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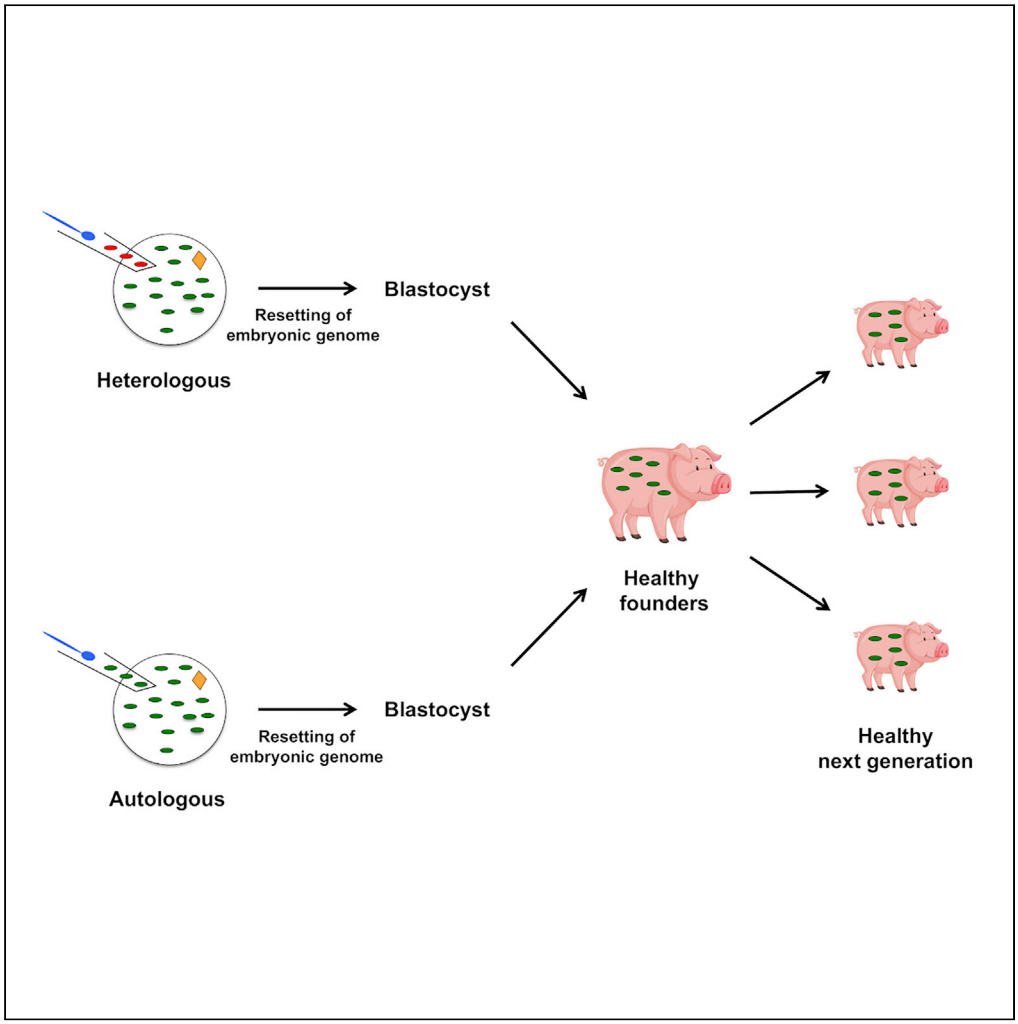

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Highlights

Adding extra mtDNA into oocytes produces healthy offspring that exhibit minor changes

Mitochondrial DNA and imprinting integrity do not appear to be impaired

The female founder population is fertile and produces normal litters

The next generation appears to modify the minor changes and is healthy

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Does supplementation of oocytes with additional mtDNA influence developmental outcome?

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SUMMARY

Introducing extra mitochondrial DNA (mtDNA) into oocytes at fertilization can rescue poor quality oocytes. However, supplementation alters DNA methylation and gene expression profiles of preimplantation embryos. To determine if these alterations impacted offspring, we introduced mtDNA from failed-to-mature sister (autologous) or third party (heterologous) oocytes into mature oocytes and transferred zygotes into surrogates. Founders exhibited significantly greater daily weight gain (heterologous) and growth rates (heterologous and autologous) to controls. In weaners, cholesterol, bilirubin (heterologous and autologous), anion gap, and lymphocyte count (autologous) were elevated. In mature pigs, potassium (heterologous) and bicarbonate (autologous) were altered. mtDNA and imprinted gene analyses did not reveal aberrant profiles. Neither group exhibited gross anatomical, morphological, or histopathological differences that would lead to clinically significant lesions. Female founders were fertile and their offspring exhibited modified weight and height gain, biochemical, and hematological profiles. mtDNA supplementation induced minor differences that did not affect health and well-being.

INTRODUCTION

Mitochondrial DNA (mtDNA) is a double stranded, circular genome that encodes 13 of the subunits of the electron transfer chain, the cell's major generator of ATP through the biochemical process of oxidative phosphorylation.[1] It also encodes 22 tRNAs, and 2 rRNAs. Normally, mtDNA is maternally-only inherited and this is the expected outcome when offspring are generated through natural mating, artificial insemination, *in vitro* fertilization and intracytoplasmic sperm injection (ICSI).[2,3] However, exceptions have been reported in mammals and Drosophila, which include the transmission of sperm mtDNA when matings between crossings of diverse genetic backgrounds take place that result in the generation of live offspring.[4–6] Likewise, the introduction of the more sophisticated assisted reproductive technologies, such as somatic cell nuclear transfer, embryonic cell nuclear transfer, metaphase II spindle transfer, pronuclear transfer, polar body transfer, cytoplasmic transfer, and mitochondrial transfer or supplementation can result in more than one population of mtDNA being transmitted to the offspring (heteroplasmy; reviewed in[7]). This form of heteroplasmy is distinct from the heteroplasmy associated with mtDNA disease where mutant and wild type molecules co-exist.[8] Nevertheless, a number of naturally occurring non-pathogenic variants (mutations and deletions) are frequently present in the female germline, which are also passed from one generation to the next, as well as spontaneous rearrangements that can take place.[9,10] In each case, their presence tends to be at low levels.[9]

Cytoplasmic transfer was proposed as a method to enhance embryo development for infertile couples whose embryos underwent repeated developmental failure.[11] Simply, cytoplasm, containing mitochondria, mtDNA and RNA amongst other factors, was taken from a young donor's oocyte and introduced into the oocytes of couples suffering from repeated embryonic developmental failure. However, the offspring possessed two populations of mtDNA,[12] which led to this approach being banned in many jurisdictions. Although a limited follow-up study on the offspring did not reveal any major concerns,[13] studies in, for example, rodent models suggested that the presence of two genetically diverse populations of mtDNA could affect an offspring's health and development and lead to serious clinical complications.[14]

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Furthermore, offspring derived from somatic cell nuclear transfer, where two populations of mtDNA often co-exist, can present with characteristics similar to mitochondrial disorders.[15]

To overcome the transmission of two or more populations of mtDNA, autologous mitochondrial supplementation has been proposed. It has been practiced in several jurisdictions resulting in the birth of children,[16,17] although in depth pre-clinical studies in appropriate large animal models have not been undertaken to determine the safety and efficacy of this approach. Furthermore, there appear to be no in-depth studies reported on the development and health of the children so far generated. The mitochondrial supplement can originate from several sources. These include the patient's own oogonial stem cells[17] or cumulus cells,[16] which ensures mtDNA genetic integrity and identity are maintained. Our approach has been to use a mitochondrial supplement derived from sister oocytes that was purified and condensed into a small bolus before its introduction into poor quality oocytes that were deficient in mtDNA as they were fertilized by ICSI, referred to as mICSI.[18] Although the amount of mtDNA is very small (approximately 780 copies), the process induces a mtDNA replication event that takes place between fertilization and the 2-cell stage, which increases mtDNA copy number four-fold and is mirrored by changes to the DNA methylation status of mtDNA-specific replication factor, POLGA.[19] To this extent, we have shown that poor quality oocytes can be rescued whereby the resultant blastocysts exhibited gene expression profiles similar to blastocysts derived from non-supplemented, good quality oocytes.[18,19] Indeed, the introduction of mtDNA into *in vitro* matured metaphase II oocytes resulted in over 2000 local genomic regions exhibiting differential patterns of DNA methylation at the blastocyst stage which, in turn, impacted on gene expression when compared with their non-supplemented counterparts.[20]

Consequently, it remains to be determined whether additional copies of mtDNA that are introduced into the oocyte at the time of fertilization would impact on offspring development, health and well-being. To do this, we have used the pig as our model, which is regarded as an excellent model to study human development because many of its organ systems and physiological and pathophysiological responses are similar to those of the human.[21] Likewise, its embryology and early development are very similar to that of the human[22]; as are its mtDNA copy number, replication and reduction events during development.[23,24] Furthermore, the pig industry and national welfare agencies have developed criteria using non-physiological and physiological measures that allow for the assessment of animal welfare, health and well-being.[25] These include the assessment of activity; the use of body condition scoring, which determines whether the pelvic bones, loins and ribs are covered to an appropriate depth by tissue; assessment of cutaneous, musculoskeletal, and gastrointestinal indicators of illness, and any other observable abnormalities; analyses of biochemical and hematological markers; and daily height and weight gain. Although these are pig specific, to a degree, they mimic the assessments performed on newborns in hospitals before departure; and growth and development assessments during childhood.

We have introduced additional copies of mtDNA isolated from either sister (autologous) or third party (heterologous) oocytes (see Figure 1) that failed to mature into mature metaphase II oocytes. In the context of human clinical practice, this approach uses a source of clinically available material that would otherwise be discarded and does not require the destruction of mature oocytes. Following zygote transfer and the birth of live offspring, we were able to assess the impact of both approaches on offspring growth and development and parameters associated with biochemistry, hematology and body condition scoring using established criteria for the pig[25]; and tissue and organ structure through histopathology. We further analyzed the DNA methylation profiles of key imprinted genes and the transmission of mtDNA in the offspring.

RESULTS

Embryo development rates

To determine if mtDNA present in isolated mitochondria from failed-to-mature oocytes impacted on fertilization and preimplantation developmental outcome, we first fertilized matured oocytes by intracytoplasmic sperm injection (ICSI) and by ICSI with extra copies of mtDNA (mICSI) derived from sister oocytes. We found that 78.2% of ICSI and 67.9% of mICSI treated oocytes survived ($p < 0.01$). However, blastocyst rates were similar at 18.5% from ICSI and 18.2% from mICSI presumptive zygotes (Table 1). Consequently, despite the introduction of extra copies of mtDNA resulting in a lower percentage of oocytes surviving compared to ICSI, both approaches had similar rates of blastocyst development. To this extent, 5.4 and 5.5 zygotes were required to generate ICSI- and mICSI-derived blastocysts, respectively (Table 1).

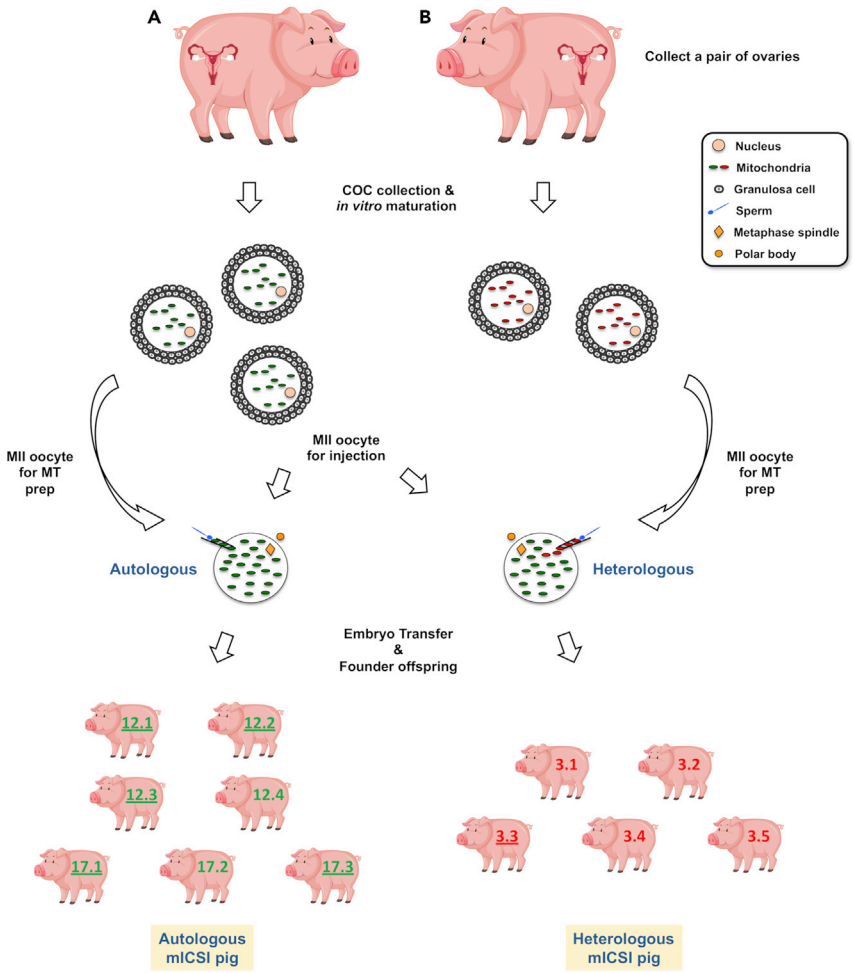

Figure 1. Schematic representation of the production of autologous and heterologous mICSI-derived offspring

Each ovary pair collected from an individual pig was treated separately from other ovary pairs throughout the experiment. Cumulus-oocyte-complexes (COC) were collected from each ovary pair and cultured *in vitro* to maturation. MII oocytes were prepared from cultured COCs for intracytoplasmic sperm injection with mitochondrial supplementation (mICSI). For autologous mICSI, the remaining 'sister' oocytes from each ovary pair (e.g. from pig 'A') were used to prepare the mitochondrial (MT) isolate, as indicated by the same color (green). For heterologous mICSI, MT isolates were prepared from the oocytes of another ovary pair (e.g., from pig 'B'), as indicated by the different color (red). Consequently, mICSI was performed using mitochondria isolated either from sister oocytes (autologous) or third party source of oocytes (heterologous). Zygotes were transferred to a recipient pig and offspring were generated as described in the STAR Methods. Three sets of mICSI founder offspring were generated as illustrated and the number on the pig indicates its ID and an underlined number represents female offspring. Images of pig and ovary were designed by brgfx/Freepik.

Generation of founders by mICSI

In order to determine if mtDNA isolated from failed-to-fertilize sister oocytes or third-party oocytes would be more developmentally efficient, we generated two sets of founders (F0), namely heterologous and autologous founders (Figure 1). In all, five heterologous founders were derived using mtDNA supplement derived from a third party (3 series pigs; Figures 1 and 2). These comprised four male (Pigs 3.1, 3.2, 3.4 and 3.5) and one female (Pig 3.3) founders from one pregnancy. In this instance, on the day of embryo production, oocytes from three ovarian sources were used to generate the founders and the third party mtDNA was derived from oocytes from a fourth ovarian source (mitochondrial preparation 3; MP3; Figure 2).

We further generated another two pregnancies (12 series and 17 series pigs; collectively referred to as autologous; Figures 1 and 3) that produced a further seven autologous founders consisting of 12.1, 12.2, 12.3 - female and 12.4 - male pigs; and 17.1, 17.3 - female and 17.2 - male pigs. On this occasion, they

Table 1. Blastocyst development rates for ICSI and mICSI

Parameters	ICSI	mICSI
Injected oocyte	284	268
Oocytes survival after injection (%)	222 (78.2%)	182 (67.9%)**
Presumptive zygotes	222	181
Blastocysts at day 7 (%)	41 (18.5%)	33 (18.2%)
Zygote: blastocyst ratio	5.4:1	5.5 : 1

**$p < 0.01$ using χ^2 test.

were generated by supplementing oocytes with mitochondria isolated from sister oocytes (MP9, MP11, MP12, MP13, MP 14, MP15, MP16 and MP17; Figure 3). Each of the founders aligned closely with mtDNA haplotypes previously identified in Australian commercial breeding lines (Figure S1). In each case, the surrogates were gilts that had undergone hormonal stimulation in preparation for receptivity and this represented their first parity.

Parturition, general health and body condition scoring

For the heterologous (3 series) founders, farrowing took place 116 days after embryo transfer. There were no complications at birth and all farrowed without intervention. The birth weights ranged from 0.72 kg (Pig 3.2) to 1.97 kg (Pig 3.4) and heights from 14 cm (Pig 3.2) to 20 cm (Pig 3.4). In the context of time to stand, three pigs (Pigs 3.1, 3.3 and 3.4) achieved this milestone within 3 min and two required over 5 min (Pigs 3.2 and 3.5). Likewise, the same three pigs found the udder within 5 min and the other two required over 5 min. Pig 3.2 also required help with feeding, whereas Pigs 3.3 and 3.5 experienced delay in feeding, with 3.3 also requiring stimulation and warmth to feed. The mobility for Pigs 3.1, 3.3 and 3.4 was judged to be healthy, whereas for Pigs 3.5 and 3.2, it was deemed to be moderately compromised and very compromised, respectively. When undergoing body condition scoring[25] at weaning, the 3 series founders exhibited a grossly normal appearance (body condition score 3) and were bright, alert and responsive. However, Pig 3.4 exhibited a dirty perineum which was likely indicative of scouring.

For the autologous cohorts, farrowing took place after 114 days for the 12 series founders without intervention. There were no complications at farrowing except for some meconium staining observed in Pigs 12.1 and 12.2 and at a higher level in Pig 12.1. The birth weights for the four offspring ranged from 1.44 to 1.74 kg. Throughout the 30 days post-birth, they demonstrated normal activity and approached within accepted time limits (<20 s). The surrogate carrying the 17 series offspring farrowed at 117 days following induction using one injection of 0.5 mL Juramate on day 116. In the context of time to stand and find the udder (suckling), there were no remarkable indications. Time to stand ranged from 1 to 2 min and time to suckle ranged from 3 min to over 20 min. This slightly extended timeline was because of the surrogate

Figure 2. Phylogenetic tree of the whole mitochondrial genomes of the heterologous founders (Pigs 3.1 to 3.5) and MP3

The phylogenetic tree was constructed using the General Time Reversible nucleotide substitution model and Neighbor Joining method with 1000 bootstrap replicates. Scale bar indicates the number of substitutions/changes per nucleotide.

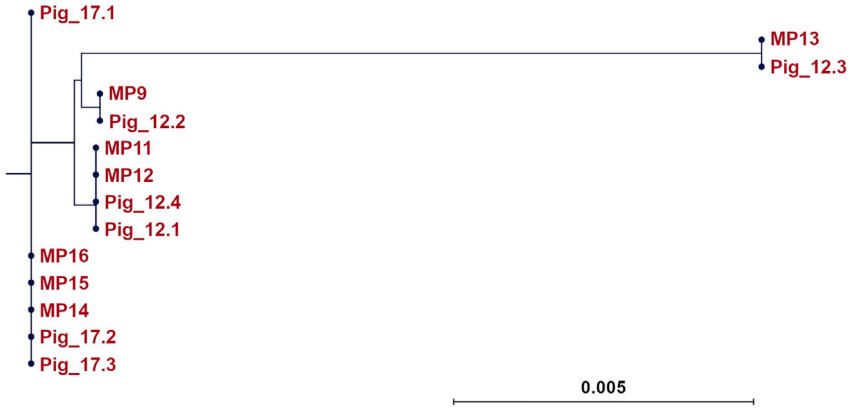

Figure 3. Phylogenetic tree of the whole mitochondrial genomes of the autologous founders (Pigs 12.1 to 12.4 and 17.1 to 17.3) and MP9 and MP11 to 16

The phylogenetic tree was constructed using the General Time Reversible nucleotide substitution model and Neighbor Joining method with 1000 bootstrap replicates. Scale bar indicates the number of substitutions/changes per nucleotide.

taking time to lie down. In terms of body condition scoring for the 12 and 17 series at weaning, there were no remarkable features with each pig being scored as healthy and exhibiting good anatomical development. Consequently, after three independent sets of mICSI embryo transfers, prenatal development did not result in any significant post-parturition events.

Height and weight from birth to sexual maturity

At weaning, compared to controls (naturally mated colony pigs), the heterologous and autologous founders were significantly heavier ($p < 0.05$; Figure 4A) and taller ($p < 0.05$; Figure 4B). However, there were no differences for body mass index (BMI; Figure 4C). In the context of overall daily weight gain, the heterologous founders were significantly heavier than controls and the autologous founders ($p < 0.001$) (Table 2). However, in terms of height, both the heterologous and autologous founders had significantly greater mean daily growth rates than controls ($p < 0.001$). Nevertheless, one of the heterologous males (Pig 3.2) was lighter in weight and shorter at birth and maintained his smaller stature throughout development. However, his growth trajectory was not stifled and he was not sufficiently small enough to be classified as a runt.

Gross anatomical analysis

On reaching sexual maturity, autologous and heterologous founders ($n = 6$) and controls ($n = 3$) were killed, then immediately autopsied, and a wide range of tissues (Data S1) collected for histopathological examination. In all pigs, the spleen was congested, but this is recognized as being passive congestion because of smooth muscle relaxation induced by the euthanasia agent. In several pigs, there were areas of pulmonary atelectasis (an artifact of immersion fixation), congestion (likely an agonal change) and mild edema with a few aggregated alveolar macrophages (a common incidental finding). Retinal detachment was commonly found, but this is an artifact of formalin fixation. It was concluded that there were no consistent morphological changes, with the exception of the euthanasia agent-induced splenic congestion, in these pigs and no histopathological changes likely to contribute to the development of later, clinically significant, lesions.

In addition, autologous founder sows 12.1, 12.2, 12.3, 17.1 and 17.3 underwent autopsy. They did not exhibit any external abnormalities. Inspection of the thorax revealed normal heart and lung appearance with normal left and right ventricular wall thickness ratios. One sow had cranioventral pulmonary consolidation indicative of previous mild *Mycoplasma hyopneumoniae* infection whereas two sows had small areas of pleural adhesion indicative of *Actinobacillus pleuropneumoniae*. Both of these infections are present within the general colony at the piggery and were, thus, classified as unremarkable. Inspection of the abdomen revealed normal liver and kidney appearance and no enteric or splenic abnormalities. However, one sow exhibited a renal cyst. In all, this suggests that mitochondrial supplementation using either sister or third party source oocytes did not impact on anatomical structure or morphology.

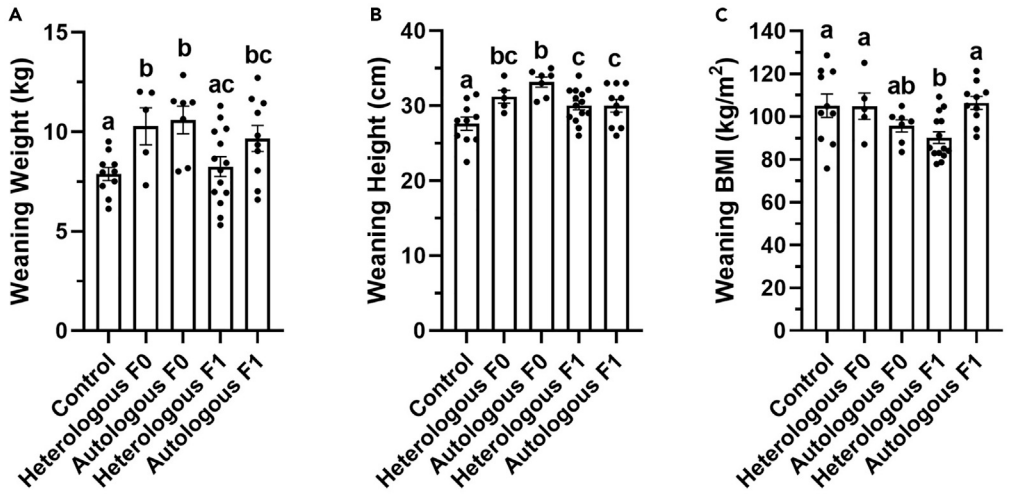

Figure 4. Piglet weight, height and body mass index

Data were collected at weaning for (A) weight, (B) height at the shoulder and (C) body mass index (BMI, kg/m^2) from control (n = 10), heterologous F0 (n = 5), autologous F0 (n = 7), heterologous F1 (n = 14), and autologous F1 (n = 10) piglets. Data expressed as mean ± standard error of the mean (SEM). Differing letters denote statistical significance of $p < 0.05$. Adapted from Table S5.

Transcriptome analysis of heart tissue for founder pigs

Because we have previously demonstrated that mitochondrial supplemented porcine embryos exhibited differential gene expression compared to controls,[18,19] we performed RNAseq on heart tissue from three control pigs (Pigs C4, 7 and 26) and five mtDNA supplemented founder pigs (Pigs 3.1, 3.3, 3.4, 12.4 and 17.2; Table S1). Multidimensional scaling (MDS) analysis revealed significant variation in Pig 3.1 (Figure 5A). This was most likely due to degradation of the RNA sample when assessed by a nucleic acid analyzer and was, thus, removed from the analysis. Female Pigs C7 and 3.3 formed a distinctive group, separated from the other male pig samples (Figure 5B); therefore, sex was included in the linear model for data correction. Over 16,000 genes were expressed and used for differential gene expression analysis (Figure 5C). In total, 54 genes showed differential expression (FDR <0.05) in the cohort of heart samples from the mtDNA supplemented founders, of which 21 were upregulated and 33 were downregulated (Table S2 and Figure 5D). Gene ontology (GO) enrichment analysis identified 16 GO terms associated with 'mitochondrial biological process' and 'cellular component' which were over-represented in the DEGs (Tables S6 and S7). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis identified three pathways associated with cardiomyopathy which were over-represented in the DEGs, namely hypertrophic ($p = 0.02$) and dilated ($p = 0.02$) cardiomyopathy in the upregulated genes, and diabetic cardiomyopathy

Table 2. Summary of estimated daily weight and height gain from linear mixed model analysis

Measurement	Comparison	Control	Heterologous F0	Heterologous F1	Autologous F0	Autologous F1
Weight (g)	Estimated mean daily growth (g), [95% CI]	673 [635-712]	793 [743-843]	665 [631-700]	685 [644-726]	625 [602-647]
	Difference from controls (g), [95% CI]		12 [6-18]	−1 [-6-4]	1 [-4-7]	−5 [-9-0]
	p value relative to control		<0.001	0.769	0.687	0.033
Height (mm)	Estimated mean daily growth (mm), [95% CI]	2.7 [2.7–2.8]	3.1 [3.0–3.1]	2.8 [2.8–2.9]	2.9 [2.8–2.9]	2.8 [2.8–2.9]
	Difference from controls (mm), [95% CI]		0.3 [0.3–0.4]	0.1 [0.0–0.1]	0.1 [0.5–0.2]	0.1 [0.0–0.1]
	p value relative to control		<0.001	0.003	<0.001	<0.001

Data presented from control n = 10, heterologous F0 n = 5, heterologous F1 n = 14, autologous F0 n = 7, autologous F1 n = 10. CI = confidence interval.

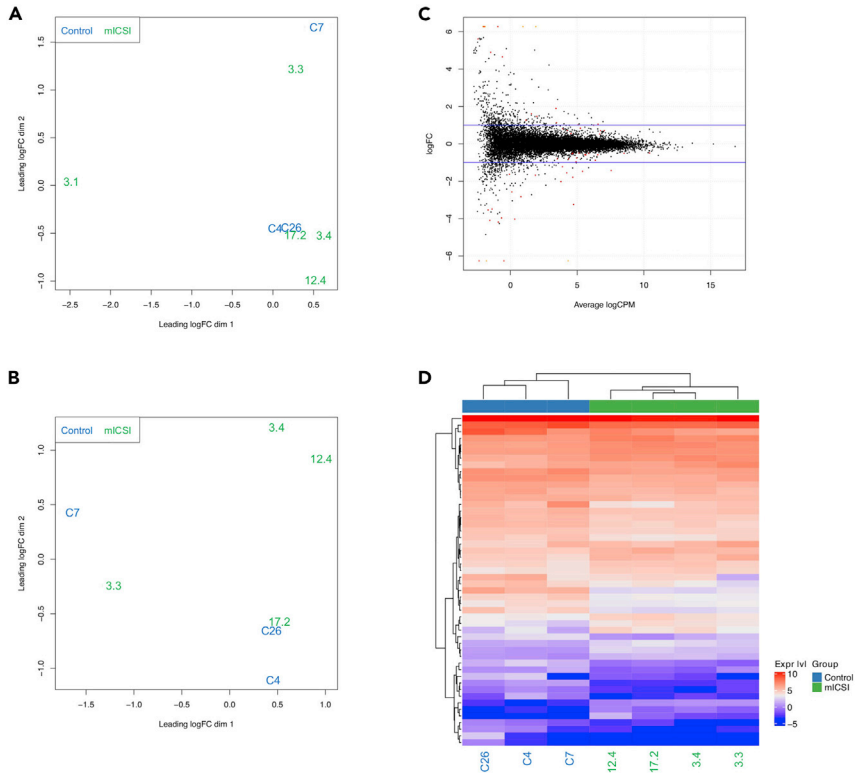

Figure 5. Transcriptome analysis of heart tissue in mICSI and control pigs

(A) Multidimensional scaling (MDS) of all RNAseq sample data.

(B) MDS plot excluding mICSI_1 sample.

(C) Smear plot showing log2-fold-change (y axis) plotted against the log2 counts per million reads (x axis). Genes showing significant differences (FDR<0.05) are shown in red.

(D) Heatmaps showing the clustering of each sample present in the comparison using the top 50 most differentially expressed genes for the comparison between control and mICSI pig RNAseq samples.

($p = 3.07 \times 10^{-7}$) in the downregulated genes (Table S8). Although histopathology and autopsy did not reveal any major defects in structure of the heart tissue, the gene expression analysis suggests the potential for cardiac problems.

Biochemical and histological assessments

Using standard clinical biochemistry and hematology assessments as described in,[25] we observed a number of differences amongst control, and autologous and heterologous founder pigs at weaning and sexual maturity. Each assessment is listed in Tables S9–S12. Of interest, in weaning age pigs, cholesterol and bilirubin (heterologous and autologous founders); and anion gap and lymphocyte count (autologous founders) were all elevated above reference or published ranges (Table 3). Differences outside of publishable ranges for sexually mature pigs included potassium (heterologous founders) which was elevated above the reference range and bicarbonate (autologous founders) which was below the reference range (Table 3).

Assessments which occurred within ranges associated with previously published datasets but significantly different from controls at the weaning stage are listed in Table S13. At the adult stage, there were again differences that were within published ranges as shown in Table S14.

Fertility of heterologous and autologous founders

To determine whether the founders were reproductively competent, we mated the female offspring with colony stud boars. The heterologous Pig 3.3 produced a litter of 14 F1 offspring (seven males and seven females). These exhibited good body condition scoring (BCS 3) when assessed at both weaning and

Table 3. Summary of Biochemical and haematological differences for heterologous and autologous weaner piglets and sexually mature pigs relative to control, which were statistically significant and outside of reference ranges within previously cited datasets and analyzing laboratory reference ranges, as indicated in Tables S9–S12

Direction of observed change	Heterologous F0	Autologous F0	Heterologous F1	Autologous F1
Weaner piglets				
Increased	Cholesterol Bilirubin	Cholesterol Anion gap[a] Bilirubin Lymphocyte count	Anion gap	MCH concentration Monocyte count
Decreased			Creatinine Potassium[b]	Creatinine
Sexually mature pigs				
Increased	Potassium			Not assessed
Decreased		Bicarbonate[b]		Not assessed

MCH, mean corpuscular hemoglobin.
[a]Test group outside of reference range compared to control; however not statistically significant.
[b]Control measurement outside of published reference ranges.

adulthood. Two of the F1 females (Pig 3.3.1 and 3.3.2) were also mated and, at euthanasia, one was carrying 15 fetuses whereas the other carried 16 fetuses, of which one was a mummy. At autopsy, the two F1 females (Pigs 3.3.1 and 3.3.2) appeared normal with no visible abnormalities present.

The four autologous founders were also mated and produced litters of 8 (12.2), 10 (17.3), 11 (12.3) and 14 (12.1) offspring. In terms of these offspring, we adopted a non-interventionist approach, where we sought to determine whether the mother and offspring would fend for each other and for themselves, as is the situation following breeding in a piggery. However, 10 of the offspring from 12.1 died as the mother crushed them. A similar occurrence took place for two of the offspring from 12.3 and one offspring was euthanized as it was not viable. In terms of 17.3, two offspring were crushed and one exhibited splay leg, which is associated with males within the colony. Four of the offspring from 12.1 died through crushing, one died seven weeks after birth, and one was euthanized because of low viability. Based on these limited data, this suggests that F1 offspring generated through mtDNA supplementation may require assisted postnatal care to ensure survivability. Nevertheless, when assessed for body condition scoring at weaning, all the remaining pigs were bright, alert and active. However, the 12.1 cohort did present with some scouring; and the 17.3 cohort exhibited mild stunting which likely also resulted from previously treated scouring. One pig had also been treated for arthritis but appeared to be unaffected when assessed for body condition scoring.

Of interest, the born alive rates for the heterologous and autologous F1s are similar to those across the five mtDNA haplotypes of commercial pigs in Australia (Figure 2). Their mean born alive rates are 9.85 $\pm$ 0.27 (mean $\pm$ SEM; haplotype A - KT279758), 10.14 $\pm$ 0.40 (haplotype B - KT261429), 11.32 $\pm$ 0.30 (haplotype C - KT279759), 10.89 $\pm$ 0.44 (haplotype D - KT279760), and 11.57 $\pm$ 0.40 (haplotype E – KT261430).[26] We further mated founder 17.1 and she had 15 fetuses present at the third trimester. These outcomes suggest that mtDNA supplementation does not impact on the resultant offspring's fertility.

Assessment of F1 heterologous and autologous offspring

Each of the F1 offspring underwent similar assessments as their founders. At weaning, the heterologous offspring were similar in weight to the controls whilst the autologous offspring were significantly heavier ($p < 0.05$). The heterologous offspring were also significantly lighter than their founders ($p < 0.05$; Figure 4A). In terms of height at weaning, the autologous and heterologous offspring were taller than controls but the autologous offspring shorter than their founders (Figure 4B). Finally, the heterologous offspring had lower BMIs at weaning compared to controls, their founders and the autologous founders. Overall weight gain and height gain (birth to sexual maturity) were fitted to linear models to compare overall weight and height gain per day across the model. In terms of daily weight gain, the heterologous offspring gained

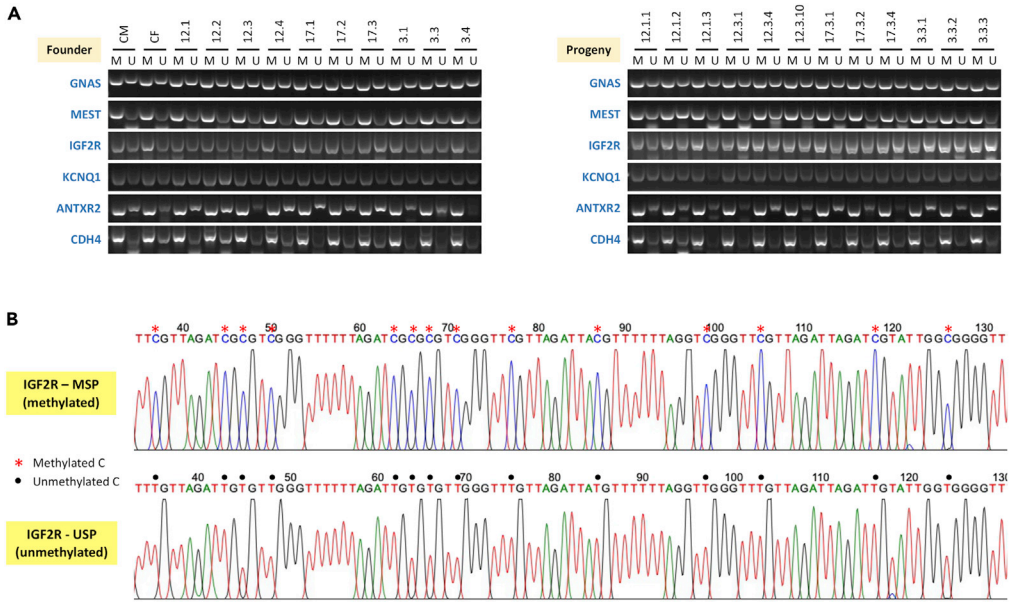

Figure 6. DNA methylation status of imprinted genes in *Sus scrofa* mICSI founders and offspring

(A) Methylation specific-PCR was carried out to investigate the DNA methylation status of tail DNA samples extracted from control male and female (CM and CF, respectively) pigs, mICSI founder pigs (top panel) and their progeny (bottom panel). Primers were designed for methylated (M) and unmethylated (U) DNA fragment amplification from bisulfite treated DNA samples for target imprinted genes (*GNAS*, *MEST*, *IGF2R* and *KCNQ1*) and control genes (*ANTXR2* and *CDH4*).

(B) Confirmation of MSP amplicons by Sanger sequencing. Methylated and unmethylated MSP amplicons of *IGF2R* were purified and sequenced by the Sanger method. BS-unconverted/methylated and converted/unmethylated cytosines were indicated by red asterisk and black dot, respectively.

weight similarly to controls, however, the autologous offspring gained weight more slowly relative to controls ($p = 0.033$; Table 2). Height gain per day was significantly greater in the heterologous offspring ($p = 0.003$) and the autologous offspring ($p < 0.001$) relative to controls (Table 2). In all, there were notable adjustments to height and weight gain in the subsequent generation that tended to normalization or control values.

Biochemical and hematological analyses of the heterologous weaner F1 cohort revealed that anion gap was elevated and creatinine and potassium were decreased and outside of previously published datasets and reference ranges (Table 3). For the autologous F1 weaners, mean corpuscular hemoglobin concentration and monocyte count were elevated and outside of their respective reference ranges and levels of creatinine were similarly decreased. At the adult stage, only the heterologous F1s were assessed, and their values appeared to have normalized to those of controls (Table 3). Consequently, the increased levels of cholesterol observed in the heterologous weaner founders appeared to have resolved by adulthood in founders and F1s.

DNA methylation patterns of imprinted genes

Because discrepancies between various assisted reproductive technologies and imprinted genes have been reported, we investigated whether the DNA methylation status of imprinted genes was conserved as a result of mtDNA supplementation in offspring and the next generation. We screened the putative imprint control regions (ICRs) of selected imprinted genes of the pig,[20] namely *GNAS*, *MEST*, *IGF2R* and *KCNQ1*, by methylation-specific PCR. Given that paternal and maternal alleles of ICRs are differentially methylated,[27–29] we expected to amplify both methylated and unmethylated fragments from the regions. Indeed, we confirmed amplification of both fragments in controls, and heterologous and autologous mtDNA supplemented founders and their offspring at similar levels (Figures 6A and S2), which was also confirmed by Sanger sequencing (Figure 6B). On the other hand, for non-imprinted gene controls, namely

ANTXR2 and *CDH4*, the methylated fragment was amplified predominantly in all the samples. Consequently, the methylation patterns of the imprinted genes appear to be conserved in DNA isolated from tail samples from founders derived through mtDNA supplementation and in their offspring.

mtDNA genotyping of the heterologous cohort

Since the introduction of cytoplasmic and heterologous mitochondrial transfer and the other more invasive assisted reproductive technologies, two or more mitochondrial genomes can be transmitted to the offspring.[7,30] Consequently, the amount of additional mtDNA that is transmitted and the resultant mtDNA genetic identity of the offspring would be major concerns. We, therefore, performed whole mtDNA genome next generation sequencing on tail samples isolated shortly after birth to determine the mtDNA genotype of each of the heterologous founders. We also isolated DNA from MP3, which was used to supplement the oocytes. Following whole mtDNA genome next generation sequencing, we constructed sequence alignments that were employed to generate a phylogenetic tree (Figure 2) to show the varying degrees of mtDNA genetic divergence amongst the cohort of oocytes used to generate the offspring and provide MP3. The mitochondrial genomes of Pigs 3.1 and 3.4 were 16,680 bp in size; Pigs 3.2 and 3.5 possessed genomes of 16681 bp in size; and Pig 3.3's mtDNA genome comprised 16,682 bp. Pig 3.4 possessed the same genotype as MP3 in terms of content and size whereas Pig 3.3 was the most divergent (Figure 2). Nevertheless, we continued to assign Pig 3.4 to the heterologous cohort as the intent was to generate heterologous offspring in the experimental design for this group of founders; and the source of oocytes used to generate MP3 originated from a third party but maternally related ovarian source. This analysis also showed that Pigs 3.2 and 3.5 were derived from the same or sister ovarian sources.

Through next generation sequencing of the tail samples, we were also able to identify variants present amongst the heterologous founders (Data S2). Initially, we applied a minimum threshold of 3% for detection of variants as previously employed.[26,31] Pigs 3.1, 3.2 and 3.5 possessed the same two variants at similar frequencies. However, Pigs 3.3 and 3.4 possessed four and five variants, respectively. We extended our analysis by decreasing the threshold to 0.5% based on the premise that Pigs 3.1, 3.2, 3.3 and 3.5 arose from a different mtDNA haplotype to MP3 and we were seeking to determine whether there was low level transmission of variants specific to MP3. This approach increased the number of variants with Pigs 3.3 and 3.4 harboring the greatest number of variants, namely 36 and 35, respectively. On the other hand, Pig 3.1 harbored 18 variants, Pig 3.2 possessed 26 variants and Pig 3.5 possessed 23 variants. The highest frequency for a variant amongst this set of founders was 19.31% (deletion at position 376) in Pig 3.3. Our analysis also showed that single nucleotide variants (SNVs) specific to MP3 were detectable in Pigs 3.1, 3.2, 3.3 and 3.5 (green font). They accounted for 2/18 variants in Pig 3.1; 3/26 in Pig 3.2; 17/36 in Pig 3.3; and 2/23 in Pig 3.5. These unique SNVs to MP3 suggest low levels of transmission of the mitochondrial supplement to the heterologous founders. The remaining variants are likely naturally occurring variants (black font) present in the female germline, as previously shown in pigs with similar mtDNA genotypes.[9,19]

We further performed similar analyses on heart, brain and calf muscle tissue from Pigs 3.1, 3.3 and 3.4 to determine if there was biased or neutral segregation of the MP3 SNVs. From Data S2 (green font), it is evident that some of the SNVs are present in one or more of the four samples analyzed. Of interest, for Pig 3.1, only one MP3 specific variant was present in heart and brain tissue whereas the remainder were naturally occurring variants (black font). However, seven of the variants present in calf muscle tissue were specific to MP3, which represents an increase from the tail sample. For Pig 3.3, there were fewer variants present in heart (15 variants), brain (19 variants) and calf muscle (25 variants) compared with tail (36 variants) tissues. Nevertheless, the MP3 specific SNV at 376 (deletion) was present at similar levels to the tail sample (range = 15.98 to 19.31; Data S2). The calf muscle sample exhibited a greater number of MP3 specific SNVs compared to brain and heart and similar to tail. Because Pig 3.4 had the same genotype as MP3, there were no specific SNVs to track in other tissues. However, the likely naturally occurring variants were considerably fewer in heart, brain, and calf muscle.

We also analyzed 10 metaphase II oocytes from Pig 3.3 (Data S2). Overall, there were more naturally occurring variants present in mature oocytes than in somatic tissues. Furthermore, many of the MP3 SNVs present in the somatic tissues were not identifiable in the oocytes suggesting biased segregation or a presence at below threshold of detection at the lowest level (0.5%). However, the MP3 derived deletion at 376 persisted at similar levels in the oocytes to the somatic tissues; and the SNVs present at 2,620 and 10,179 were present in the oocytes but not the somatic tissues. Analysis of Pig 3.3's 14 offspring generated

through natural mating demonstrated an interesting pattern of transmission. A few of the MP3 SNVs that were present in the somatic tissues or oocytes of Pig 3.3 also persisted in the offspring (green font) with only the deletion at 376 and the SNV at 16,141 persisting in all the offspring. In addition, 14 other MP3 SNVs were also identified (yellow-orange font) that were neither present in the oocytes or somatic tissues of Pig 3.3. However, most of these were confined to three offspring. A number of additional naturally occurring variants were also identified in the offspring (red font) that were not identified in the mother's somatic tissues or oocytes.

mtDNA genotyping of the autologous cohorts

Our phylogenetic analysis of the autologous founders showed that Pig 12.1 and 12.4 exhibited the same mtDNA genotype at 16,681 bp in size (Figure 3) whist 12.2 (16,681 bp) and 12.3 (16,680 bp) exhibited different genotypes. Pigs 12.1 and 12.4 were generated through either MP11 or 12 whilst Pig 12.3 was generated with MP13 and Pig 12.2 with MP9. Pigs 17.1, 17.2 and 17.3 all originated from the same mtDNA genotype, which was 16,680 bp in size, and were generated using one of MP14, 15 or 16. Again, we undertook variant analysis using both 3% and 0.5% thresholds. Variant analysis of Pigs 12.1 and 12.4 revealed a number of common and different variants between the two offspring (Data S2). At a 3% threshold, Pig 12.3 harbored two variants, Pig 2.1 four variants and Pigs 12.2 and 12.4 four and five variants, respectively. Using a 0.5% threshold, Pig 12.1 harbored 25 naturally occurring variants whereas Pig 12.4 harbored 19. Many of these were present at less than 1% whereas five were present at between 4 and 7%. However, one variant, 13,748, was present at 1.82% in Pig 12.1 but at 33.89% in Pig 12.4. Pig 12.2 harbored 20 variants, again many present at less than 1% and the highest at 5.45%. However, the number of variants increased to 56 after the sequencing of 10 metaphase II oocytes. Overall, these naturally occurring variants tended not to be uniformly distributed across the oocytes with only several common to the majority of oocytes and the tail sample. These included the deletions at positions 376, 1253, 5188, 10,456 and 15,505; and the insertions at positions 1253 and 5188. Pig 12.3 harbored 21 naturally occurring variants which ranged in frequency from 0.52 to 5.25%. In this instance, the number variants present in 10 metaphase II oocytes decreased and were primarily indicative of deletions and insertions.

The 17 series founders harbored two (Pigs 17.2 and 17.3) and three variants (Pig 17.1) when assessed at the 3% threshold. However, they tended to harbor higher numbers of variants when assessed at 0.5% with Pigs 17.1, 17.2 and 17.3 possessing 32, 55 and Pig 54 variants, respectively (Data S2). They were also present at a frequency ranging from 0.51 to 25.52 for Pig 17.1, 0.52 to 5.13 for Pig 17.2 and 0.51 to 5.07 for Pig 17.3.

Consequently, the vast majority of variants identified in the autologous and heterologous founder somatic tissues and oocytes were at low levels and representative of underlying variants often observed in individuals and that mtDNA supplementation did not appear to affect the number of naturally occurring variants.

DISCUSSION

The pig has become one of the favored species to conduct human pre-clinical assessments and for the generation of models of human disease.[32] Studies in non-human primates are limited by the availability of species and their use is considered to be more contentious whereas rodents offer limited capability for translation of basic knowledge into clinical practice. The pig is frequently used to undertake toxicology studies and has a physiology and pathophysiology similar to the human.[21,22] It is also a model used to study oocyte and embryonic development given its closeness to the human[33]; and it is becoming an increasingly accepted model to study epigenetic regulation during human development including DNA methylation.[34,35] Consequently, by using oocytes from Australian commercial breeding lines of pigs that encompass the five mtDNA haplotypes we had previously identified,[26,36] we were able to generate offspring using a mtDNA supplement derived from failed-to-fertilize oocytes and assess the effectiveness of this approach for couples seeking to have children using more sophisticated assisted reproductive approaches.

When developing new assisted reproductive technologies, it is important that the outcomes are as close as possible to those from natural conception (humans) or natural matings (large animals) especially when assessing safety and efficacy. Indeed, children generated as a result of assisted reproduction are normally monitored and assessed through developmental milestones associated with the general population and not against specific milestones set against various assisted reproductive technologies. These assessments

take place just after birth, before departure from hospital, and at various stages during childhood. Although ICSI comprises a component of the mtDNA supplementation process, it is primarily a process designed to overcome male factor infertility and is not more successful than *in vitro* fertilization in achieving implantation and live births for non-male factor infertility.[37–39] Our approach of mtDNA supplementation with similar approaches using third party donor ooplasm,[11,40] cytoplast fusions,[14] or autologous supplementation using egg precursor[17] or cumulus[16] cell mitochondria are designed to overcome poor oocyte quality where oocytes either fail to fertilize or, if they fertilize, arrest during preimplantation development and are, thus, indicative of female factor infertility. Therefore, mtDNA supplementation should be regarded as an integrated treatment and not an add-on to conventional ICSI, as there are currently no available methods to transfer mitochondria into oocytes apart from the untested process of oocyte-oocyte fusion. When considering mitochondrial supplementation as a single intervention, the choice of control group would be a non-intervention group. Consequently, a natural conception group is the most appropriate control group when evaluating the effects of assisted reproductive technologies on the wellbeing of human offspring.[41–44]

Using values from natural matings, we were able to determine how close the founder population was to the desired outcomes or if there was a degree of divergence from the norm using animals from our colony and values from other studies that used natural matings. Furthermore, by assessing a second generation of offspring that were produced through natural mating, we were again able to use values from natural matings to assess whether any modifications persisted in that generation or whether there was a shift to the norm. A similar strategy was also employed when assessing the first monkeys generated through metaphase II spindle transfer and their subsequent offspring,[45] and to assess DNA methylation and gene expression profiles[46] and nuclear-mtDNA interactions[47] of cloned pigs. This proved a valuable strategy especially when we assessed the biochemical and hematological values in both generations and observed, for example, how the aberrant values for cholesterol were modified in the next generation. Consequently, as the aim of our work was to determine if autologous and heterologous mtDNA supplementation, using mtDNA from failed-to-mature oocytes, was safe and efficacious, we compared outcomes to naturally mated controls. This should provide prospective patients and clinicians with a degree of assurance about anticipated outcomes in the context of the norm. Had our work focused on identifying the mechanisms of action of the extra mtDNA alone, it would have been pertinent to compare outcomes to another assisted reproductive technology, for example ICSI, as we previously did when assessing the molecular mechanisms associated with mtDNA supplementation.[18,20]

Although our gross anatomical (body condition scoring and pathology) and histopathological assessments did not raise any major concerns, we note that there were some postnatal developmental differences. For example, the heterologous founder population tended to be statistically heavier than the control population whereas both sets of founders had statistically greater growth rates. In the next generation, the heterologous offspring normalized their weight gain to that of controls whereas the autologous cohort exhibited reduced weight. Both groups also maintained their increased daily growth rates. Perhaps it is not unexpected to observe differences in weight and size given that other forms of oocyte reconstruction, for example, somatic cell nuclear transfer, are sometimes associated with mild and more severe forms of large offspring syndrome[48,49] with the next generation exhibiting greater similarities to naturally mated controls,[50] likely because of the resetting of the oocyte's epigenome, as discussed in.[51] Nevertheless, the offspring in this study were not affected to the extent that they exhibited large offspring syndrome. Likewise, there were a few differences for the biochemical and hematological assessments amongst the weaners once adjusted against values reported for a broad range of pigs. We chose to make these adjustments given that our offspring were generated from randomly acquired oocytes and sperm and were not from a restricted or inbred lineage. Although some of the differences normalized on the offspring reaching sexual maturity, a very few persisted. On reaching sexual maturity, both sets of founders were fertile and produced litters following natural mating. The litter sizes appeared within normal range for the associated mtDNA haplotypes.[26] However, it is evident that some of the female founders did not exhibit the care that might be expected of a mother with a number of instances of crushing. Piglet mortality because of crushing is common in the pig industry and is mainly attributed to housing and environmental causes.[52] However, the incidence of crushing in the female founders is unusually high. It is highly unlikely that this situation would occur in human clinical practice given the extent to which couples using this technology would have engaged with assisted reproduction in order to have a child and the degree of postnatal care that is available to patients.

Our RNA-Seq analysis of heart tissue revealed a number of genes that were differentially expressed in the mtDNA supplemented cohorts compared to controls, which were predicted to modulate other biological processes and pathways associated with several forms of cardiomyopathy. These included hypertrophic and dilated cardiomyopathy where gene expression was upregulated. However, there appeared to be greater protection against diabetic cardiomyopathy as genes associated with this pathway were downregulated. Nevertheless, as stated above, the pathology and histopathology examinations of the heart did not suggest any defects that might manifest later in life. However, the gene expression outcomes might warrant observation in offspring if this approach were to be used clinically.

From an epigenetic perspective, it is important to determine that parental DNA methylation (imprinting) patterns are not compromised when assessing new technologies. We know from our previous work that although a number of differentially methylated regions were observed at the blastocyst stage following mtDNA supplementation, imprinted regions were not affected.[20] However, imprinting anomalies have been associated with some assisted reproductive technologies[53–55] and incomplete reprogramming of the somatic cell following nuclear transfer.[56] In samples isolated from tail, we did not observe any potential imprinting defects from the regions we analyzed which demonstrates consistency with the setting of early imprinting programming in the mtDNA supplemented embryo. It is, however, feasible that the differential gene expression observed in the heart tissue of mtDNA supplemented founders could have resulted from discrete tissue specific differences in potential differentially methylated regions of their respective nuclear genomes. However, the aberrant patterns of DNA methylation and gene expression observed in blastocysts did not appear to have a significant impact on offspring health and well-being. This suggests that the aberrant patterns could have been modified during development when multiple waves of DNA de/methylation and epigenetic reorganization take place,[57,58] or they were simply not sufficiently disruptive. This is reassuring given the reconstruction that the oocyte has undergone and the karyotype abnormalities reported with ooplasmic transfer[40] and epigenetic modifications associated with nuclear transfer.[59,60]

The addition of third party mtDNA into oocytes to produce offspring has been of major concern for quite some time[14,61] and has led to the banning of such approaches in some jurisdictions.[62] This arose from the birth of offspring[11] that were carriers of two distinct populations of mtDNA, namely from the mother's oocyte and from the oocyte of the donor ooplasm.[12] A subsequent, brief report documented a case of pervasive development disorder in one infant and two incidences of XO syndrome, one of which spontaneously aborted and the other was selectively aborted.[40] A more recent follow study of 13 children, when between the ages of 13 and 18, indicated that they had generally met age-related developmental milestones with occasional anomalies,[13] although no in-depth genetic analysis was conducted and the ongoing degree of donor mtDNA transmission was not reported. The matter is further compounded by heteroplasmic mice that carry two different mtDNA genotypes through embryo-oocyte fusion, which exhibited severe metabolic syndrome and other associated pathophysiology disorders[14], whereas growth[63] and genomic integrity[64] are affected in mice generated through ooplasmic transfer.

Our analysis of the mitochondrial patterns of transmission in the heterologous founders and their offspring suggested that some of the mtDNA supplement could have been transmitted to the next generation when assessed using the lower threshold of 0.5%. We specifically sought the variants associated with another mtDNA haplotype, as identified in previous studies[26] and are, therefore, less likely to be indicative of background noise. Nevertheless, their presence was at very low levels or was undetectable except for one single base deletion (position 376) present in the large non-coding D loop region of the mitochondrial genome. On the whole, the levels of SNVs associated with the mtDNA supplement were not above the levels associated with the naturally occurring variants observed in tail, heart, brain or calf muscle samples of the heterologous founders. Of interest though, there was biased segregation, for example, in Pig 3.1, where the calf muscle tissue appeared to harbor more SNVs that were not detectable in brain or heart samples. Indeed, in the clinical context of mtDNA disease, mtDNA mutations and deletions are not present in all tissues nor are they uniformly segregated.[65]

Biased segregation might be a cause for concern as it results from preferential replication of mtDNA in some tissues. When a small bolus of mtDNA of approximately 780 copies is introduced into an oocyte, it has the potential to segregate to all blastomeres, just a few, or even one. This process may be influenced by whether the bolus disaggregates or remains tightly packed both of which will affect how these molecules segregate. Given that a fertilizable oocyte possesses more than 200,000 copies of mtDNA,[23,24] the

small bolus introduced represents no more than 0.39% of the total content. Consequently, if the molecules disaggregate, its presence in any given somatic tissue is indicative of a selective replication event otherwise it would have simply been diluted out. On the other hand, if it remains in an aggregated or partly aggregated form in one or more cells and is present as mtDNA replication is initiated post-gastrulation then it would be a major contributor to those cells. However, for a mtDNA rearrangement to manifest clinically, it is common that the presence of 60–85% mutation or deletion load is required.[66,67] The levels of variants observed in the heterologous founders (either as mtDNA supplemented SNVs or naturally occurring variants), and autologous founders (naturally occurring variants only), are far removed from these levels and would most likely not result in a pathology. However, this does not preclude a major selective replication event taking place, as observed in embryonic stem cell lines derived through metaphase II spindle or pronuclear transfer, or in offspring derived from embryonic or somatic cell nuclear transfer. In each case, a small amount of mtDNA (when compared with the oocyte's mtDNA content) accompanies the spindle,[68] the pronuclei,[69] or embryonic[70] or donor cell[71] into the recipient oocyte and can, on occasions, be detected at high levels; and can even outcompete the recipient oocyte's contribution. This process is often referred to as reversion. Nevertheless, our analysis of tail DNA samples from the offspring of the heterologous female founder suggested that although some of the SNVs associated with the mtDNA supplement, including those present in the founder and her oocytes and those not previously observed in those samples, were present in the offspring, the levels remained persistently low and below clinically relevant levels. The one exception was the deletion at position 376 which maintained similar levels in the founder's tissues, oocytes and offspring. This suggests that, in this instance, there is not a predisposition for selective replication. In all, the majority of SNVs and naturally occurring variants were not at levels higher than for sperm mtDNA that is transmitted when strains or breeds are crossed.[4–6] Nevertheless, in the context of metaphase II spindle and pronuclear transfer, which have been proposed as technologies to overcome poor oocyte quality in terms of infertility and the transmission of mtDNA disease (referred to as Mitochondrial Donation), the carryover of fewer than 780 copies of mtDNA is unlikely to result in preferential selection of these molecules. Although current levels of mtDNA carryover tend to be higher, the reduction of carryover to fewer than 800 copies offers the potential to prevent the transmission of two mtDNA genotypes to offspring in the case of infertility or mutant molecules in the context of Mitochondrial Donation.

Mitochondrial supplementation has, in the main, been regarded as an approach that provides extra units of energy to deficient oocytes.[72,73] We sourced purified populations of mitochondria isolated from failed to fertilize oocytes and, in our previous work, we have shown that there are slight differences in the levels of ATP generated by these mitochondria compared to mitochondria derived from oocytes that have fertilisation capacity.[18] In this respect, mitochondria from oocytes with fertilization capacity are more quiescent whereas those of less competent oocytes have more active mitochondria. However, when compared with somatic cells, the difference is marginal. Nevertheless, we have also previously argued that the amount of mtDNA (approximately 780 copies) introduced into an oocyte is minimal.[18] The fertilizable oocyte possesses large numbers of mitochondria, approximately 100,000, which is significantly more than somatic cells, and they are naive and produce characteristically low levels of ATP.[74] If the oocyte has 1 to 2 copies of mtDNA per mitochondrion,[75–77] which is far fewer than for somatic cells, 400 additional mitochondria are not going to necessarily make a significant difference to mitochondrial and oocyte function. In our previous work, we showed that the supplementation of additional mtDNA into mtDNA deficient oocytes induced a mtDNA replication event that increased mtDNA copy number 4-fold by the 2-cell stage.[18] This was matched by changes to the DNA methylation profile of the mtDNA-specific replication factor. This represents a significant genomic event, which is unlikely to be influenced by carried over RNA given we used purified populations of mitochondria, and that altering the content of one genome could impact on the other genome requiring the interactions between the two genomes to be reestablished,[78] as demonstrated in tumor models.[79,80] Indeed, it is now thought that the cofactors generated through the metabolic pathways in the mitochondrion are major contributors to the regulation of DNA methylation.[81] Consequently, there are important global genomic and epigenomic events that need to be considered if mtDNA supplementation protocols are to be continued to be practiced clinically. For example, the differential gene expression identified in the heart tissue could result from this effect, although the significant changes appear to take place in the preimplantation embryo and do not necessarily impact on tissue morphology and structure.

By performing autologous and heterologous mtDNA supplementation with mitochondria isolated from failed to fertilize oocytes, we have seen changes associated with height and weight gain and some

biochemical and hematological markers with subsequent adjustments in the next generation. There were also notable changes in gene expression in the heart tissue of founder offspring. In all, the effects are not major and do not appear to influence offspring health and well-being, although extra oversight might be a clinical consideration when monitoring children. In addition, the use of low levels of either heterologous or autologous populations of mtDNA that can mediate epigenetic and genomic changes in the preimplantation embryo do not appear to affect the inheritance of mtDNA. Indeed, the epigenomic and genomic changes observed in blastocyst stage embryos appear to correct later during development. The use of oocytes that failed to reach metaphase II provides a source of mtDNA that would otherwise be wasted but, in the context of autologous supplementation, would ensure the genetic integrity of the offspring could be maintained. Consequently, it appears that mtDNA supplementation could be used to rescue failed to fertilize oocytes, as previously shown,[18] with the potential to produce healthy offspring. Nevertheless, it would be appropriate to repeat these experiments to increase the number of animals investigated and to further validate our outcomes either using a similar or another experimental large animal model. Ultimately, as with the introduction of Mitochondrial Donation, society must decide if it wishes to embrace this form of technology irrespective of whether reproductive laws require amendment for its introduction or not.

Limitations of the study

This study has generated three sets of offspring using a large animal model to test the safety and efficacy of mtDNA supplementation. Overall, it appeared that there were a few differences in the founder population when compared with the control population, which were modified in a second generation. However, it would be beneficial to repeat the approach in a similar model to determine if the outcomes were similar or other, so far unidentified problems arose. In this context, it would also be beneficial to include offspring generated through ICSI in addition to natural matings as controls to determine the effects that arose through the ICSI procedure and those arising solely from the extra copies of mtDNA.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

J.C.S. designed the experiments, supervised the research, performed the mtDNA next generation sequencing analysis, wrote the article, and obtained funding for the work; S.M. performed oocyte retrieval and microinjection, embryology, assisted with embryo transfers, assisted with collection of tissues from animals and co-wrote the article; S.O'L. performed embryo transfers, oversaw births and collected animal measurement data, performed autopsies, collected tissues from animals, and co-wrote the article; T.O. prepared samples for and analyzed the RNA sequencing, performed the imprinting analysis, assisted with collection of tissues from animals, and co-wrote the article; A.P. analyzed the height and weight gain data, assisted with collection of tissues from animals and co-wrote the article; V.H.T.N. prepared samples for mtDNA next generation sequencing, co-analyzed the mtDNA sequencing data and co-wrote the article; L.M. performed animal husbandry, monitored animals, collected measurement data from animals, assisted with collection of tissues from animals and co-wrote the article; S-Y.H. performed oocyte retrieval and microinjection, embryology, and co-wrote the article; E.A. analyzed the oocyte and embryo data and co-wrote the article; J.F. performed histopathology, analyzed the data, and co-wrote the article; R.K. monitored animals, performed veterinarian duties, performed autopsies and co-wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
0.22% altrenogest solution	MERCK Animal Health	Cat# 57926-100
0.9% NaCl solution	BAXTER	Cat# AHF7124
CircoFLEX®	Boehringer Ingelheim	N/A
eCG/Folligon®	Intervet Schering-Plough Animal Health	Cat# 369397
FERON 200 + B12 INJECTABLE IRON DEXTRAN COMPLEX + VITAMIN B12	Bayer	Cat# FERR0020000100IN
hCG/Chorulon®	Intervet Schering-Plough Animal Health	Cat# 377384
Hepes-TCM199®	Gibco	Cat# 12340-030
TCM199	Gibco	Cat# 11150059
PlatinumTM Taq DNA Polymerase High Fidelity	Thermo Fisher Scientific	Cat# 12340-030
prostaglandin 2α/Lutalyse®	Zoetis	Cat# 40034684
Respisure®	Zoetis	N/A
Thiopentone sodium	Jurox Pty Ltd	Cat# 503690
Toltrazuril/Baycox®	Bayer	N/A
Pentobarbitone (Lethabarb)	Virbac	N/A
Insulin	Sigma	I2643
EGF	Sigma	E9644
Cysteamine	Sigma	M9768
Sodium Chloride	Sigma	S5586
Potassium Chloride	Sigma	P5405
Potassium Phosphase	Sigma	P5655
Magnesium Sulphate Heptahydrate	Sigma	M7774
Sodium Bicarbonate	Sigma	S5136
Calcium Chloride Dihydrate	Sigma	C7902
Penicillin-G	Sigma	P3032
Streptomycin Sulphate	Sigma	S9137
Glutamine	Sigma	G5763
Taurine	Sigma	T7416
Hypotaurine	Sigma	H1384
Bovine Serum Albumin (Bovostar)	Bovogen	BSAS 0.1
D-Glucose	Sigma	G6152
Sodium Pyruvate	Sigma	P2256
Sodium Lactate	Sigma	L7900
Calcium Lactate	Sigma	L4388
Foetal Bovine Serum	Gibco	10099141
HEPES	Sigma	H6147
Mannitol	Sigma	63359
Sucrose	Sigma	57903
EDTA	Sigma	E6758
Nonessential amino acids	Sigma	11140-050
MEM amino acids	Sigma	11130-051

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hyaluronidase	Sigma	H2126
Critical commercial assays		
DNeasy Blood & Tissue Kit	QIAGEN	Cat# 69504
EZ DNA Methylation-Gold Kit	Zymo Research	Cat# D5005
ISOLATE II PCR and Gel Kit	Meridian Bioscience	Cat# BIO-52060
Nextera Flex Library Preparation Kit	Illumina Inc	Cat# 20018705
NovaSeq 6000 S2 Reagent Kit v1.5	Illumina Inc	Cat# 20028314
RNeasy Mini Kit	QIAGEN	Cat# 74106
TruSeq SBS Kit v3-HS	Illumina Inc	Cat# FC-401-3001
Deposited data		
Heart RNAseq	In this study	NCBI BioProject: PRJNA823749
mtDNA sequences	In this study	NCBI BioProject: PRJNA749323
Experimental models: Organisms/strains		
Pig: Large-White x Landrace cross-breed	The University of Adelaide	N/A
Oligonucleotides		
Methylation-specific PCR primers	In this study	Table S3
Primers for amplification of *Sus scrofa* mtDNA	In this study	Table S4
Software and algorithms		
CLC Genomics Workbench v22.0	QIAGEN	https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/qiagen-clc-genomics/
edgeR v3.2.1	Robinson et al., 2010[82]	N/A
featureCounts v1.5.3	Dobin et al., 2013[83]	http://subread.sourceforge.net/
IBM SPSS Statistics v28	IBM Corp.	https://www.ibm.com/au-en/products/spss-statistics
limma v3.46.0	Ritchie et al., 2015[84]	N/A
MethPrimer	Li and Dahiya, 2002[85]	N/A
STAR v2.5.3a	Dobin et al., 2013[83]	N/A
Stringtie v2.1.4	Kovaka et al., 2019[86]	http://ccb.jhu.edu/software/stringtie/
Sus scrofa genome assembly Sscrofa11.1	NCBI	https://www.ncbi.nlm.nih.gov/assembly/GCF_000003025.6/
TrimGalore	Krueger, 2012[87]	https://github.com/FelixKrueger/TrimGalore

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Justin C. St. John (jus.stjohn@adelaide.edu.au).

Materials availability

DNA samples generated in this study are available on request from the lead contact, Justin C. St. John (jus.stjohn@adelaide.edu.au).

Data and code availability

- Heart RNA-seq data and mtDNA sequence data have been deposited at NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this manuscript is available from the lead contact upon request

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal experiments

All animal and husbandry procedures were approved by The University of Adelaide Animal Ethics Committee (Approval number 32293). *Sus scrofa* zygotes were generated by mtDNA supplementation and intracytoplasmic sperm injection, as previously described[18] and shown in Figure 1. Zygotes were then transferred to post-pubertal Large White x Landrace gilts at 26 weeks of age as recipients for embryo transfer (ET). Pregnancy was confirmed by the presence of visible amniotic vesicles. Pregnant recipients were fed a commercial gestation diet formulated to supply all nutrient requirements until farrowing at approximately 114 days post ET. They were housed in pens until 4 days prior to expected farrowing and then transferred into a farrowing crate. Farrowing of recipients was supervised and, if necessary, was induced on day 116 post ET. Piglet birthweight and shoulder height were measured, and all piglets were processed as per standard colony protocol. Piglets and recipient gilts were housed in a temperature-controlled room, piglets received a vaccination and were allowed access to a commercial standard diet. Piglets were assessed weekly prior to weaning and monthly until sexual maturity by recording weights and heights, time to approach and scored for activity. Biochemical and haematology assessments were undertaken at weaning and again at sexual maturity. Natural mating was allowed at oestrus detection and gilts approximately 22 week of age were provided with direct contact with a sexually mature boar.

METHOD DETAILS

Cumulus-oocyte-complexes collection and *in vitro* maturation

Gilt ovaries were collected in pairs from a local abattoir and transported to the laboratory in warm 0.9% NaCl solution (Baxter,Old Toongabbie, NSW, Australia). Cumulus-oocyte-complexes (COCs) from each ovary pair were aspirated from follicles with diameters of 3–6 mm using an 18 G needle. Once isolated, the COCs were washed three times in handling media (25 mM Hepes-TCM199; Gibco) supplemented with 10% sow follicular fluid (SFF). The COCs from each ovary pair were cultured in individual wells for 42 to 44 h in 500 μL pre-equilibrated *in vitro* maturation (IVM) media (TCM199 media supplemented with 0.80 mM Na-pyruvate, 0.61 mM L-glutamine, 0.88 M cysteamine, 5 μg/mL insulin, 10 IU/mL PMSG, 10 IU/mL hCG, and 0.10 μg/mL EGF and 10% SFF) at 38.5 °C in a humidified incubator with 5% CO_2 in air.

To collect MII oocytes, expanded COCs from each ovary pair were transferred in 200 μL handling media supplemented with 5 μg/mL hyaluronidase to a 1.5 mL Eppendorf tube. The COCs were then vortexed for 5 min and briefly spun down. The samples were transferred to a dish and denuded oocytes were washed using a narrow glass pipette to ensure complete removal of all cumulus cells. MII oocytes, which presented with a polar body, were held in a microdroplet until required and then transferred to a 20 μL droplet in readiness for microinjection.

Isolation of mitochondria from *Sus scrofa* oocytes

Mitochondrial fractions were prepared from *Sus scrofa* oocytes as described previously.[18] Briefly, *in vitro* failed-to-mature oocytes were denuded from cumulus oocyte complexes and 10 to 15 oocytes were resuspended in 5 mL of mitochondrial isolation buffer (20 mM Hepes pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA) containing 2 mg/mL BSA. Oocytes were homogenised by 10 strokes of a drill-fitted Potter-Elvehjem tissue grinder set (VWR International, PA, USA) on ice. The oocyte homogenate was centrifuged at 800 g for 10 min at 4°C to remove cell debris. Mitochondrial supernatants were centrifuged at 10,000 g for 20 min at 4°C to pellet the mitochondria. The mitochondrial pellet was resuspended in 700 μL of mitochondrial isolation buffer, then further centrifuged at 10,000 g for 20 min. The supernatant was removed, and the mitochondrial pellet was resuspended in 5 μL of mitochondrial isolation buffer and used for mICSI and DNA extraction.

Generation of ICSI and mICSI-derived blastocysts

Sus scrofa zygotes were generated by mICSI, as previously described[18] and shown in Figure 1, using either mtDNA from sister oocytes (i.e. the same ovary) or a third party source of oocytes (another ovary pair). Zygotes were then transferred to a portable incubator for transport in readiness for embryo transfer.

Preparation and synchronisation of pigs

Post-pubertal Large White x Landrace gilts were selected at 26 weeks of age as recipients for embryo transfer (ET). For each planned ET, two gilts were selected to undergo oestrus synchronisation. The gilt displaying the strongest standing reflex and the greater external signs of oestrus with increased vulva swelling was then used as the recipient. Oestrus was synchronised by oral administration of 0.22% altrenogest (Regumate; Merck Animal Health; Summit, New Jersey) for 16 days. At 24 h after the last altrenogest dose, gilts received 750 IU of eCG (Folligon; Intervet Australia, Bendigo, Vic). This was followed by 750 IU of hCG (Chorulon; Intervet Australia, Bendigo, Vic) 104 h later to induce ovulation. ET was performed approximately 24 h following administration of hCG, approximately 12–18 h before expected ovulation.

Surgery and embryo transfer

Synchronised gilts were fasted overnight until the time of surgery. They were then anesthetized with thiopentone sodium intravenously at 15 mg/kg bodyweight (Thiobarb; Jurox Pty, Ltd, Rutherford, NSW) and maintained within a closed-circuit system using isoflurane (3.5%–5%) while placed in a supine position on a surgical table. A 5 cm ventral midline incision was made between the two pairs of distal nipples. Blunt dissection and entry into the peritoneal cavity was made to isolate one uterine horn to exteriorise the ovary and oviduct. Putative zygotes (circa 20 h old) were introduced at approximately 10–15 cm into the oviduct via the infundibulum using a 11.4 cm Argyle catheter.

Post-operative monitoring, care and ultrasound checks

The recipient gilt was transferred to a single stall and given antibiotic and analgesic coverage for 48 h post-surgery using 150 mg/mL amoxycillin trihydrate and 50 mg/mL flunixin meglumine, respectively. On day 10 post ET, intramuscular administration of 1000 IU eCG followed by 1000 IU of hCG on day 13 were given to generate accessory corpora lutea for maintenance of pregnancy.[88] Pregnancy status was determined by real-time ultrasound between day 28 and day 45 post ET. Pregnancy was confirmed by the presence of visible amniotic vesicles.

Farrowing and assessment at birth

Pregnant recipients were fed 2.5 kg/d of a commercial gestation diet formulated to supply all nutrient requirements until farrowing at approximately 114 days post ET. They were housed in pens until 4 days prior to expected farrowing and then transferred into a farrowing crate. Farrowing of recipients was supervised and, if necessary, was induced using prostaglandin 2α (Lutalyse; Zoetis, NJ, USA) on day 116 post ET if natural farrowing had not occurred beforehand. Piglet birthweight and shoulder height (from the base of the front trotter to the highest point on shoulder) were measured as soon as possible after birth. Time to reach the nipple was recorded as a measure of piglet viability.[25] If a supervised farrowing was not possible, the piglets were weighed and assessed for viability within 24 h.

Assessments at 12 to 24 h post-birth

At 24 h post farrowing, all piglets were processed as per standard colony protocol. Their tails were docked to prevent biting from littermates and DNA was extracted for mtDNA genotype and imprinting analyses; their canine teeth clipped to prevent damage to littermates and the sow's udders; and they were ear tagged and tattooed for identification. They also received an intramuscular iron injection (1 mL of Fr + B12; Feron 200 + B12; Bayer, Pymble, NSW, Australia).

Body condition scoring

Body condition scoring was undertaken at weaning and on reaching sexual maturity by an experienced pig veterinarian using established criteria.[25] Briefly, a pig with a BCS of 1 was deemed to be very compromised (VC), typified by the pelvic bones being prominent; loin narrow and flank hollow; vertebrae prominent; and individual ribs prominent. A BCS of 2 was categorised as moderately compromised (MC). This was characterised by the pelvic bones being obvious; loin narrow and flank narrow; vertebrae visible; and rib cage less apparent with each rib difficult to see. A BCS of 3 or greater was described as healthy (HY) whereby the pelvic bones were covered; vertebrae somewhat covered; and ribs covered but could be felt. The veterinarian then assessed the pigs for clinical abnormalities. These included cutaneous, musculoskeletal, and gastrointestinal indicators of illness, and any other observable abnormalities.

Husbandry pre-weaning, weaning and post-weaning

Piglets and recipient gilts were housed in a temperature-controlled room maintained at approximately 22°C. On day 4, piglets received a vaccination against *M. hyopneumoniae* (Respisure®, Zoetis, Parkville, Vic) and an oral drench of Toltrazuril (Baycox®, Bayer, Pymble, NSW) and on day 14 vaccinated against porcine circovirus-2 (Circoflex®, Boehringer, Macquarie Park, NSW). Piglets were allowed access to a commercial creep feed from 21 d until weaned into an outside straw-based pen at 28 d. From weaning to 10 wk, piglets were allowed *ad libitum* access to a commercial weaner diet and then a commercial grower diet until 20 wk, and then a finisher diet. Piglets were assessed weekly prior to weaning and monthly until sexual maturity by recording weights and heights, time to approach and scored for activity.[25] Statistical analyses for height and weight were performed by fitting a linear mixed effect model, with overall slope estimated to determine weight/height gain per day as a fixed effect, which was compared to each individual animal's deviation from this slope as a random effect to establish the rate of weight/height gain per day. Daily weight gains were compared using a linear mixed model with the study group as the fixed effect and the mother of each piglet fitted as a random effect to control within litters.

Body mass index (BMI) at weaning was calculated using the following formula: weight at weaning/(measurement from base of the front trotter to the highest point of the shoulder)^2 and expressed as kg/m^2. Statistical analysis was performed using a general linear model alongside individual statistical analysis for height and weight at weaning.

Biochemical and haematology assessments

At weaning and again at sexual maturity, 10 mL blood samples were obtained by jugular venepuncture into each of an EDTA and plain vacuum blood collection tubes. Serum and plasma samples were delivered within 1 h of collection to the University of Adelaide Veterinary Diagnostic Laboratory for biochemistry and haematology analyses with biochemistry testing using the Beckman Coulter AU480 (Beckman-Coulter, Brea, California, USA), and haematology tested using the ADVUA 2120 (Siemens, Erlangen, Germany).

Biochemistry measurements were recorded for sodium, potassium, chloride, bicarbonate, sodium:potassium ratio, anion gap, serum glucose, urea, creatinine, calcium, phosphate, calcium:phosophate ratio, total protein, albumin, globulin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, glutamate dehydrogenase, gamma glutamyl transferase, creatine kinase, magnesium, cholesterol, and beta hydroxybutyrate concentration.

Haematology measurements included red blood cell count, hemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell distribution width, platelet count, white blood cell count, neutrophil count, lymphocyte count, monocyte count, eosinophil count, basophil count, fibrinogen concentration, and plasma total protein concentration.

Statistical analyses for biochemistry and haematology were performed using IBM SPSS Statistics Version 28. Normality was assessed using Kolmogorov-Smirnov normality tests alongside quantile comparison (Q-Q) plots. Where data appeared approximately normal, differences between groups were measured using general linear models with pairwise comparisons adjusted by least significant difference (i.e. not adjusted), and if not approximately normal, data were analyzed using independent sample Kruskal-Wallis tests.

Histopathology

Tissues from each animal were collected, immersion-fixed in 10% neutral buffered formalin, paraffin-embedded, and 6 μm sections were cut and stained with haematoxylin and eosin for histopathological examination.

Mating

Oestrus was stimulated and identified in gilts from approximately 22 weeks of age by providing direct contact with a sexually mature boar. Natural mating was allowed at oestrus detection and again 24 h later if still exhibiting oestrus.

RNA extraction from heart, RNAseq library construction and next generation sequencing

Total RNA was extracted from approximately 10 mg of *Sus scrofa* heart tissue using the RNeasy Mini Kit (QIAGEN, VIC, Australia), according to the manufacturer's instructions. RNA quality was assessed using a LabChipGX Nucleic Acid Analyzer (PerkinElmer Inc) by measuring 28S, 18S and 5S rRNA and RNAseq libraries were constructed and next generation sequencing was performed by the Australian Genome Research Facility (Melbourne, VIC, Australia). Briefly, heart RNA samples from control pigs (n = 3) and mICSI-derived founder pigs (n = 5) were used to generate RNAseq libraries. Depletion of rRNA in the RNA-seq library was conducted using the Ribo-zero stranded protocol (Illumina Inc. CA, USA). Next generation sequencing libraries were sequenced by the Illumina NovaSeq S1 platform using 150 bp paired-end sequencing chemistry TruSeq SBS Kit v3 reagents.

RNAseq data analysis and identification of differentially expressed genes (DEG)

RNAseq raw fastq files were trimmed and quality filtered using Trim Galore (https://github.com/FelixKrueger/TrimGalore).[87] Trimmed paired-end reads were aligned to the *Sus scrofa* genome assembly *Sscrofa11.1* (https://www.ncbi.nlm.nih.gov/assembly/GCF_000003025.6/) using '*STAR*' (version 2.5.3a)[83] with default parameters. The counts of reads mapping to each known gene were summarised at gene level using the featureCounts v1.5.3 utility of the subread package (http://subread.sourceforge.net/)[83] with default parameters. The transcripts were assembled using the *Stringtie* tool v2.1.4 (http://ccb.jhu.edu/software/stringtie/) and reads aligned to *Sscrofa11.1* using the reference annotation based assembly option (RABT). Summary statistics for the RNAseq data are shown in Table S1.

DEGs were identified between control and mICSI pig heart samples using edgeR version 3.2.1[82] run in R version 4.0.3. The Trimmed Mean of M-values (TMM) normalisation method was applied to normalise read counts according to differences in library size between samples. Multidimensional scaling (MDS) was conducted to visualise the summary of gene expression for all sample libraries. The gender of each pig was included in the linear mixed model as a covariate and batch effect was successfully corrected in the expression data. Genes were considered to be differentially expressed if their FDR (false discovery rate)/adjusted p value was <0.05 (Table S2). DEGs were visualized by smear plot and heatmap using R packages. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis results were generated in '*limma*' version 3.46.0[84] using the DEGs (*adj.p*-val <0.05) identified in the differential expression analysis as input.

Methylation specific-PCR

The DNA methylation status of imprinted genes was investigated by methylation specific-PCR (MSP).[89] Total DNA was extracted from *Sus scrofa* tail samples using the DNeasy Blood & Tissue Kit (QIAGEN, VIC, Australia), according to the manufacturer's instructions. Tail DNAs were bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research, CA, USA). Primers specific to methylated- and unmethylated-DNA fragments were designed by MethPrimer[85] and listed in Table S3. Target DNA fragments were amplified in an MJ Research PTC-200 Thermal Cycler (Marshall Scientific, NH, USA) using Platinum *Taq* DNA Polymerase High Fidelity (Cat No. 11304011, Thermo Fisher Scientific, MA, USA) by denaturing at 95°C for 2 min followed by 40–45 cycles of 95°C for 15 s, 55–59°C for 20 s, 65°C for 30 s (annealing temperatures are documented in Table S3). Amplified PCR products underwent gel electrophoresis on 2% agarose gels. To validate the specificity of MSP primers, gel bands were excised, purified using the ISOLATE II PCR and Gel Kit (Meridian Bioscience, TN, USA) and sequenced using a standard Sanger sequencing protocol.[90]

mtDNA next generation sequencing

mtDNA next generation sequencing was undertaken following long PCR amplification with four primer sets (see Table S4) to produce four overlapping fragments using Platinum *Taq* High Fidelity (Thermo Fisher Scientific, MA, USA), as described.[91] Amplified mtDNA fragments were purified from gel excised bands using the ISOLATE II PCR and Gel Kit (Meridian Bioscience, TN, USA). The four mtDNA fragments were mixed in the same molar ratio and used for next generation sequencing. Next generation sequencing library construction and sequencing was carried out by Australian Genome Research Facility. Briefly, Nextera Flex Library Prep (Illumina Inc. CA, USA) was used for construction of mtDNA amplicon libraries which were sequenced by the Illumina NovaSeq S1 platform using NovaSeq 6000 S2 Reagent Kit v1.5 (200 or 300 cycles).

mtDNA mapping

Sequences were aligned to a reference pig mitochondrial genome[92] using CLC Genomics Workbench Version 22.0, as described.[26] Briefly, reads were trimmed by one nucleotide from each end; and filtered to exclude those of <15 bp in length. Only reads that surpassed a Phred quality score of 15 were accepted for mapping. The following mapping parameters were applied: a match score of 1; a mismatch cost of 2; an insertion/deletion cost of 3; and a similarity fraction of 0.80. More than 98% of the DNA fragments from the library aligned to the reference genome. Duplicate reads were then removed. For sample specific variant calling, a consensus sequence (reference genome for each sample) was obtained for each sample. The raw data for each sample were then remapped to their consensus sequence using the same criteria as above. Phylogenetic trees were constructed after consensus sequences had been aligned using the Create Alignment tool (CLC Genomics Workbench) employing a gap cost of 10; and gap extension cost of 1. The alignments were then imported into the Maximum Likelihood Phylogeny Version 1.3 tool (CLC Genomics Workbench) and trees generated using the Neighbor Joining method; the General Time Reversible nucleotide substitution model; and bootstrapping of 1000 replicates.

Analysis of SNVs and naturally occurring variants

Using the reads mapped to an individual's consensus sequence, variant calling was conducted using CLC Genomic Workbench's Low Frequency Variant Detection module (Version 2.3) with the following parameters applied: exclusion of all duplicate reads; presence of all variants on forward and reverse reads; and a minimum of 50 reads for each variant. A minimum threshold of 0.5% was set for a variant to be called.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using a general linear model alongside individual statistical analysis for height and weight at weaning. Statistical analyses for biochemistry and haematology were performed using IBM SPSS Statistics Version 28. Normality was assessed using Kolmogorov-Smirnov normality tests alongside quantile comparison (Q-Q) plots. Where data appeared approximately normal, differences between groups were measured using general linear models with pairwise comparisons adjusted by least significant difference. If data did not appear approximately normal, data were analysed using independent sample Kruskal-Wallis tests. In all tests, $p < 0.05$ was considered as statistically significant.