58	8.06	32.68	5.82E-08	0.00091
ART3	5.02	7.09	29.85	2.12E-07	0.00166
ENOX2	-3.79	7.59	24.35	2.14E-06	0.00850
KIAA1217	-2.39	9.50	23.69	2.88E-06	0.00850
LRRFIP2	4.04	7.25	23.67	2.91E-06	0.00850
PPFIBP2	4.04	7.52	23.21	3.57E-06	0.00850
ZFP2	4.55	6.86	23.20	3.81E-06	0.00850
YTHDF3	-3.60	7.66	22.49	4.94E-06	0.00964
PHLDB2	-3.71	7.75	22.15	5.76E-06	0.00982
ZNF655	4.41	6.80	22.07	6.29E-06	0.00982
DTNA	4.45	6.82	21.50	8.13E-06	0.01154
B3GALT5	3.79	7.14	21.10	9.29E-06	0.01209
PRICKLE1	-3.71	6.87	19.31	2.11E-05	0.02462
HYKK	4.21	6.72	19.31	2.21E-05	0.02462
EMSY	3.77	6.90	18.67	2.85E-05	0.02965
ZNF329	3.96	6.63	18.27	3.55E-05	0.03467
CDKL5	-4.03	6.65	17.75	4.53E-05	0.03888
B3GALNT1	3.80	6.87	17.67	4.53E-05	0.03888
CCDC158	-3.64	7.10	17.58	4.73E-05	0.03888
AKAP11	2.50	8.16	17.15	5.80E-05	0.04526
ST7L	-3.74	6.55	16.99	6.46E-05	0.04694
AHNAK2	3.49	7.01	16.87	6.61E-05	0.04694
RFX3	3.63	6.83	16.75	6.99E-05	0.04746
NEXN	3.74	6.56	16.65	7.59E-05	0.04771
NIN	-3.41	6.98	16.46	8.00E-05	0.04771
CELF1	2.50	8.32	16.40	8.24E-05	0.04771
GIT2	3.14	7.13	16.40	8.25E-05	0.04771
RASGRP3	3.72	6.55	16.31	8.90E-05	0.04791
JADE3	3.01	7.14	16.20	9.08E-05	0.04791
WHSC1L1	3.48	6.77	16.17	9.20E-05	0.04791
HFM1	3.73	6.56	16.02	1.02E-04	0.05012
PPRC1	-3.17	7.04	15.88	1.06E-04	0.05012
HERC1	2.71	7.79	15.76	1.12E-04	0.05012
ZNF583	3.60	6.52	15.81	1.13E-04	0.05012
SEMA6D	3.28	6.89	15.74	1.13E-04	0.05012
DGKE	-3.74	6.56	15.76	1.16E-04	0.05012
TRPS1	-3.53	6.77	15.56	1.23E-04	0.05193
TFDP2	3.01	7.16	15.31	1.39E-04	0.05695
FIP1L1	2.74	7.50	15.18	1.48E-04	0.05915
NPR2	3.59	6.52	15.12	1.56E-04	0.05958
ZNF691	-3.43	6.46	15.12	1.56E-04	0.05958
CTNNA2	-3.56	6.51	15.05	1.61E-04	0.06002
PCMTD1	2.62	7.69	14.94	1.66E-04	0.06033
TEAD1	3.21	6.89	14.88	1.71E-04	0.06061
CFLAR	-3.07	7.04	14.79	1.79E-04	0.06199
AHI1	3.22	6.84	14.65	1.91E-04	0.06427
GUCY1A3	3.59	6.52	14.59	2.01E-04	0.06427
ITPR1	3.45	6.77	14.54	2.02E-04	0.06427
SIRT4	3.37	6.45	14.59	2.02E-04	0.06427
CAB39L	-3.22	6.87	14.34	2.22E-04	0.06814
DAP3	3.33	6.73	14.33	2.22E-04	0.06814
KCNAB1	2.64	7.14	14.00	2.61E-04	0.07355
NCAM1	-2.76	7.48	14.00	2.62E-04	0.07355
ANK3	3.50	6.50	14.01	2.66E-04	0.07355
ZCCHC6	3.01	7.01	13.96	2.67E-04	0.07355
CDC27	2.79	7.47	13.94	2.68E-04	0.07355
CNOT6L	-2.73	7.48	13.91	2.73E-04	0.07355

MTSS1	3.17	6.67	13.79	2.90E-04	0.07548
ERC1	3.17	6.64	13.74	2.96E-04	0.07548
RND2	-3.21	6.68	13.73	2.98E-04	0.07548
NR3C1	3.30	6.72	13.72	3.00E-04	0.07548
KDM5B	-3.00	6.78	13.68	3.04E-04	0.07548
CCDC150	3.53	6.51	13.62	3.22E-04	0.07852
XPNPEP3	-3.19	6.41	13.55	3.32E-04	0.07903
CXHXorf23	-3.14	6.81	13.48	3.37E-04	0.07903
SLC26A2	-3.27	6.70	13.45	3.41E-04	0.07903
KIF27	2.08	8.53	13.43	3.44E-04	0.07903
GAS2L3	3.32	6.45	13.41	3.57E-04	0.07915
CPED1	3.03	6.62	13.30	3.68E-04	0.07915
LOC105612555	3.24	6.69	13.28	3.72E-04	0.07915
TMEM232	3.19	6.68	13.27	3.72E-04	0.07915
DLG1	-3.36	6.69	13.24	3.79E-04	0.07915
GOLGA1	-3.23	6.42	13.26	3.82E-04	0.07915
CIB1	-2.87	6.71	13.20	3.85E-04	0.07915
GBF1	-2.46	7.50	13.20	3.85E-04	0.07915
SCN3A	3.25	6.41	13.05	4.25E-04	0.08241
CEP57	-3.06	6.61	12.99	4.27E-04	0.08241
ADARB1	-3.20	6.42	13.00	4.34E-04	0.08241
TENM1	3.34	6.46	12.99	4.37E-04	0.08241
PSTPIP1	3.34	6.45	12.98	4.38E-04	0.08241
PRMT3	-2.81	6.81	12.93	4.40E-04	0.08241
TPRKB	-2.66	6.94	12.93	4.41E-04	0.08241
KIF21A	-2.09	8.88	12.91	4.45E-04	0.08241
ABLIM1	2.82	7.34	12.89	4.48E-04	0.08241
LGALS8	2.58	7.46	12.82	4.64E-04	0.08430
CFAP44	-2.89	6.89	12.76	4.79E-04	0.08436
BTBD16	3.27	6.43	12.80	4.80E-04	0.08436
PLK4	-2.98	6.75	12.75	4.81E-04	0.08436
STAU2	2.97	6.96	12.73	4.86E-04	0.08436
TBL1X	-2.52	7.49	12.68	4.99E-04	0.08558
RBM23	2.44	7.64	12.60	5.18E-04	0.08802
ZNF484	3.23	6.43	12.55	5.42E-04	0.09107
GLTSCR1L	-2.69	6.82	12.39	5.73E-04	0.09519
VPS13B	2.61	7.02	12.35	5.87E-04	0.09646
CHRD1L	-3.03	6.37	12.36	5.96E-04	0.09673
C15H11orf74	-3.03	6.38	12.34	6.01E-04	0.09673
AKAP13	3.02	6.82	12.25	6.16E-04	0.09814
CEP63	2.93	6.74	12.21	6.28E-04	0.09899
ACTR6	-2.91	6.35	12.23	6.34E-04	0.09899

Supplementary Data 2. Differential expression data for the 50 genes with lowest *p*-values. LogFC = Log2 fold change between experimental conditions. LogCPM = Log2 counts per million. FDR = False discovery rate.

523 Supplementary Data 3

Category	GO Term	Fold Enrichment	p-value
Cellular Component	Centrosome	4.03	0.0156
Molecular Function	Nucleic acid binding	2.96	0.0160
Biological Process	Spermatid development	9.60	0.0379
Cellular Component	Node of Ranvier	47.01	0.0412
Molecular Function	Guanylate cyclase activity	45.21	0.0427
Biological Process	Regulation of transcription, DNA-templated	2.95	0.0498

524 *Supplementary Data 3. GO terms identified as enriched in the list of 99 DEGs with FDR < 0.1 by DAVID.*

525

526 Supplementary Data 4

All_correlations
(Sup Data 2).pdf

527

528 *Supplementary Data 4. Correlations between DEGs from biosolid vs control DGE analysis which were identified as having*
529 *enriched GO terms, against the geometric mean of germ cell markers.*

530

531 Supplementary Data 5

Name of Data Set	p-value	Number of Positively Correlating Genes	Number in Common	Number of Genes Combined
Feig et al (2007) DOI: 10.1093/molehr/gal097				520
Testicular biopsies of azoospermia patients with Sertoli-cell-only syndrome _vs_ full spermatogenesis	3.57E-18	338	105	
Testicular biopsies of azoospermia patients with Sertoli-cell-only _vs_ spermatid stage arrest	2.43E-16	274		
Testicular biopsies of azoospermia patients with Sertoli-cell-only _vs_ spermatocyte stage arrest	2.21E-10	144		
Spiess et al (2012) DOI: 10.1093/humrep/dem292				
Testis from infertile man + Johnsen score 3.2 _vs_ full spermatogenesis	3.44E-07	482	465	
Testis from infertile man + Johnsen score 2 _vs_ full spermatogenesis	8.16E-06	542		
GSE6023				
SCOS patient testicular biopsy - Johnsen Score 10 _vs_ Score 2	2.80E-05	93	47	
SCOS patient testicular biopsy - Johnsen Score 9 vs Score 2	4.48E-07	98		

Supplementary Data 5. DEG data sets identified as having significant positive correlation with the biosolid DEG data.

532

533

534 Supplementary Data 6

All_basespace_correlations (Sup Data 3).

535

536 *Supplementary Data 6. Correlations between common DEGs from biosolid vs control DGE analysis and BaseSpace*
537 *correlations which were identified as having enriched GO terms or pathways, against the geometric mean of germ cell*
538 *markers.*

539

540

541 Supplementary Data 7

Gene	Forward Primer	Reverse Primer
CCND1	GATGCCAACCTCCTCAACGA	TGAACTTCACGTCTGTGGCA
ACLY	GCCCCAAGATTCAGTCCCAA	CGATGGTCCGGATCTGAGTG
ACOX1	TGGCTGCATTATCCAAAGGA	ATCAGGCTTCACCTGGGCAT
VEGFA	CTTGCCTTGCTGCTCTACCT	GCCTCGGCTTGTCACATTTTTC
ACAD11	ACATCCCTGCCATGAACCAG	GCTACATCGGGCTCTGTCAT
ACACA	GAAGTCCCTCAGACTCTTAACC	AACCAGACATGCTGGATCTCAT
LPL	GACTCCAACGTCATCGTGGT	TGTGGAAACTTCAGGCAGGG
PDPK1	GACGACGAGGACTGCTATGG	CAGGCAGAGAACCTCAAGGG
LDHA	GGCCCATTAGGGCATCTCT	TGCTGTTACATTATAGTCTTTGCCA
HK1	ACCAAGTCAAAAAGATTGACAAGT	TTGGCATCATAGTCCCCACG
FASN	GCCTCACTGCCTTCCAGATT	GACTCGGGGCTGATGTCAAT
CPT1A	GCGCCGGGTGCATTC	CTCCCACCACTCACTCACATAA
FABP4	CATGAAAGAAGTGGGTGTGGG	GGTAGCAGTGACACCGTTCAT
BIRC5	ACCAGATGACGACCCTATAGAAGA	CAATGGCACAGCGGACTTTC

542 *Supplementary Data 7. Primer details for qPCR.*