

1 **Origins of lifetime health around the time of conception: causes**  
2 **and consequences**

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36 **Abstract (200 words)**

37 **Parental environmental factors including diet, body composition, metabolism and**  
38 **stress affect the health and chronic disease risk of people throughout their lives, as**  
39 **captured in the ‘Developmental Origins of Health and Disease’ (DOHaD) concept.**  
40 **Research across epidemiological, clinical and basic science fields has identified the**  
41 **period around conception as being critical in the processes mediating parental**  
42 **influences on the next generation’s health. During this time, from the maturation of**  
43 **gametes through to early embryonic development, parental lifestyle can adversely**  
44 **influence long-term risks of offspring cardiovascular, metabolic, immune and**  
45 **neurological morbidities, often termed ‘developmental programming’. We review**  
46 **‘periconceptual’ induction of disease risk from four broad exposures: maternal**  
47 **overnutrition and obesity; maternal undernutrition; related paternal factors; and from**  
48 **the use of assisted reproductive treatment. Human studies and animal models**  
49 **demonstrate the underlying biological mechanisms, including epigenetic, cellular,**  
50 **physiological and metabolic processes. A novel meta-analysis of mouse paternal and**  
51 **maternal protein undernutrition indicate distinct parental periconceptual**  
52 **contributions to postnatal outcomes. We propose that the evidence for**  
53 **periconceptual effects on lifetime health is now so compelling that it calls for new**  
54 **guidance on parental preparation for pregnancy, beginning before conception, to**  
55 **protect the health of offspring.**

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57

## 58 Introduction

59 The notion that maternal physiology, body composition, diet and lifestyle during pregnancy  
60 have profound and enduring effects on offspring long-term health and disease risk into  
61 adulthood has received strong evidential support across epidemiological, medical and basic  
62 science fields<sup>1-3</sup>. Thus, the ‘Developmental Origins of Health and Disease’ (DOHaD) concept  
63 has emerged, proposing that poor developmental experience can provoke increased risk of  
64 non-communicable disease in later life, particularly cardiovascular and metabolic  
65 comorbidities such as hypertension, obesity and type-2 diabetes, atopic conditions and some  
66 forms of cancer, as well as neurological impairment. A recent focus in DOHaD research has  
67 been to probe **when** during pregnancy the conceptus is most vulnerable to such adverse  
68 influences, thereby informing targeted protection and possible intervention. Increasing  
69 evidence points to the importance of the time around conception (=periconceptual period).

70

### Box 1: Key messages

Whilst evidence for developmental origins of later disease can be found throughout gestation and beyond, there is a growing consensus from both human and animal studies that a critical period is around conception and hence merits particular attention.

As we review, preconception maternal overnutrition and obesity, maternal undernutrition, related paternal factors, and assisted reproductive treatments all may change the phenotype and potential of gametes and early embryos, with enduring consequences across the lifespan.

Our new data reveal that suboptimal maternal and paternal nutrition around conception have similar effects on offspring weight, but differing effects on offspring blood pressure.

These critical influences on lifetime health occurring so early in development may reflect perturbations or adaptations in epigenetic, cellular, metabolic and/or physiological mechanisms. Defining these mechanisms and the exposures that drive them is critical to the characterisation of more specific recommendations for preconception health.

**This emerging knowledge has significant societal and medical implications. In particular, it provides the basis for a new emphasis on preparation for pregnancy, before conception, to safeguard public health and as a means of disease prevention.**

## 71 Periconceptual developmental conditioning

72 The **periconceptual period** has been variously defined, but for DOHaD processes the key  
73 events broadly cover the completion of meiotic maturation of oocytes, differentiation of  
74 spermatozoa, fertilisation and resumption of mitotic cell cycles in the zygote, marking the

75 transition from parental to embryonic genomes<sup>4</sup> and the onset of morphogenesis up to  
76 implantation<sup>5</sup>. This represents a period of a few weeks, dependent upon mammalian species,  
77 and is characterised by extensive change in morphology (emergence of distinct embryonic  
78 and placental cell lineages); genomic re-organisation (epigenetic modifications such as DNA  
79 methylation to regulate lineage-specific gene expression in the conceptus); and changes in  
80 metabolism (setting homeostatic regulators for growth and energy supply). **See Figure 1 for**  
81 **a résumé of key events**. It is however recognised that influences at every stage from earliest  
82 childhood can shape preconception health and thereby influence eventual pregnancy and birth  
83 outcomes.

84 Adverse developmental processes around the time of conception have been demonstrated in  
85 human and animal models in response to diverse environmental situations. In vivo, the quality  
86 of a mother's diet, both overnutrition and obesity<sup>6</sup> or undernutrition<sup>7</sup>, and/or other aspects of  
87 her physiological status including hyperglycemia/lipidemia<sup>8</sup>, may affect embryo potential with  
88 consequences for offspring disease risk over the lifetime. Paternal lifestyle and phenotype can  
89 similarly influence long-term offspring health, mediated either through the sperm or seminal  
90 plasma<sup>9</sup>. Periconceptual parental influences may have particular and differing effects on  
91 male and female offspring<sup>10</sup>. In addition, more babies are being born as a result of assisted  
92 reproductive treatments (ART) some of which involve embryo culture and exposure to  
93 potentially inappropriate environmental factors, which may alter offspring phenotype<sup>10,12</sup>.  
94 Long-term outcomes are consistent with the DOHaD concept, including cardiometabolic,  
95 immunological and neurological non-communicable disorders.

96 To some the concept of 'periconceptual' origins of lifetime health may not be intuitive. Why  
97 should this short window at the very start of development have such profound consequences  
98 for the rest of our lives? Critically, the essential steps in reproduction over this period occur  
99 when the few cells involved are fully exposed to environmental conditions, making them  
100 **vulnerable** to disturbance of epigenetic mechanisms and an altered profile of embryonic gene  
101 expression that persists through subsequent cell cycles and drives an altered developmental  
102 programme. Metabolic and cellular homeostatic characteristics of the embryo, including  
103 mitochondrial activity, can also change in response to nutrient availability. Conversely,  
104 periconceptual sensitivity to environmental cues also raises the possibility that this window  
105 is one of **opportunity**, providing the embryo with capacity to respond to prevailing conditions  
106 and to optimise development to best suit survival and fitness<sup>7</sup>. Thus, periconceptual  
107 developmental plasticity (induction of different phenotypes from a single genotype) may  
108 facilitate setting of suitable growth and metabolic parameters to match the perceived

109 environment but which, if environmental conditions change, may become maladaptive and  
110 lead to later disease<sup>3</sup>.

111 This article focuses on four broad periconceptional environmental exposures shown to induce  
112 adverse effects in humans and animal models (Figure 2), and discusses mechanistic causes  
113 and consequences. We also report new data on the relative contributions of maternal and  
114 paternal influences to long-term periconceptional influences in an established low protein diet  
115 model of parental undernutrition.

116

### 117 **Periconceptional developmental conditioning through maternal overnutrition** 118 **and obesity**

119 The global rise in maternal obesity is associated with reduced female fertility and heightened  
120 risk of obesity in the offspring<sup>2</sup>. Adverse effects of high maternal body mass index (BMI) on  
121 the offspring may reflect elevated maternal glucose and insulin concentrations driving fetal  
122 growth and adiposity, resulting in increased birth and childhood weight, but may also include  
123 shared lifestyle factors within families<sup>6</sup>. Impaired offspring metabolism may also be associated  
124 with increased risk of allergic and atopic conditions, revealing the complexity in phenotype<sup>2</sup>.  
125 Maternal obesity models in animals have confirmed the link with offspring cardiovascular and  
126 metabolic disease risk<sup>6,13</sup>.

127 Why might the periconceptional period be causal for obesity-related conditioning? Obese  
128 women have higher circulating concentrations of inflammatory cytokines<sup>14</sup>, and of hormones  
129 and metabolites which accumulate within the ovarian follicular fluid and can affect oocyte  
130 maturation and potential adversely. Thus, maternal BMI is positively associated with increased  
131 follicular fluid insulin, lactate, triglycerides, leptin and other metabolic regulators<sup>15</sup>. This rich  
132 follicular fluid compromises the developmental competence of exposed animal oocytes in  
133 experimental models, reducing embryo quality<sup>16</sup>. Moreover, oocytes from obese women are  
134 smaller and produce blastocysts with increased triglycerides and reduced glucose  
135 consumption, markers of poorer potential<sup>17</sup>.

136 In addition to metabolite overexposure, maternal obesity in mice induces defects in the  
137 mitochondrial phenotype of eggs, including abnormal morphology and cristae structure<sup>18</sup>,  
138 altered membrane potential and distribution<sup>18</sup> and increased mitochondrial DNA content<sup>18,19</sup>,  
139 all markers of disturbed mitochondrial function and energy homeostasis. Oocytes from obese

140 dams also exhibit increased oxidative stress and spindle abnormalities suggesting increased  
141 risk of aneuploidy<sup>18,19</sup>.

142 These mitochondrial defects in oocytes may derive from the elevated lipid content and inherent  
143 insulin resistance caused by high maternal adiposity. Oocyte hyperlipidaemia in turn leads to  
144 impaired metabolic regulation and endoplasmic reticulum stress **in mice**<sup>16</sup>, a condition where  
145 proteins misfold during biosynthesis and which contributes to metabolic and cardiovascular  
146 disease. Bovine and murine *in vitro* oocyte maturation models demonstrate that elevated fatty  
147 acid concentrations perturb follicular physiology, reduce oocyte developmental competence,  
148 including altered transcriptome and epigenome profiles in blastocysts, and lead to early  
149 embryos with compromised metabolism and lower potential<sup>12</sup>.

150 The combination of metabolic, mitochondrial and chromosomal alterations in oocytes and  
151 embryos from obese mothers has important implications for subsequent development. In  
152 mice, obese mothers have smaller fetuses and pups which develop overgrowth, adiposity and  
153 glucose intolerance after birth<sup>20</sup>. Transfer of mouse blastocysts from obese mothers to normal  
154 recipients produces similarly growth-restricted fetuses with associated malformations despite  
155 the absence of gestational maternal obesity<sup>18</sup>. Similarly, in sheep, female offspring from  
156 embryos of obese natural mothers transferred to non-obese mothers exhibit increased  
157 adiposity, with dysregulation in liver and muscle insulin signalling and hepatic fatty acid  
158 oxidation<sup>21</sup>. These changes are associated with epigenetic perturbations in the liver, including  
159 upregulation of microRNAs regulating insulin signalling<sup>21</sup>. Similarly, mouse embryos  
160 transferred from diabetic mothers to control recipients exhibit fetal growth retardation and  
161 congenital anomalies resembling natural diabetic pregnancies<sup>8</sup>; such structural changes are  
162 in keeping with clinical practice, in which pre/periconceptual folic acid supplementation and  
163 improved diabetes control reduce the incidence of anomalies.

164 The periconceptual effects of maternal obesity are also apparent in ART pregnancies.  
165 Fertility declines with increasing BMI in women receiving donor oocytes, as in non-donated  
166 pregnancies, suggesting reduced uterine receptivity<sup>22</sup>. However, in other studies, recipient  
167 BMI had no effect on donor oocyte pregnancy success, whilst **donor** BMI was negatively  
168 associated<sup>23</sup>, indicating that pre-conception oocyte quality is influenced by maternal adiposity.

169

## 170 **Periconceptual developmental conditioning through maternal undernutrition**

### 171 **Human studies**

172 Poor nutrition in utero and low birth weight remain highly prevalent in low and middle income  
173 countries and are associated with increased risks of chronic diseases in later life across  
174 diverse human populations, particularly if followed by accelerated weight gain during  
175 infancy<sup>1,3</sup>. Similar human cardiometabolic and neurological consequences arise from maternal  
176 exposure to famine, e.g. the Dutch Hunger Winter of 1944/45. In human studies it is difficult  
177 to pinpoint gestational windows when heightened sensitivity to maternal undernutrition occurs,  
178 but the Dutch famine analyses suggest a poorer prognosis for those offspring **conceived**  
179 during the famine rather than experiencing it later in gestation<sup>24</sup>. Similarly, individuals exposed  
180 in utero, particularly during the first trimester, to the Chinese Great Famine (1959-61) have  
181 increased risk of hypertension in adulthood<sup>25</sup>. Exposure during the periconceptual period of  
182 the Dutch famine is reported to cause epigenetic dysregulation resulting in reduced DNA  
183 methylation of the imprinted growth-regulating IGF2 gene persisting into adulthood, along with  
184 differential methylation in the regulatory regions of genes affecting growth and metabolism<sup>24</sup>.

185 In another important human study, dramatic seasonal variation in maternal nutrient  
186 consumption in The Gambia affected perinatal outcomes including birth weight, adult health  
187 and mortality<sup>26</sup>. By studying genomic regions where methylation patterns are highly correlated  
188 across tissues derived from all three germ lines it has been possible to demonstrate that  
189 maternal nutrition at conception alters the epigenome prior to gastrulation, with the effects  
190 persisting, at minimum, well into childhood and adolescence<sup>27</sup>. This periconceptual legacy  
191 coincided with seasonal changes in maternal plasma methyl-donor biomarkers which, along  
192 with BMI, are also predictive of childhood methylation patterns<sup>28</sup>. So far, significant deviations  
193 in the methylation patterns of loci predictive of immune function, tumour suppression<sup>29</sup> and  
194 obesity<sup>30</sup> have been noted.

## 195 **Animal models**

196 Animal models have been essential for investigating mechanisms involved in the multistep  
197 processes linking periconceptual maternal undernutrition with later-life disease risk. In  
198 rodents, feeding a low protein diet (LPD) - specifically during the periconceptual period,  
199 either exclusively during the final 3 days of oocyte maturation<sup>31</sup> or the 3-4 day window of  
200 preimplantation embryo development (Emb-LPD)<sup>32,33</sup>, with normal nutrition at all other times -  
201 is sufficient to induce an altered growth trajectory and cardiovascular, metabolic and neuro-  
202 behavioural dysfunction in adulthood. Such targeted dietary models commonly show  
203 hypertension in adult offspring, coupled with increased adiposity<sup>7,31-33</sup>. Similar findings have  
204 been reported in sheep<sup>34</sup>.

205 Rodent and sheep models of maternal periconceptional undernutrition suggest that impaired  
206 regulation of fetal development may underlie co-morbidities. For example, studies in sheep  
207 have shown that the late gestation fetal cardiovascular response to hypoglycaemia is modified  
208 by prior peri-implantation undernutrition<sup>35</sup>. Moreover, peri-implantation and late gestation  
209 maternal undernutrition affect fetal sheep skeletal muscle development differentially<sup>36</sup>, and  
210 maternal undernutrition in early gestation alters gestation length and fetal and postnatal  
211 growth<sup>37</sup>.

## 212 **Induction and response mechanisms**

213 The mouse embryonic period low protein diet (Emb-LPD) model has helped reveal how  
214 periconceptional maternal undernutrition may initiate adverse effects during early  
215 embryogenesis<sup>7</sup>. Emb-LPD reduces circulating maternal insulin and amino acid  
216 concentrations, including reduced branched-chain amino acids (BCAAs) within the uterine  
217 luminal fluid that bathes early embryos before implantation<sup>38</sup>. BCAAs act as targets for embryo  
218 nutrient sensors, enabling nutrient status to be sensed by blastocysts via the mammalian  
219 target of rapamycin complex 1 (mTORC1) growth-regulating signalling pathway, inducing an  
220 altered growth trajectory from before implantation<sup>38</sup> (see below), and shown by embryo  
221 transfer to be induced within the blastocyst<sup>33</sup>. Altered induction by Emb-LPD in mice activates  
222 compensatory responses that are distinct between extra-embryonic (trophectoderm; primitive  
223 endoderm) and embryonic (epiblast) lineages of the blastocyst (**Figure 1**). The Emb-LPD  
224 trophoctoderm becomes more proliferative, adopts a more invasive migratory phenotype at  
225 implantation, and activates increased endocytosis of maternal uterine luminal fluid proteins as  
226 an alternative source of nutrients, leading to a placenta that is more efficient in nutrient transfer  
227 to the fetus<sup>38-40</sup>. Similarly, the primitive endoderm activates compensatory responses to  
228 enhance nutrient delivery via the yolk sac placenta, mediated through epigenetic  
229 mechanisms<sup>40,41</sup>.

230 In response to Emb-LPD, changes in embryonic lineages may help set the embryonic and  
231 fetal growth trajectory to match prevailing nutrient availability. The embryonic lineages utilise  
232 preimplantation nutrient sensing to regulate growth across somatic organs (e.g., liver and  
233 kidney) through adaptations in the rate of ribosome biogenesis<sup>42</sup>. In essence, rRNA expression  
234 is suppressed during periods of maternal dietary restriction but is increased, beyond that of  
235 the control rate, when the dietary challenge is removed. This mechanism modulates the level  
236 of DNA methylation at the rDNA promoter, thereby mediating RNA polymerase I interaction  
237 with the promoter to regulate ribosome biogenesis and growth<sup>42,43</sup>. Interestingly, rDNA has  
238 also been found to be a genomic target for growth regulation in models of maternal high-fat or

239 obesogenic diets<sup>43</sup>. This exquisite lifetime mechanism, activated in the preimplantation  
240 embryo, is likely to be responsive to uterine luminal fluid nutrient concentrations and appears  
241 to utilise a nutrient-sensing ribosome factor, Rrn3, to mediate the rDNA responses<sup>42</sup>. The  
242 growth-regulating role of the embryonic lineages is critical since perinatal weight associates  
243 with adult disease risk<sup>33</sup>.

244

245

## 246 **Paternal origin of periconceptual developmental programming**

247 Whilst the connection between a mother's diet and the long-term health of her offspring has  
248 been studied in detail, our understanding of how a father's diet impacts his offspring remains  
249 limited. However, links are now emerging between paternal lifestyle, sperm quality and  
250 impaired offspring health<sup>9</sup>. Here, both direct (sperm quality, epigenetic status, DNA integrity)  
251 and indirect (seminal fluid composition) paternal mechanisms have been identified, with the  
252 potential to affect **mouse** offspring development across multiple generations<sup>44</sup>.

253 Mirroring female reproductive fitness, male fertility is closely linked to nutrition and body  
254 composition. In humans and rodents, elevated BMI is associated with reduced sperm  
255 motility<sup>45</sup>, increased sperm abnormality<sup>46</sup>, increased sperm reactive oxygen species levels,  
256 reduced serum testosterone and increased oestradiol concentrations<sup>47</sup>. Consumption of a  
257 'Western-style' diet high in sugar, fat and processed food associates with reduced sperm  
258 motility in men<sup>48</sup>, while consumption of energy-dense diets in men and rodents is associated  
259 with poor sperm motility, morphology and DNA integrity<sup>49</sup>. Reduced sperm DNA integrity, as  
260 occurs in obesity and diabetes, correlates with reduced **human** embryonic development and  
261 decreased pregnancy rates<sup>50</sup>. In men undergoing IVF treatment, obesity is associated with  
262 reduced blastocyst development and live birth rates<sup>51</sup>. In rodents, paternal obesity induced by  
263 high-fat diet increases sperm DNA damage<sup>52</sup>, reduces blastocyst development and  
264 implantation rates<sup>53</sup> and causes sub-fertility in male and female offspring for up to two  
265 generations<sup>54</sup>. Interestingly, these negative effects on offspring development can be prevented  
266 through paternal dietary and exercise interventions **in mice**<sup>55</sup>, indicating that sperm-mediated  
267 effects may be transient and even reversible. In rats, a paternal high-fat diet for 10 weeks  
268 before mating affected female (but not male) offspring pancreatic  $\beta$ -cell function and increased  
269 body weight, glucose intolerance and impaired insulin secretion<sup>56</sup>. Offspring of male mice over-  
270 nourished during neonatal life demonstrate glucose intolerance, fasting hyperglycaemia and  
271 insulin resistance, mirroring the metabolic disturbance seen in their fathers<sup>57</sup>.

272 Similar to the impacts of paternal obesity, paternal LPD in mice induces the expression of  
273 genes involved in offspring hepatic lipid and cholesterol biosynthesis<sup>58</sup>. Analysis of offspring  
274 hepatic epigenetic status revealed genome-wide changes in DNA methylation, including the  
275 key lipid regulator *PPARα*. In adulthood, offspring from male mice fed LPD have higher birth  
276 weight, a reduced male:female offspring ratio, increased adult adiposity, hypotension, glucose  
277 intolerance and elevated serum TNF-α levels<sup>59</sup>. Furthermore, paternal LPD also affects  
278 blastocyst *AMPK* gene expression, placental size, fetal growth and skeletal development<sup>60</sup>.

279 As for maternal periconceptional nutrition models, epigenetic mechanisms are likely mediators  
280 of effects of paternal phenotype and exposures on offspring development<sup>61</sup>. Changes in  
281 patterns of sperm histone modifications (methylation, acetylation), DNA methylation and/or  
282 RNA content are prime candidates for such paternal periconceptional programming. Sperm  
283 from infertile men display significant changes in histone populations<sup>62</sup>, with enrichment of  
284 active histone marks (i.e. H3K27me3) at key developmental and pluripotency genes in human  
285 and mouse sperm<sup>62</sup>. Studies have also revealed that sperm-derived histones are transferred  
286 into the oocyte and incorporate into zygotic chromatin following **human** fertilisation<sup>63</sup>. However,  
287 whether any of the 2-15% histones retained within the mammalian sperm contribute directly  
288 to zygotic gene expression regulation is unknown. Human sperm also contain several  
289 thousand coding RNA transcripts<sup>64</sup> and altered expression is linked with infertility<sup>65</sup>. Recent  
290 studies have shown that levels of sperm tRNA-derived small RNAs (tsRNAs) are altered by  
291 paternal diet in mice<sup>66</sup>. Interestingly, offspring generated by injecting zygotes with sperm  
292 tsRNA taken from male mice fed a HFD showed impaired glucose tolerance and insulin  
293 secretion<sup>66</sup>. While such studies highlight the role of RNA populations in intergenerational  
294 programming<sup>67</sup>, the significance of these sperm-derived RNA molecules remains to be  
295 elucidated.

296 Apart from sperm-specific mechanisms of developmental programming, seminal plasma  
297 composition, (e.g. granulocyte-macrophage colony-stimulating factor) influences **mouse**  
298 embryonic, placental and offspring development<sup>68</sup> and initiates maternal reproductive tract  
299 immunological responses, essential in the establishment and maintenance of **human**  
300 pregnancy<sup>69</sup>. In mice, paternal seminal fluid impacts on the maternal uterine environment,  
301 altering blastocyst development, placental size and adult offspring glucose tolerance,  
302 adiposity and blood pressure<sup>70</sup>.

303

304 **Defining the parental contribution to periconceptional developmental effects**

305 Shared maternal and paternal dietary and other lifestyle influences may potentially combine  
306 for greater impact on periconceptual development. However, most research models to date  
307 are uniparental in design and the combined effects of both parents are unknown. Whether the  
308 impact of poor paternal diet on offspring development and wellbeing is of equivalent  
309 significance to that of poor maternal diet is also unknown. As a first step, Box 2 and Figure 3  
310 show a meta-analysis of our mouse maternal and paternal LPD diet models using published  
311 data for offspring weight at birth, adult systolic blood pressure (SBP) and adult heart:body  
312 weight ratio (a measure of heart capacity) including datasets covering maternal intervention  
313 restricted to the periods of oocyte maturation, preimplantation development or the entirety of  
314 gestation<sup>31,33,59</sup>. The use of the same robust, statistical random effects regression analysis  
315 across each of these studies strengthens our comparison of parental effects in the current  
316 analysis. However, such rigorous statistical approaches are rarely adopted, especially in  
317 animal model studies, and so we have restricted our analysis to data from these three studies  
318 alone. Offspring birth weight was increased in response to maternal LPD during the terminal  
319 stages of oocyte development (Egg-LPD) and during preimplantation development (Emb-LPD) (**Figure 3a**). Overall, the pooled estimate demonstrated parental  
320 LPD increased offspring birth weight. Our second analysis explored the impact of parental  
321 LPD on adult offspring SBP. Here, all maternal challenges resulted in offspring hypertension  
322 (**Figure 3b**), while paternal LPD resulted in a trend towards lower blood pressure in the adult  
323 offspring. Our final analysis examined the impact of parental diet on adult heart:body weight  
324 ratio (**Figure 3c**). Only paternal LPD had a significant effect, reducing offspring heart:body  
325 weight ratio. These new data demonstrate differential effects from paternal and maternal  
326 periconceptual developmental exposures on offspring phenotype. It is essential that further  
327 studies define the precise impacts and underlying mechanisms by which parental diet regimes  
328 affect offspring development and wellbeing. Studies examining concurrent paternal and  
329 maternal interventions on shared offspring outcomes are also warranted.  
330

## Box 2: Analysis of parental contribution effect

- Data for offspring phenotype were taken from Watkins et al 2008a<sup>31</sup>, 2008b<sup>33</sup> and 2014<sup>59</sup>. Each study used the same NPD and LPD formulation fed to either female or male mice for distinct periconceptional durations.
- All three studies employed the same rigorous random effects regression analysis to account for the hierarchical nature of the studies in the statistical analyses.
- Raw data on individual offspring weight at birth, adult tail-cuff systolic blood pressure measurement and adult heart:body weight ratio for all groups were used for the analyses.
- Raw mean differences between experimental and study-specific control group (normalised to a value of 0) offspring were calculated ( $\Delta = \mu_1 - \mu_2$ ) for birth weight, systolic blood pressure (SBP) and heart:body weight ratio parameters.
- Weight (%) refers to the individual contribution (by number of animals) of each study to the total Pooled Estimate. Heterogeneity (i.e. variation in outcomes between studies) was assessed using  $\chi^2$  test on Cochran's Q-statistic and by calculating  $I^2$  (i.e. percentage of variation across studies attributed to heterogeneity rather than chance). As heterogeneity was significant for all analyses, pooled estimates were calculated by the random effects (Mantel-Haenszel) method.
- The largest effect on offspring birth weight was in response to maternal preimplantation (Emb-LPD) diet (raw mean difference: 0.18g, 95% CI 0.11 – 0.24;  $P < 0.0001$ ) (**Figure 3a**). Maternal LPD restricted to the terminal stages of oocyte maturation (Egg-LPD) also resulted in increased birth weight (raw mean difference: 0.09g, 95% CI 0.05 – 0.13;  $P < 0.0001$ ). However, maternal LPD throughout gestation had no impact (raw mean difference: 0.04g,  $P = 0.26$ ) on offspring birth weight (likely reflecting fetal growth regulation during gestation, discussed above), as did paternal LPD (raw mean difference 0.03g,  $P = 0.09$ ). Overall we observe a significant pooled estimate effect of parental LPD on offspring weight at birth (raw mean difference: 0.1g, 95% CI 0.07 – 0.13;  $P < 0.0001$ ) representing an increase in LPD offspring weight of 7.8%.
- Analysis of offspring SBP revealed all maternal LPD groups had elevated SBP (raw mean difference: Egg-LPD 6.92mmHg, 95% CI 4.95 – 8.90;  $P < 0.0001$ ; Emb-LPD 5.60mmHg, 95% CI 3.63 – 7.56;  $P < 0.001$ ; LPD 5.54mmHg, 95% CI 3.66 – 7.42;  $P < 0.0001$ ) (**Figure 3b**). In contrast, paternal LPD resulted in a trend towards the programming of lower offspring blood pressure (raw mean difference: -3.49mmHg, 95% CI -7.62 – 0.63;  $P = 0.096$ ). The differential parental effect on offspring SBP meant the pooled estimate showed no overall difference (raw mean difference: -0.36mmHg, 95% CI -1.75 – 1.02;  $P = 0.61$ ).
- Our final analysis examined the impact of parental diet on adult heart:body weight ratio. All groups displayed either a negative impact or no effect (**Figure 3c**). The largest size effect was observed in response to maternal Emb-LPD (raw mean difference: -0.05, 95% CI -0.1 – 0.01  $P = 0.073$ ). Only the paternal LPD offspring heart:body weight ratio reached significance (raw mean difference: -0.03, 95% CI -0.07 – -0.01;  $P = 0.038$ ) (**Figure 3c**). Overall, the pooled effects indicated a reduction in adult heart:body weight ratio following parental, both maternal and paternal, LPD (raw mean difference: -0.03, 95% CI -0.05 – -0.01;  $P = 0.0035$ ).

## 332 **Periconceptual developmental programming and ART**

333 Direct evidence for human periconceptual effects comes from assisted reproductive  
334 treatments (ART) in which mature gametes and the preimplantation embryo are exposed to  
335 precisely timed in vitro manipulations. Several million apparently healthy ART children have  
336 now been born worldwide, but relatively little is known about the possible impact of the  
337 technology-associated exposures during conception and very early development on their  
338 health status during childhood and later life. The spectrum of human demographic  
339 confounders (including parental infertility), changes and improvements in ART techniques  
340 over time, and the relative sample sizes used make analyses complex and the reported  
341 outcomes need to be interpreted with caution. Nevertheless, it is well established that  
342 singleton ART pregnancies have increased risk of low birth weight, congenital abnormalities  
343 and higher mortality rate, although disentangling confounding by parental infertility is difficult<sup>71</sup>.  
344 Human embryo culture media have changed over time and the predominant current practice  
345 is to use commercially sourced media of proprietary (unspecified) composition (discussed  
346 in<sup>12</sup>). Comparison of perinatal outcome following use of different commercial media, including  
347 a multicentre randomised controlled trial, has indicated that birth weight is significantly  
348 affected<sup>72</sup>, with effects on growth still manifest at age 2 years<sup>73</sup>.

349 Compared with naturally conceived offspring, the cardiovascular phenotype of IVF children  
350 and adolescents reveals increased risk of high blood pressure<sup>11,74</sup>, vascular dysfunction with  
351 abnormal blood flow and vessel thickness<sup>75</sup> and evidence of cardiovascular remodelling during  
352 development *in utero* affecting heart shape and chamber size<sup>74</sup>. Metabolic consequences  
353 include increased fasting glucose and peripheral insulin resistance<sup>11,76</sup>, raised plasma lipids,  
354 and obesity<sup>76</sup>. A systematic review found no difference in cognitive outcomes among children  
355 conceived with conventional IVF and those conceived naturally, but did identify conflicting  
356 findings that require clarification among studies of children conceived with intracytoplasmic  
357 sperm injection<sup>77</sup>.

358 Collectively, current evidence suggests that ART, like the in vivo nutritional models discussed  
359 above, may alter the development and growth trajectory of human embryos, and increase the  
360 risk of postnatal chronic cardiometabolic dysfunction. This legacy is unlikely to be due to  
361 parental infertility in isolation since controls in some studies comprise those naturally  
362 conceived offspring from sub-fertile parents<sup>11,75</sup>. Moreover, ART animal models demonstrate  
363 similar long-term consequences to human studies, despite normal parental fertility<sup>78</sup>. Thus,  
364 IVF embryo culture and transfer in mice results in offspring with altered growth trajectory,  
365 relative hypertension, cardiovascular abnormalities and glucose/insulin dysfunction<sup>78</sup>.

366 ART-associated adverse effects on long-term health appear to have an epigenetic origin  
367 induced during the periconceptual period. ART children have an increased risk of rare  
368 imprinting disorders associated with DNA methylation errors on imprinted genes<sup>79</sup> and  
369 aberrant methylation of imprinted *H19* gene has been reported in human cultured embryos<sup>80</sup>.  
370 In mouse models, embryo culture may cause imprinted genes to lose their allele-specific  
371 expression (particularly at the growth regulating *H19/IGF2* locus) together with aberrant  
372 methylation patterning in embryos, placental and fetal tissues<sup>81</sup>. ART-induced aberrant  
373 epigenetic profiles may also be propagated during human pregnancy in fetal and placental  
374 tissues and persist into childhood affecting genes regulating growth such as the *IGF2/H19*  
375 locus<sup>82</sup>. Media composition, particularly albumin or serum components or ammonium ion  
376 accumulation from amino acid catabolism, may contribute to altered mouse epigenetic  
377 status<sup>83</sup>. Importantly, even a very limited culture period is sufficient to induce epigenetic  
378 changes<sup>81</sup>. Embryo culture exposure also modifies expression and methylation of non-  
379 imprinted genes in mice and alters expression of DNA methyltransferases<sup>84</sup>. For example, in  
380 mouse models ART affects the endothelial nitric oxide synthase (*eNOS*) gene implicated in  
381 vascular dysfunction and modification of culture media composition may prevent this effect<sup>85</sup>.  
382 Although provocative, more studies in both animal models and humans are required in order  
383 to replicate findings to date.

384

### 385 **Diversity and commonality in periconceptual effects**

386 The evidence reviewed above reveals that periconceptual experience can induce lifelong  
387 changes in phenotype, affecting disease risk. Beyond these nutritional and ART conditions,  
388 studies in rodents show broader examples of periconceptual effects, such as from maternal  
389 stress<sup>86</sup>. Moreover, maternal alcohol consumption exclusively around conception induced  
390 metabolic dysfunction in rat adult offspring with evidence of epigenetic disturbance<sup>87</sup>. In the  
391 case of mouse maternal systemic inflammation at conception, whilst not affecting  
392 cardiometabolic health, suppressed adult offspring innate immunity after challenge, possibly  
393 to protect 'self' in a predicted pathogenic postnatal environment<sup>88</sup>. In addition, mouse embryo  
394 transfer experiments suggest that advanced maternal age may adversely affect offspring  
395 cardiometabolic health<sup>89</sup>, but the mechanisms underlying this age-associated effect are  
396 unknown.

397 The diversity of periconceptual induction conditions identified across mammalian species,  
398 coupled with clear evidence of both maternal and paternal pathways, implicates an early  
399 window when environmental exposures, combined with an inherent capacity for

400 developmental plasticity, may confer advantage when the offspring are exposed to a similar  
401 environment postnatally. During the periconceptual period there is rapid and radical  
402 molecular, cellular and morphogenetic restructuring; the signalling pathways that control these  
403 processes are sensitive to multiple molecules and other factors within the cellular environment  
404 and may provide a mechanistic underpinning for this concept<sup>90</sup>. However, as we have  
405 described, the periconceptual setting of metabolic homeostasis may become maladaptive if  
406 conditions change or if nutrient levels induce perturbations in metabolism, generating the  
407 circumstances underlying adverse health risk. A consistent mechanism identified across  
408 conditions and species has been epigenetic variation, a plausible pathway to 'biological  
409 embedding' of early life exposures and transmission of phenotypic effects throughout life. This  
410 has been demonstrated directly through manipulation of maternal one-carbon (1-C)  
411 metabolism during early embryogenesis, potentially reducing the availability of methyl donor  
412 groups necessary for DNA and histone methylation<sup>91</sup>, but such epigenetic changes are not  
413 necessarily linked directly with changes in gene expression<sup>92</sup>. Thus, a periconceptual  
414 maternal diet deficient in 1-C metabolite substrates and cofactors (vitamin B<sub>12</sub>, folate,  
415 methionine) in sheep modified offspring DNA methylation and led to adverse cardiometabolic  
416 and immune dysfunction<sup>93</sup>. Similarly, folate addition to rodent maternal LPD can rescue normal  
417 expression and DNA methylation of metabolic regulators in offspring which underlie  
418 cardiovascular dysfunction<sup>94</sup>. A mouse paternal low folate diet altered sperm DNA methylation  
419 profile, changed the placental transcriptome and resulted in offspring with craniofacial and  
420 musculoskeletal malformations<sup>95</sup>. Moreover, the negative impact of mouse paternal  
421 undernutrition on sperm quality, testicular oxidative stress, fertility and offspring fat  
422 accumulation and dyslipidaemia are reversed through vitamin and antioxidant  
423 supplementation<sup>96</sup>. As with ART, additional studies are warranted to define the critical  
424 window(s) and pathways linking perinatal one-carbon metabolism, epigenetic variation and  
425 programming of later offspring health.

426

## 427 **Conclusion: Protecting health of the next generation and the way forward**

428 We propose there is now sufficient evidence from human and animal research that the  
429 periconceptual period is a key window during which poor maternal and paternal physiology,  
430 body composition, metabolism and diet can induce increased risk of chronic disease in  
431 offspring, a lifetime legacy and major driver of health burden in the 21st century. The evidence  
432 that similar consequences can result from ART practices sharpens the focus on this window.  
433 Environmental factors may perturb gametes or early embryos, affecting homeostatic

434 mechanisms, or may induce adaptations to developmental environmental signals with  
435 consequences persisting into adulthood.

436 This evidence calls for a major re-examination of public health policy to protect against future  
437 disease risk through societal advice on, and greater provision of, preconception care<sup>97</sup> as also  
438 promoted in the two accompanying reviews in this series (Stephenson et al, submitted; Barker  
439 et al, submitted). Whilst a preconception focus on parental risk factors such as smoking and  
440 excess alcohol intake is wise and well established, new drives to prepare nutritionally for  
441 pregnancy are critical, including healthy body composition, physical activity and diet for both  
442 parents<sup>98</sup>. Further definition of the underlying epigenetic, cellular, metabolic and/or  
443 physiological mechanisms and the exposures that drive them, is an important research  
444 agenda that is pivotal to the characterisation of more specific recommendations for  
445 preconception health.

446

447

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470

471 **Contributors**

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474 manuscript.

475

476 **Figure legends**

477 **Figure 1. Biological events underpinning periconceptual conditioning**

478 The periconceptual period is one of extensive cellular change comprising the completion of  
479 meiotic maturation of oocytes, differentiation of spermatozoa, fertilisation and resumption of  
480 mitotic cell cycles in the zygote, marking the transition from parental to embryonic genomes<sup>4</sup>  
481 and the onset of morphogenesis<sup>5</sup>. Periconceptual biology is indeed 'busy' – the  
482 morphological and cellular changes occurring during the switch from parental to embryonic  
483 generations leading to blastocyst formation are driven by pronounced sub-cellular and  
484 molecular processes. These include global restructuring of the epigenome (mainly DNA  
485 methylation and histone modifications that control gene expression), such that expression  
486 from the new embryonic genome is distinct from the parental genomes<sup>99</sup>. Epigenetic  
487 reorganisation allows the embryo to first exhibit *totipotency*, a naïve cellular state conferring  
488 the ability to construct both true embryonic (future fetal) cell lineages and the extra-  
489 embryonic (placental) lineages that become evident in the blastocyst. Subsequently,  
490 epigenetic modifications underpin embryo *pluripotency*, the capacity to generate all three  
491 germ layers (ectoderm, mesoderm, endoderm) once gastrulation has taken place.  
492 Morphogenesis of the blastocyst is followed by embryo hatching from the zona pellucida  
493 coat and implantation mediated through adhesion of the outer trophoderm layer of the  
494 blastocyst to the uterine endometrium and subsequent invasion and decidualisation.  
495 Activation of the new embryonic genome before implantation not only permits de novo gene  
496 expression distinct from parental genomes but also involves establishment of the embryo's  
497 metabolism that matures over time<sup>100</sup>.

498

499 **Figure 2. Summary of periconceptual developmental conditioning from the four**  
500 **areas reviewed with main mechanisms highlighted in the progression of disease risk.**  
501 **ICSI = intracytoplasmic sperm injection, IVF = in vitro fertilization.**

502

503 **Figure 3. Defining the relative influence of maternal and paternal factors during**  
504 **periconceptual conditioning in mice following parental low protein diet (LPD; 9 %**  
505 **casein).**

506 The effect of parental LPD on **(A)** offspring weight at birth, **(B)** adult offspring systolic blood  
507 pressure (SBP), and **(C)** adult offspring heart:body weight ratio are shown when compared

508 with offspring from normal protein diet (NPD; 18% casein) fed parents. Analysis of 4 studies  
509 involving female MF1 mice being fed LPD exclusively during the terminal stages of oocyte  
510 maturation (3.5 days prior to mating; Egg-LPD), exclusively during preimplantation embryo  
511 development (Emb-LPD) or throughout gestation (LPD). Forest plots also include offspring  
512 data in response to a paternal low protein (Pat-LPD) fed to C57BL6 males prior to mating.  
513 For Egg-NPD n = 189–80 from 19 litters; Egg-LPD n = 201-67 from 19 litters; NPD n = 131-  
514 76 from 19 litters; LPD n = 116-85 from 19 litters; Emb-LPD n = 134-78 from 19 litters; Pat-  
515 NPD n = 85-76 from 16 litters; Pat-LPD n = 73-62 from 16 litters. **A.** Plots present differences  
516 between means ( $\pm$  95% CI) of birth weight (grams) to study specific NPD group. Data  
517 combining all LPD and all NPD treatment groups is used to determine the Pooled Estimate.  
518 Heterogeneity ( $\chi^2$ ) between studies = 1.96 (3 df),  $I^2$  = 33%. **B.** Plots present differences  
519 between means ( $\pm$  95% CI) of adult SBP (mmHg) to study specific NPD group. Data  
520 combining all LPD and all NPD treatment groups is used to determine the Pooled Estimate.  
521 Heterogeneity ( $\chi^2$ ) between studies = 1.05 (4 df),  $I^2$  = 39%. **C.** Plots present differences  
522 between means ( $\pm$  95% CI) of heart:body weight ratio to study specific NPD group. Data  
523 combining all LPD and all NPD treatment groups is used to determine the Pooled Estimate.  
524 heterogeneity ( $\chi^2$ ) between studies = 1.86 (3 df),  $I^2$  = 61%.

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528 **References**

- 529 1. Barker DJ, Thornburg KL. The obstetric origins of health for a lifetime. *Clinical obstetrics and*  
530 *gynecology*. 2013; **56**(3): 511-9.
- 531 2. Godfrey KM, Reynolds RM, Prescott SL, Nyirenda M, Jaddoe VW, Eriksson JG, et al. Influence  
532 of maternal obesity on the long-term health of offspring. *The lancet Diabetes & endocrinology*. 2017;  
533 **5**(1): 53-64.
- 534 3. Hanson MA, Gluckman PD. Early developmental conditioning of later health and disease:  
535 physiology or pathophysiology? *Physiol Rev*. 2014;94:1027-76.
- 536 4. Li L, Lu X, Dean J. The maternal to zygotic transition in mammals. *Molecular aspects of*  
537 *medicine*. 2013; **34**(5): 919-38.
- 538 5. Bedzhov I, Graham SJ, Leung CY, Zernicka-Goetz M. Developmental plasticity, cell fate  
539 specification and morphogenesis in the early mouse embryo. *Philos Trans R Soc Lond B Biol Sci*.  
540 2014; **369**(1657).
- 541 6. Nicholas LM, Morrison JL, Rattanatray L, Zhang S, Ozanne SE, McMillen IC. The early origins  
542 of obesity and insulin resistance: timing, programming and mechanisms. *Int J Obes (Lond)*. 2016;  
543 **40**(2): 229-38.
- 544 7. Fleming TP, Watkins AJ, Sun C, Velazquez MA, Smyth NR, Eckert JJ. Do little embryos make  
545 big decisions? How maternal dietary protein restriction can permanently change an embryo. *Reprod*  
546 *Fertil Dev*. 2015.
- 547 8. Wyman A, Pinto AB, Sheridan R, Moley KH. One-cell zygote transfer from diabetic to  
548 nondiabetic mouse results in congenital malformations and growth retardation in offspring.  
549 *Endocrinology*. 2008; **149**(2): 466-9.
- 550 9. Sinclair KD, Watkins AJ. Parental diet, pregnancy outcomes and offspring health: metabolic  
551 determinants in developing oocytes and embryos. *Reprod Fertil Dev*. 2013; **26**(1): 99-114.
- 552 10. Hansen PJ, Dobbs KB, Denicol AC, Siqueira LG. Sex and the preimplantation embryo:  
553 implications of sexual dimorphism in the preimplantation period for maternal programming of  
554 embryonic development. *Cell and tissue research*. 2016; **363**(1): 237-47.
- 555 11. Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE, Delemarre-van de Waal  
556 HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin*  
557 *Endocrinol Metab*. 2008; **93**(5): 1682-8.
- 558 12. Sunde A, Brisson D, Dumoulin J, Harper J, Lundin K, Magli MC, et al. Time to take human  
559 embryo culture seriously. *Hum Reprod*. 2016; **31**(10): 2174-82.
- 560 13. Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, et al.  
561 Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and  
562 insulin resistance: a novel murine model of developmental programming. *Hypertension*. 2008; **51**(2):  
563 383-92.
- 564 14. Ruebel ML, Cotter M, Sims CR, Moutos DM, Badger TM, Cleves MA, Shankar K, Andres A.  
565 Obesity modulates inflammation and lipid metabolism oocyte gene expression: a single cell  
566 transcriptome perspective. *J Clin Endocrinol Metab*. 2017 ;102:2029-2038.
- 567 15. Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, et al. Obese women  
568 exhibit differences in ovarian metabolites, hormones, and gene expression compared with  
569 moderate-weight women. *The Journal of clinical endocrinology and metabolism*. 2009; **94**(5): 1533-  
570 40.
- 571 16. Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ, et al. Exposure to lipid-rich follicular  
572 fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-  
573 oocyte complexes. *Fertil Steril*. 2012; **97**(6): 1438-43.
- 574 17. Leary C, Leese HJ, Sturmey RG. Human embryos from overweight and obese women display  
575 phenotypic and metabolic abnormalities. *Hum Reprod*. 2015; **30**(1): 122-32.
- 576 18. Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, et al. High fat diet induced  
577 developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain  
578 defects. *PLoS One*. 2012; **7**(11): e49217.

- 579 19. Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchon MR, et al. Maternal diet-  
580 induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PLoS*  
581 *One*. 2010; **5**(4): e10074.
- 582 20. Jungheim ES, Schoeller EL, Marquard KL, Loudon ED, Schaffer JE, Moley KH. Diet-induced  
583 obesity model: abnormal oocytes and persistent growth abnormalities in the offspring.  
584 *Endocrinology*. 2010; **151**(8): 4039-46.
- 585 21. Nicholas LM, Rattanatray L, MacLaughlin SM, Ozanne SE, Kleemann DO, Walker SK, et al.  
586 Differential effects of maternal obesity and weight loss in the periconceptional period on the  
587 epigenetic regulation of hepatic insulin-signaling pathways in the offspring. *FASEB J*. 2013; **27**(9):  
588 3786-96.
- 589 22. Bellver J, Pellicer A, Garcia-Velasco JA, Ballesteros A, Remohi J, Meseguer M. Obesity reduces  
590 uterine receptivity: clinical experience from 9,587 first cycles of ovum donation with normal weight  
591 donors. *Fertil Steril*. 2013; **100**(4): 1050-8.
- 592 23. Cardozo ER, Karmon AE, Gold J, Petrozza JC, Styer AK. Reproductive outcomes in oocyte  
593 donation cycles are associated with donor BMI. *Hum Reprod*. 2016; **31**(2): 385-92.
- 594 24. Tobi EW, Goeman JJ, Monajemi R, Gu H, Putter H, Zhang Y, et al. DNA methylation signatures  
595 link prenatal famine exposure to growth and metabolism. *Nature communications*. 2014; **5**: 5592.
- 596 25. Wang PX, Wang JJ, Lei YX, Xiao L, Luo ZC. Impact of fetal and infant exposure to the Chinese  
597 Great Famine on the risk of hypertension in adulthood. *PLoS One*. 2012; **7**(11): e49720.
- 598 26. Rayco-Solon P, Fulford AJ, Prentice AM. Differential effects of seasonality on preterm birth  
599 and intrauterine growth restriction in rural Africans. *Am J Clin Nutr*. 2005; **81**(1): 134-9.
- 600 27. Waterland RA, Kellermayer R, Laritsky E, Rayco-Solon P, Harris RA, Travisano M, et al. Season  
601 of conception in rural gambia affects DNA methylation at putative human metastable epialleles.  
602 *PLoS genetics*. 2010; **6**(12): e1001252.
- 603 28. Dominguez-Salas P, Moore SE, Baker MS, Bergen AW, Cox SE, Dyer RA, et al. Maternal  
604 nutrition at conception modulates DNA methylation of human metastable epialleles. *Nature*  
605 *communications*. 2014; **5**: 3746.
- 606 29. Silver MJ, Kessler NJ, Hennig BJ, Dominguez-Salas P, Laritsky E, Baker MS, Coarfa C,  
607 Hernandez-Vargas H, Castelino JM, Routledge MN, Gong YY, Herceg Z, Lee YS, Lee K, Moore SE,  
608 Fulford AJ, Prentice AM, Waterland RA. Independent genomewide screens identify the tumor  
609 suppressor VTRNA2-1 as a human epiallele responsive to periconceptional environment. *Genome*  
610 *Biol*. 2015;16:118.
- 611 30. Kühnen P, et al. Interindividual Variation in DNA Methylation at a Putative POMC Metastable  
612 Epiallele Is Associated with Obesity. *Cell Metab*. 2016;24:502-9.
- 613 31. Watkins AJ, Wilkins A, Cunningham C, Perry VH, Seet MJ, Osmond C, et al. Low protein diet  
614 fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular  
615 abnormalities in offspring. *J Physiol*. 2008; **586**(8): 2231-44.
- 616 32. Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the  
617 preimplantation period of rat development causes blastocyst abnormalities and programming of  
618 postnatal hypertension. *Development*. 2000; **127**(19): 4195-202.
- 619 33. Watkins AJ, Ursell E, Panton R, Papenbrock T, Hollis L, Cunningham C, et al. Adaptive  
620 responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult  
621 onset disease. *Biol Reprod*. 2008; **78**(2): 299-306.
- 622 34. Torrens C, Snelling TH, Chau R, Shanmuganathan M, Cleal JK, Poore KR, et al. Effects of pre-  
623 and periconceptional undernutrition on arterial function in adult female sheep are vascular bed  
624 dependent. *Exp Physiol*. 2009; **94**(9): 1024-33.
- 625 35. Burrage D, Braddick L, Cleal J, Costello P, Noakes D, Hanson M, Green L. The late gestation  
626 fetal cardiovascular response to hypoglycaemia is modified by prior peri-implantation undernutrition  
627 in sheep. *J Physiol* 2009;587:611.

- 628 36. Costello PM, Rowleron A, Astaman NA, Anthony FE, Sayer AA, Cooper C, Hanson MA, Green  
629 LR. Peri-implantation and late gestation maternal undernutrition differentially affect fetal sheep  
630 skeletal muscle development. *J Physiol* 2008;586:2371-9.
- 631 37. Cleal JK, Poore KR, Newman JP, Noakes DE, Hanson MA, Green LR. The effect of maternal  
632 undernutrition in early gestation on gestation length and fetal and postnatal growth in sheep. *Ped*  
633 *Res* 2007;62:422-7.
- 634 38. Eckert JJ, Porter R, Watkins AJ, Burt E, Brooks S, Leese HJ, et al. Metabolic induction and  
635 early responses of mouse blastocyst developmental programming following maternal low protein  
636 diet affecting life-long health. *PLoS One*. 2012; **7**(12): e52791.
- 637 39. Watkins AJ, Lucas ES, Marfy-Smith S, Bates N, Kimber SJ, Fleming TP. Maternal nutrition  
638 modifies trophoblast giant cell phenotype and fetal growth in mice. *Reproduction*. 2015; **149**(6):  
639 563-75.
- 640 40. Sun C, Velazquez MA, Marfy-Smith S, Sheth B, Cox A, Johnston DA, et al. Mouse early extra-  
641 embryonic lineages activate compensatory endocytosis in response to poor maternal nutrition.  
642 *Development*. 2014; **141**(5): 1140-50.
- 643 41. Sun C, Denisenko O, Sheth B, Cox A, Lucas ES, Smyth NR, et al. Epigenetic regulation of  
644 histone modifications and *Gata6* gene expression induced by maternal diet in mouse embryoid  
645 bodies in a model of developmental programming. *BMC developmental biology*. 2015; **15**(1): 3.
- 646 42. Denisenko O, Lucas ES, Sun C, Watkins AJ, Mar D, Bomsztyk K, et al. Regulation of ribosomal  
647 RNA expression across the lifespan is fine-tuned by maternal diet before implantation. *Biochimica et*  
648 *biophysica acta*. 2016; **1859**(7): 906-13.
- 649 43. Holland ML, Lowe R, Caton PW, Gemma C, Carbajosa G, Danson AF, Carpenter AA, Loche E,  
650 Ozanne SE, Rakyan VK. Early-life nutrition modulates the epigenetic state of specific rDNA genetic  
651 variants in mice. *Science*. 2016;353:495-8.
- 652 44. Croy JE, Eaton SA, Aiken A, Young PE, Giannoulatou E, Ho JW, et al. Male-lineage  
653 transmission of an acquired metabolic phenotype induced by grand-paternal obesity. *Molecular*  
654 *metabolism*. 2016; **5**(8): 699-708.
- 655 45. Hammoud AO, Gibson M, Stanford J, White G, Carrell DT, Peterson M. In vitro fertilization  
656 availability and utilization in the United States: a study of demographic, social, and economic factors.  
657 *Fertil Steril*. 2009; **91**(5): 1630-5.
- 658 46. Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, et al. Impact of body  
659 mass index values on sperm quantity and quality. *Journal of andrology*. 2006; **27**(3): 450-2.
- 660 47. Tunc O, Bakos HW, Tremellen K. Impact of body mass index on seminal oxidative stress.  
661 *Andrologia*. 2011; **43**(2): 121-8.
- 662 48. Gaskins AJ, Colaci DS, Mendiola J, Swan SH, Chavarro JE. Dietary patterns and semen quality  
663 in young men. *Hum Reprod*. 2012; **27**(10): 2899-907.
- 664 49. Agbaje IM, Rogers DA, McVicar CM, McClure N, Atkinson AB, Mallidis C, et al. Insulin  
665 dependant diabetes mellitus: implications for male reproductive function. *Hum Reprod*. 2007; **22**(7):  
666 1871-7.
- 667 50. Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in  
668 ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril*.  
669 2004; **82**(2): 378-83.
- 670 51. Bakos HW, Henshaw RC, Mitchell M, Lane M. Paternal body mass index is associated with  
671 decreased blastocyst development and reduced live birth rates following assisted reproductive  
672 technology. *Fertility and sterility*. 2011; **95**(5): 1700-4.
- 673 52. Bakos HW, Mitchell M, Setchell BP, Lane M. The effect of paternal diet-induced obesity on  
674 sperm function and fertilization in a mouse model. *Int J Androl*. 2011; **34**(5 Pt 1): 402-10.
- 675 53. Mitchell M, Bakos HW, Lane M. Paternal diet-induced obesity impairs embryo development  
676 and implantation in the mouse. *Fertil Steril*. 2011; **95**(4): 1349-53.
- 677 54. Fullston T, Ohlsson Teague EM, Palmer NO, DeBlasio MJ, Mitchell M, Corbett M, et al.  
678 Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete

679 penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA  
680 content. *FASEB journal : official publication of the Federation of American Societies for Experimental*  
681 *Biology*. 2013; **27**(10): 4226-43.

682 55. Palmer NO, Bakos HW, Owens JA, Setchell BP, Lane M. Diet and exercise in an obese mouse  
683 fed a high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol*  
684 *Endocrinol Metab*. 2012; **302**(7): E768-80.

685 56. Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA, Morris MJ. Chronic high-fat diet in fathers  
686 programs beta-cell dysfunction in female rat offspring. *Nature*. 2010; **467**(7318): 963-6.

687 57. Pentinat T, Ramon-Krauel M, Cebria J, Diaz R, Jimenez-Chillaron JC. Transgenerational  
688 inheritance of glucose intolerance in a mouse model of neonatal overnutrition. *Endocrinology*. 2010;  
689 **151**(12): 5617-23.

690 58. Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, et al. Paternally induced  
691 transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell*.  
692 2010; **143**(7): 1084-96.

693 59. Watkins AJ, Sinclair KD. Paternal low protein diet affects adult offspring cardiovascular and  
694 metabolic function in mice. *American journal of physiology Heart and circulatory physiology*. 2014;  
695 **306**(10): H1444-52.

696 60. Watkins AJ, Sirovica S, Stokes B, Isaacs M, Addison O, Martin RA. Paternal low protein diet  
697 programs preimplantation embryo gene expression, fetal growth and skeletal development in mice.  
698 *Biochim Biophys Acta*. 2017; **1863**(6): 1371-81.

699 61. Zeybel M, Hardy T, Wong YK, Mathers JC, Fox CR, Gackowska A, Oakley F, Burt AD, Wilson  
700 CL, Anstee QM, Barter MJ, Masson S, Elsharkawy AM, Mann DA, Mann J. Multigenerational  
701 epigenetic adaptation of the hepatic wound-healing response. *Nat Med*. 2012; **18**:1369-77.

702 62. Hammoud SS, Nix DA, Hammoud AO, Gibson M, Cairns BR, Carrell DT. Genome-wide analysis  
703 identifies changes in histone retention and epigenetic modifications at developmental and imprinted  
704 gene loci in the sperm of infertile men. *Human reproduction*. 2011; **26**(9): 2558-69.

705 63. van der Heijden GW, Ramos L, Baart EB, van den Berg IM, Derijck AA, van der Vlag J, et al.  
706 Sperm-derived histones contribute to zygotic chromatin in humans. *BMC developmental biology*.  
707 2008; **8**: 34.

708 64. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal  
709 fertile men. *Lancet*. 2002; **360**(9335): 772-7.

710 65. Jodar M, Kalko S, Castillo J, Balleca JL, Oliva R. Differential RNAs in the sperm cells of  
711 asthenozoospermic patients. *Hum Reprod*. 2012; **27**(5): 1431-8.

712 66. Chen Q, Yan M, Cao Z, Li X, Zhang Y, Shi J, et al. Sperm tsRNAs contribute to  
713 intergenerational inheritance of an acquired metabolic disorder. *Science*. 2016; **351**(6271): 397-400.

714 67. Sharma U, Conine CC, Shea JM, Boskovic A, Derr AG, Bing XY, Belleannee C, Kucukural A,  
715 Serra RW, Sun F, Song L, Carone BR, Ricci EP, Li XZ, Fauquier L, Moore MJ, Sullivan R, Mello CC,  
716 Garber M, Rando OJ. Biogenesis and function of tRNA fragments during sperm maturation and  
717 fertilization in mammals. *Science*. 2016 Jan 22; **351**(6271):391-6.

718 68. Sjoblom C, Roberts CT, Wikland M, Robertson SA. Granulocyte-macrophage colony-  
719 stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and  
720 placental morphogenesis. *Endocrinology*. 2005; **146**(5): 2142-53.

721 69. Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA. Seminal plasma differentially  
722 regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells.  
723 *Molecular human reproduction*. 2007; **13**(7): 491-501.

724 70. Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. Maternal tract factors  
725 contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the*  
726 *National Academy of Sciences of the United States of America*. 2014; **111**(6): 2200-5.

727 71. Qin JB, Sheng XQ, Wu D, Gao SY, You YP, Yang TB, et al. Worldwide prevalence of adverse  
728 pregnancy outcomes among singleton pregnancies after in vitro fertilization/intracytoplasmic sperm

729 injection: a systematic review and meta-analysis. Archives of gynecology and obstetrics. 2017;  
730 **295**(2): 285-301.

731 72. Kleijkers SH, Mantikou E, Slappendel E, Consten D, van Echten-Arends J, Wetzels AM, et al.  
732 Influence of embryo culture medium (G5 and HTF) on pregnancy and perinatal outcome after IVF: a  
733 multicenter RCT. Hum Reprod. 2016; **31**(10): 2219-30.

734 73. Kleijkers SH, van Montfoort AP, Smits LJ, Viechtbauer W, Roseboom TJ, Nelissen EC, et al. IVF  
735 culture medium affects post-natal weight in humans during the first 2 years of life. Hum Reprod.  
736 2014; **29**(4): 661-9.

737 74. Valenzuela-Alcaraz B, Crispi F, Bijmens B, Cruz-Lemini M, Creus M, Sitges M, et al. Assisted  
738 reproductive technologies are associated with cardiovascular remodeling in utero that persists  
739 postnatally. Circulation. 2013; **128**(13): 1442-50.

740 75. Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, Garcin S, et al. Systemic and  
741 pulmonary vascular dysfunction in children conceived by assisted reproductive technologies.  
742 Circulation. 2012; **125**(15): 1890-6.

743 76. Gkourogiani A, Kosteria I, Telonis AG, Margeli A, Mantzou E, Konsta M, et al. Plasma  
744 metabolomic profiling suggests early indications for predisposition to latent insulin resistance in  
745 children conceived by ICSI. PLoS One. 2014; **9**(4): e94001.

746 77. Rumbold AR, Moore VM, Whitrow MJ, Oswald TK, Moran LJ, Fernandez RC, Barnhart KT,  
747 Davies MJ. The impact of specific fertility treatments on cognitive development in childhood and  
748 adolescence: a systematic review. Hum Reprod. 2017 May 4:1-19.

749 78. Watkins AJ, Platt D, Papenbrock T, Wilkins A, Eckert JJ, Kwong WY, et al. Mouse embryo  
750 culture induces changes in postnatal phenotype including raised systolic blood pressure. Proc Natl  
751 Acad Sci U S A. 2007; **104**(13): 5449-54.

752 79. Lazaraviciute G, Kauser M, Bhattacharya S, Haggarty P. A systematic review and meta-  
753 analysis of DNA methylation levels and imprinting disorders in children conceived by IVF/ICSI  
754 compared with children conceived spontaneously. Hum Reprod Update. 2014; **20**(6): 840-52.

755 80. Chen SL, Shi XY, Zheng HY, Wu FR, Luo C. Aberrant DNA methylation of imprinted H19 gene  
756 in human preimplantation embryos. Fertil Steril. 2010; **94**(6): 2356-8, 8 e1.

757 81. Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. Manipulations of mouse  
758 embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of  
759 development. Human molecular genetics. 2008; **17**(1): 1-14.

760 82. Turan N, Katari S, Gerson LF, Chalian R, Foster MW, Gaughan JP, et al. Inter- and intra-  
761 individual variation in allele-specific DNA methylation and gene expression in children conceived  
762 using assisted reproductive technology. PLoS genetics. 2010; **6**(7): e1001033.

763 83. Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos  
764 affects fetal development and the expression of imprinted genes. Biol Reprod. 2001; **64**(3): 918-26.

765 84. Horii T, Suetake I, Yanagisawa E, Morita S, Kimura M, Nagao Y, et al. The Dnmt3b splice  
766 variant is specifically expressed in in vitro-manipulated blastocysts and their derivative ES cells. J  
767 Reprod Dev. 2011; **57**(5): 579-85.

768 85. Rexhaj E, Pireva A, Paoloni-Giacobino A, Allemann Y, Cerny D, Dessen P, et al. Prevention of  
769 vascular dysfunction and arterial hypertension in mice generated by assisted reproductive  
770 technologies by addition of melatonin to culture media. American journal of physiology Heart and  
771 circulatory physiology. 2015; **309**(7): H1151-6.

772 86. Prasad S, Tiwari M, Pandey AN, Shrivastav TG, Chaube SK. Impact of stress on oocyte quality  
773 and reproductive outcome. J Biomed Sci. 2016 ;23:36.

774 87. Gårdebjer EM, Anderson ST, Pantaleon M, Wlodek ME, Moritz KM. Maternal alcohol intake  
775 around the time of conception causes glucose intolerance and insulin insensitivity in rat offspring,  
776 which is exacerbated by a postnatal high-fat diet. FASEB J. 2015; **29**(7):2690-701.

777 88. Williams CL, Teeling JL, Perry VH, Fleming TP. Mouse maternal systemic inflammation at the  
778 zygote stage causes blunted cytokine responsiveness in lipopolysaccharide-challenged adult  
779 offspring. BMC Biol. 2011; **9**: 49.

- 780 89. Velazquez MA, Smith CG, Smyth NR, Osmond C, Fleming TP. Advanced maternal age causes  
781 adverse programming of mouse blastocysts leading to altered growth and impaired cardiometabolic  
782 health in post-natal life. *Hum Reprod.* 2016; **31**(9): 1970-80.
- 783 90. Basson MA. Signaling in cell differentiation and morphogenesis. *Cold Spring Harb Perspect*  
784 *Biol.* 2012 ;4:1-21.
- 785 91. Steegers-Theunissen RP, Twigt J, Pestinger V, Sinclair KD. The periconceptual period,  
786 reproduction and long-term health of offspring: the importance of one-carbon metabolism. *Hum*  
787 *Reprod Update.* 2013; **19**(6): 640-55.
- 788 92. McKay JA, Adriaens M, Evelo CT, Ford D, Mathers JC. Gene promoter DNA methylation  
789 patterns have a limited role in orchestrating transcriptional changes in the fetal liver in response to  
790 maternal folate depletion during pregnancy. *Mol Nutr Food Res.* 2016;60:2031-42.
- 791 93. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, et al. DNA methylation,  
792 insulin resistance, and blood pressure in offspring determined by maternal periconceptual B  
793 vitamin and methionine status. *Proceedings of the National Academy of Sciences of the United*  
794 *States of America.* 2007; **104**(49): 19351-6.
- 795 94. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of  
796 pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic  
797 gene expression in the offspring. *The Journal of nutrition.* 2005; 135(6): 1382-6.
- 798 95. Lambrot R, Xu C, Saint-Phar S, Chountalos G, Cohen T, Paquet M, et al. Low paternal dietary  
799 folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes.  
800 *Nature communications.* 2013; **4**: 2889.
- 801 96. McPherson NO, Fullston T, Kang WX, Sandeman LY, Corbett MA, Owens JA, et al. Paternal  
802 under-nutrition programs metabolic syndrome in offspring which can be reversed by  
803 antioxidant/vitamin food fortification in fathers. *Scientific reports.* 2016; **6**: 27010.
- 804 97. Barker D, Barker M, Fleming T, Lampl M. Developmental biology: Support mothers to secure  
805 future public health. *Nature.* 2013; **504**(7479): 209-11.
- 806 98. Hanson M, Godfrey K, Poston L, Bustreo F, Stephenson J. Preconception health. Annual  
807 Report of the Chief Medical Officer 2014, The Health of the 51%: Women. Ed Davies SC. London:  
808 Department of Health, 2015, Chapter 5, 53-66.
- 809 99. Messerschmidt DM, Knowles BB, Solter D. DNA methylation dynamics during epigenetic  
810 reprogramming in the germline and preimplantation embryos. *Genes & development.* 2014; **28**(8):  
811 812-28.
- 812 100. Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev.* 2015; **27**(4): 638-54.

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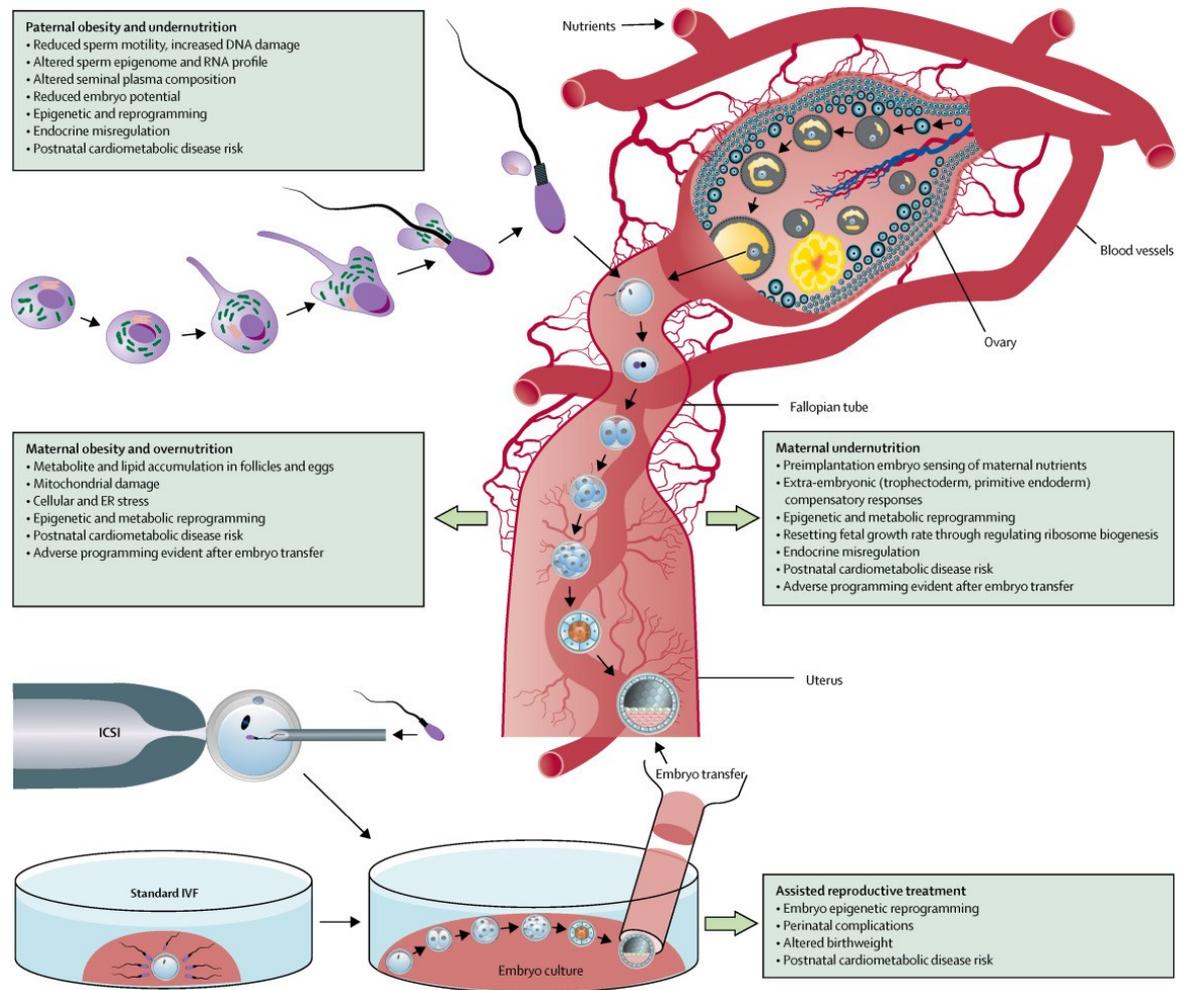
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823 **Figure 1**

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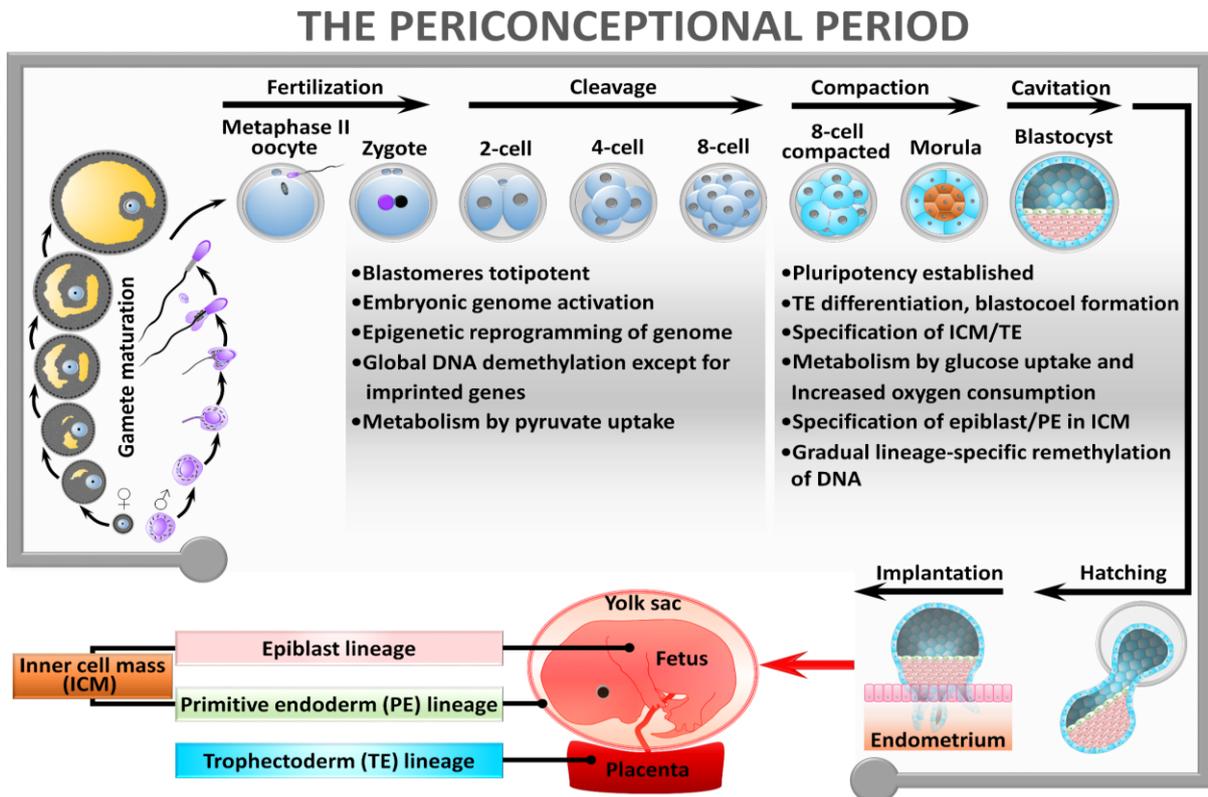
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842 Figure 3

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