Why AMPK agonists not known to be stressors may surprisingly contribute to miscarriage or hinder IVF/ART

Elizabeth E. Puscheck¹ · Alan Bolnick^{1,2} · Awoniyi Awonuga¹ · Yu Yang^{1,3} · Mohammed Abdulhasan¹ · Quanwen Li¹ · Eric Secor⁴ · Erica Loudon⁵ · Maik Hüttemann⁶ · Daniel A. Rappolee^{1,3,6,7,8,9}

Received: 6 January 2018 / Accepted: 16 May 2018 / Published online: 7 June 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

Abstract

Here we examine recent evidence suggesting that many drugs and diet supplements (DS), experimental AMP-activated protein kinase (AMPK) agonists as well as energy-depleting stress, lead to decreases in anabolism, growth or proliferation, and potency of cultured oocytes, embryos, and stem cells in an AMPK-dependent manner. Surprising data for DS and drugs that have some activity as AMPK agonists in in vitro experiments show possible toxicity. This needs to be balanced against a preponderance of evidence in vivo that these drugs and DS are beneficial for reproduction. We here discuss and analyze data that leads to two possible conclusions: First, although DS and drugs that have some of their therapeutic mechanisms mediated by AMPK activity associated with low ATP levels, some of the associated health problems in vivo and in vitro fertilization/assisted reproductive technologies (IVF/ART) may be better-treated by increasing ATP production using CoQ10 (Ben-Meir et al., *Aging Cell* 14:887–895, 2015). This enables high developmental trajectories simultaneous with solving stress by energy-requiring responses. In IVF/ART, it is ultimately best to maintain handling and culture of gametes and embryos in the quietest state with low metabolic activity (Leese et al., *Mol Hum Reprod* 14:667–672, 2008; Leese, *Bioessays* 24 (9):845–849, 2002) using back-to-nature or simplex algorithms to identify optima (Biggers, *Reprod Biomed Online* 4 Suppl 1:30–38, 2002). Stress markers, such as checkpoint proteins like TRP53 (aka p53) (Ganeshan et al., *Exp Cell Res* 358:227–233, 2017); Ganeshan et al., *Biol Reprod* 83:958–964, 2010) and a small set of kinases from the protein kinome that mediate enzymatic stress responses, can also be used to define optima. But, some gametes or embryos may have been stressed in vivo prior to IVF/ART or IVF/ART optimized for one outcome may be suboptimal for another. Increasing nutrition or adding CoQ10 to increase ATP production (Yang et al., *Stem Cell Rev* 13:454–464, 2017), managing stress enzyme levels with inhibitors (Xie et al., *Mol Hum Reprod* 12:217–224, 2006), or adding growth factors such as GM-CSF (Robertson et al., *J Reprod Immunol* 125:80–88, 2018); Chin et al., *Hum Reprod* 24:2997–3009, 2009) may increase survival and health of cultured embryos during different stress exposure contexts (Puscheck et al., *Adv Exp Med Biol* 843:77–128, 2015). We define “stress” as negative stimuli which decrease normal magnitude and speed of development, and these can be stress hormones, reactive oxygen species, inflammatory cytokines, or physical stimuli such as hypoxia. AMPK is normally activated by high AMP, commensurate with low ATP, but it was recently shown that if glucose is present inside the cell, AMPK activation by low ATP/high AMP is suppressed (Zhang et al., *Nature* 548:112–116,

✉ Daniel A. Rappolee
drappole@med.wayne.edu

¹ CS Mott Center for Human Growth and Development, Department of Ob/Gyn, Reproductive Endocrinology and Infertility, Wayne State University School of Medicine, 275 East Hancock, Detroit, MI 48201, USA

² Present address: Department of Obstetrics and Gynecology, Kaleida Women’s and Children’s Hospital Buffalo New York, Buffalo, NY, USA

³ Department of Physiology, Wayne State University School of Medicine, Detroit, MI 48201, USA

⁴ Department of Medicine, Integrative Medicine, Hartford Hospital and University of Connecticut, Hartford, CT 06102, USA

⁵ Present address: Augusta University of Health Sciences, Augusta, GA, USA

⁶ Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI, USA

⁷ Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, USA

⁸ Institutes for Environmental Health Science, Wayne State University School of Medicine, Detroit, MI, USA

⁹ Department of Biology, University of Windsor, Windsor, ON N9B 3P4, Canada

2017). As we discuss in more detail below, this may also lead to greater AMPK agonist toxicity observed in two-cell embryos that do not import glucose. Stress in embryos and stem cells increases AMPK in large stimulation indexes but also direness indexes; the fastest AMPK activation occurs when stem cells are shifted from optimal oxygen to lower or high levels (Yang et al., J Reprod Dev 63:87–94, 2017). CoQ10 use may be better than risking AMPK-dependent metabolic and developmental toxicity when ATP is depleted and AMPK activated. Second, the use of AMPK agonists, DS, and drugs may best be rationalized when insulin resistance or obesity leads to aberrant hyperglycemia and hypertriglyceridemia, and obesity that negatively affect fertility. Under these conditions, beneficial effects of AMPK on increasing triglyceride and fatty acid and glucose uptake are important, as long as AMPK agonist exposures are not too high or do not occur during developmental windows of sensitivity. During these windows of sensitivity suppression of anabolism, proliferation, and stemness/potency due to AMPK activity, or overexposure may stunt or kill embryos or cause deleterious epigenetic changes.

Keywords Drugs · Metformin · Aspirin · Diet supplements · BR-DIM · Stress · AMPK agonist · AMPK · EMBRYOS · Blastocysts · TSC · ESC · iPSC · OOCYTES · Potency · Differentiation · Anabolism · Catabolism · Warburg metabolism · Oxidative phosphorylation · Mitochondria · Electron transport chain

Introduction. Recent examples of compounds that have some activity as AMPK agonists; diet supplements, and drugs that have negative effects on reproduction or development

Recent research shows that AMP-activated protein kinase (AMPK) mediates or is associated with stress-driven loss of potency factor proteins in cultured embryonic stem cells (ESCs), placental trophoblast stem cells (TSCs), and AMPK agonists that block reprogramming which produces induced pluripotent stem cells (iPSCs) by preventing switch to Warburg metabolism and blocking Oct4 transcription [1–3]. AMPK activity is associated with decreased potency in oocytes [4], and AMPK activity causes potency loss in two-cell embryos [5–7] and blastocysts [5, 7, 8]. AMPK inhibitors, compound C and AraA, block stress-forced potency loss in ESCs, TSC blastocysts, and two-cell embryos suggesting that AMPK mediates potency loss.

Several reports prove that non-stressful activators of AMPK such as drugs (aspirin, metformin) and diet supplements (BioResponse 3,3'-Diindolylmethane [BR-DIM]) cause growth arrest or decrease and potency decrease in cultured two-cell embryos and ESCs tested in high throughput screen (HTS) [6, 9–11]. The benefit of the HTS is that it enables higher numbers of doses and rapid testing of effects of single or multiple stimuli with simultaneous outcomes of growth and stemness or differentiation. It was also shown that diet supplements (DS) and drugs that have some activity as AMPK agonists retard and arrest stem cell growth soon after exposure of cultured two-cell embryos ~2–3 days before the blastocyst stage. However, it has not been shown yet which stage of embryo, two-cell or blastocyst, is most sensitive to stress-, DS-, or drug-caused potency or growth decrease. In the bovine model, cultured two-cell stage embryos are more sensitive to metformin than maturing oocytes for growth and developmental arrest [10]. In the mouse model, the two-cell

stage embryo is more sensitive than the blastocyst stage for several AMPK-dependent outcomes [5]. This may support the hypothesis that early post-fertilization events are susceptible to precise energy management, but in vivo data are required to confirm relevance of in vitro outcomes. This hypothesis is supported by the observation that most AMPK α 1 null mutants survive the two- to four-cell stage in vivo and survive to birth, whereas most cultured AMPK α 1 null embryos die at the two- to four-cell stage [12]. The authors of this study also suggest that metformin, partially through AMPK activity, can have negative epigenetic and spermatogenic effects [13, 14]. Together, the data suggest a need to test whether embryos (especially two-cell embryos) use AMPK to adapt to stress, but when over-activated, AMPK can also be dangerous for embryo health. There may be conditions of AMPK activation in vivo that would have the same negative effects observed in vitro, but this remains to be tested.

Compounds with some benefits due to AMPK agonism: there is evidence for efficacy but not all therapeutic mechanisms are AMPK-dependent

The negative experimental data are surprising since most reports of DS and drugs that have some activity as AMPK agonists show beneficial exposures effects. What are the reasons AMPK agonist improve oocyte and embryo progression in vitro? Are there specific AMPK agonist dose and exposure ranges that are beneficial? Are the effects beneficial only when compared with a control that is unhealthy (i.e., when embryos are isolated from diabetic mothers or are cultured under intrinsically stressful conditions)? Are the embryos or oocytes that are improved during culture already compromised in vivo to the point where AMPK agonists are needed to rescue poor health?

Oocyte stress by culture alone or by several stressors such as arsenite and hyperosmotic sorbitol is rescued for meiotic resumption by AMPK agonists; however, these effects are a rescue and do not emulate the speed of magnitude of oocyte maturation in vivo [15–17] and these studies have not culminated with successful fertilization, implantation, and parturition from oocytes treated with AMPK agonists. Reports of improvement are mostly for treatments in vivo, but oocytes and embryos from diabetic or experimentally obese females are improved by metformin and other drugs that have some activity as AMPK agonists [18–20]. It should be noted that obese women have other problems such as reactive oxygen species that can disturb one carbon metabolism and the status of DNA methylation [21]. However, these oocytes and embryos from obese women are also likely to have AMPK activity at abnormally low levels (e.g., suppressed by high ATP when high glucose and triglyceride uptake occurs in obese mothers) or require AMPK function in increasing uptake of glucose and triglycerides and fatty acids when they are insulin resistant. Moreover, oocytes and embryos from *normal* mothers may suffer if exposed to AMPK agonists from many sources, for example due to stress, drugs, or DS or due to high doses.

The reasons why there is a lack of a literature on AMPK agonist toxicity were discussed previously in the first report of AMPK agonist that affects on arrest of two-cell embryo development [6]. The drugs and DS discussed here are known mostly for other mechanisms than AMPK agonism. For example, metformin is used to improve ovulation in infertile women with polycystic ovarian syndrome (PCOS) [22–24], or type 2 diabetes (T2D). Metformin overcomes insulin resistance to enable ovulation in infertile women largely by blocking glucagon-induced cAMP and inhibiting protein kinase A [25–27], but rosiglitazone counteracts insulin resistance and increases androgens in women with PCOS [28]. Aspirin is an anti-inflammatory, antipyretic, and analgesic with inhibitory effects on prostaglandin production and irreversibly inhibits cyclooxygenase (COX) 1/2 activity and is used by fertile and infertile women [23, 29–39]. Thus, although metformin [40] and Asa [41] have therapeutic mechanisms partly through AMPK activity, there are many other effects that are AMPK-independent. In some drugs and DS, AMPK-dependent mechanisms may be additively or synergistically less important because other therapeutic mechanisms are more important. But, the possibility of AMPK agonism overexposure exists because of the preponderance of DS, with some drugs that have some AMPK agonism.

The drug DS BR-DIM can improve maternal fertility and diabetic metabolism. BR-DIM is a DS derived from yellow cruciferous vegetables and is a proprietary derivative of DIM that is absorbed better from the intestines [42]. DIM and BR-DIM act through mechanisms as an androgen antagonist and as a histone deacetylase inhibitor [43, 44]. However, BR-DIM

diminishes growth or kills prostate cancer cells that have some stem cell properties, via AMPK-dependent mechanisms [45] in vivo and in vitro. Although diminishing growth in cancer stem cells is beneficial, diminishing growth in stem cells of early embryos would be detrimental if in vitro results repeat during gestational exposures. Most DS are mild mitochondrial poisons and lower ATP and increase AMP due to poorer mitochondrial function. Like metformin and aspirin, diet supplements like BR-DIM mediate some of their activities through AMPK as well as other, sometimes better known mechanisms.

Commonly used drugs that have some activity as AMPK agonists include *metformin* and *phenformin*, aspirin, *rosiglitazone*, and *troglicizone* and diet supplements such as *resveratrol*, *green/black tea*, *berberine*, and BR-DIM [1]. These italicized drugs and DS, with the exception of aspirin which directly contacts and decreases activity of the AMPK-negative regulatory β -subunit (decreasing the off-rate), are mitochondrial inhibitors which decrease ATP, increase AMPK, and activate the γ -subunit [46] (Fig. 1). For example, metformin was shown to bind to and inhibit complex I of the electron transport chain (ETC) of mitochondrial oxidative phosphorylation resulting in highly efficient ATP production, which also leads to an increase in mitochondrial superoxide production [47], modulating redox-regulated signaling pathways. Additional mitochondrial targets exist including glycerophosphate dehydrogenase, which metformin binds leading to enzyme inhibition and thus contributes to an increase in the cellular redox state [48]. The guanide phenformin has an even broader inhibitory spectrum by targeting ETC complexes II and IV in addition to complex I [49] as well as ATP synthase [50]. As a result, ATP levels drop leading to an increase of the AMP/ATP ratio and consequent activation of AMPK. However, metformin may also activate AMPK through direct contact in some circumstances [51]. Thus, these drugs increase the on-rate. In cells with mutant AMPK γ -subunits, these underlined drugs and DS do not have activated AMPK or cause their AMPK-dependent effects. Interestingly, the DS CoQ10 is exceptional in increasing electron transport chain activity and thus mitochondrial ATP production and may improve reproductive efficiency [52] but obviate the need to use AMPK agonists.

Generally, metformin is considered to be safe during pregnancy and its role in correcting metabolic derangement makes it beneficial for patients with diabetes during pregnancy. Animal studies using dosages up to 600 mg/kg daily did not show teratogenic effects and extremely high dosages between 900 and 1500 mg/kg also failed to induce carcinogenicity [53]. Using ex vivo perfusion of the placental cotyledon model, Kovo et al. demonstrated that the transport of metformin through placenta is mediated by carrier and at 1 mg/ml, the transport of metformin is saturated [54]. The potential competition between metformin and other drugs in the use of transporter can also limit fetal exposure.

Fig. 1 The AMPK heterotrimer is activated at the γ -subunit indirectly by Met, BR-DIM. The negative regulator β -subunit is deactivated (off-rate) and AMPK activated directly by Asa or A769662 which bind ser108

However, it should be noted that the beneficial effects of metformin are partly conveyed by activating AMPK to counteract stress [55]. AMPK can also be activated by multiple other stressors. Over-activation of AMPK by both environmental stress and drugs may contribute to adverse outcomes. It has been reported that stimulation of AMPK in embryos in diabetic rodents can lead to neural tube defects through suppression of PAX3 [56]. We do not have sufficient data to exclude the possibility that human embryos may be subjected to the negative effect of metformin. The current evidences support the efficacy and safety of metformin in regard to immediate pregnancy outcomes [57]. It is important to follow the offspring in order to determine whether metformin treatment of diabetes during pregnancy results in many changes in the risk of metabolic disorders through epigenetic programming [58].

So what are the mechanisms that prescribed or elective drugs and/or diet supplements use that modulate ATP and AMPK?

Of the > 500 protein kinases in the kinome [59, 60], AMPK is unique in regulating nearly all the catalytic and anabolic pathways of intermediary carbon metabolism that enable synthesis of carbon polymers: polynucleotides or DNA and RNA, fatty acids, starches such as glycogen, and proteins [61, 62]. As the cellular energy sensor, AMPK homologs are present in the earliest forms of life and are present in nearly all types of cells, including gametes and stem cells.

When ATP is low (and thus AMP is high), AMPK downregulates anabolic metabolism that consumes ATP and upregulates catabolic metabolism that produces ATP, thus rebalancing ATP equilibrium [62]. AMPK also increases cellular uptake of triglycerides and fatty acids, glucose, and other nutritional molecules to increase ATP. It is this later AMPK function that makes it important in the treatments of T2D, PCOS [12, 63], hypertriglyceridemia, and obesity that negatively affect maternal health and reproductive capacity.

The fact that AMPK also favors catabolic over anabolic function also makes diet supplements and drugs that are AMPK agonists attractive to women who wish to keep their weight low. AMPK favors oxidative phosphorylation over glycolytic metabolism as the former produces 38 ATP/glucose and the latter only 2 ATP/glucose [64, 65]. This means that AMPK brings glucose and triglycerides and fatty acids into cells, but tends to burn them to CO₂ in the mitochondria rather than use them in anabolic pathways that would lead to weight gain and production of glycogen and fat as well as RNA/DNA and protein.

This may be one global reason why all diet supplements listed at the US Office of Dietary Supplements (<https://ods.od.nih.gov/>) studied to date (i.e., > 50 of > 70 DS listed) activate AMPK, and many have substantial therapeutic activity through an AMPK-mediated switch from anabolic to catabolic pathways [66].

As mentioned previously, many drugs activate AMPK as a mitochondrial blocker (e.g., metformin) or by directly interacting with the AMPK heterotrimer and activating it (e.g., aspirin). This also suggests the potential for synergism

between diet supplements and drugs may include the possibility that the AMPK heterotrimer may be hyper-activated by increasing enzyme on-rate through the γ -subunit and decreasing the off-rate through the β -subunit [66–68] (Fig. 1).

Many drugs and diet supplements that activate AMPK are in clinical trials to block cancer growth because AMPK's properties lead to decreases in anabolic and increase in catabolic pathways. Also, cancer stem cells proliferate more rapidly because they favor aerobic glycolysis (glycolysis even when oxygen is present) which is also called “Warburg metabolism” [69]. These proliferating cells have suppressed Krebs's tricarboxylic acid pathway (TCA) that in postmitotic cells produces high ATP through oxidative phosphorylation (OxPhos) but “wastes” carbon polymers from nutritional sources and converting these to CO_2 . In summary, AMPK blocks Warburg and favors OxPhos as well as favoring catabolic over anabolic pathways. However, from the blastocyst stage onwards, stem cells of the implanting embryo use Warburg metabolism to enable rapid growth at the start of the exponential growth phase of embryogenesis [64].

Miscarriage is likely to have a metabolic link as stunting of embryonic and stem cell growth is linked to insufficient embryonic function to continue pregnancy [1, 70]. Most of miscarriage happens soon after implantation and about one third (35%) occurs after chemical (hCG; 20%) or clinical (ultrasound; 15%) detection of pregnancy. It is estimated that half of miscarriages are due to non-genetic outcomes. But genetic anomalies that slow anabolism and stem cell doubling rates are like stressors or AMPK agonists; they slow cell growth rates that may trigger compensatory mechanisms of forced differentiation. Thus, miscarriage may have a common mediator; genetically or environmentally caused insufficient stem cell proliferation in the stem cell “pipeline” required to produce early sufficient differentiated function.

The chances of overexposure in vitro and in vivo

Since DS and drugs are generally cleared in hours in vivo, in vitro stimulations tend to be overexposures in regard to biological effects measured in days [5, 6]. However, AMPK-dependent decreases in anabolism and potency occur within one hour before the peak blood dose is reached and therefore these exposures are not artifacts of in vitro overexposures. Consequently, these mechanisms may lead to growth arrest or decreases in proliferation.

There are two possibilities for underestimates in studies in vivo. These underestimates are based on in vitro studies discussed above; most studies test drugs or DS as single variables, but DS and drugs may be exposed in pairs or sets and at different times of the day increasing *duration* of DS and drugs that have some activity as AMPK agonists. In addition, the *magnitude* of AMPK activity can be increased by multiple DS and drugs and by activators of the on-rate of the γ -subunit

simultaneously with decreased off-rate of the α -subunit. These two possible mechanisms for AMPK agonist toxicity have not been widely tested in vivo.

What should be done next?

In the IVF setting, metformin has a beneficial effect by increasing pregnancy rates and decreasing miscarriages [71]. We have discussed the concept before that augmenting energy production might be better than inhibiting it with drugs that act as inhibitors on mitochondrial function such as the biguanidine metformin. However, it should be noted that cellular energy levels can be well maintained as long as there is enough glucose. This is so because ATP production via substrate level phosphorylation in glycolysis, although less efficient, is faster than ATP production through oxidative phosphorylation [72]. Therefore, as an alternate possibility, it should be considered that cellular energy levels may not be that central for potency and growth during early embryogenesis at the blastocyst stage when glucose usage increases [73]. As long as glucose is plentiful, energy levels can be low; this concept has increasingly gained acceptance in the cancer field (reviewed in [74]), with cellular growth rates similar to those seen in the early embryo. Inhibition of mitochondrial catabolic activity—paralleled by a reprogrammed mitochondrial metabolism—may thus augment the generation of biosynthetic intermediates including NADPH, amino acids, cytosolic acetyl-CoA, and five-carbon sugars, all of which the proliferating cell heavily relies on. Future selective experimental manipulation of cellular energy versus metabolic intermediates may shed light on their respective importance for potency and embryonic growth. It should also be noted that it was recently reported that high levels of glucose maintain low AMPK activity and low glucose levels increase AMPK activity, independent of AMP levels [75, 76]. Thus, AMPK responds directly, but independently to glucose or AMP. The embryonic niche at 2% oxygen, coupled with sufficient glucose to sustain low glycolytic ATP production but high conservation of carbon for anabolic proliferation, and sufficient microfluidics flow at the blastocyst stage [64] should improve blastocyst culture.

At the two-cell embryo stage in mouse, bovine and human pyruvate usage is highest and supports development through the blastocyst stage [73, 77–79] better than glucose and this occurs at a time when pentose phosphate pathway usage is highest [79]. The pentose phosphate pathway is significant in producing reducing power through NADPH for fatty acid synthesis (as well as other macromolecular synthesis) and prevention of oxidative stress, as well as nucleic acid synthesis. However, the higher pyruvate uptake and lower level of glucose uptake may lead to increasing AMPK activation that is prevented by glucose [75].

As a possible outcome, direct *partial* metabolic inhibition at the level of the mitochondrial ETC by metformin and other compounds may prevail over *mild* AMPK-mediated activation of catabolism. In contrast, stress-induced AMPK hyperactivation would overcome this effect leading to potency loss as we and others have shown in vitro [3–8, 10, 64, 80–83]. The concepts of additive or synergistic negative AMPK effects in vitro [67, 84] and of repeated doses modeled in vitro and in vivo [85] need more in vivo testing with regard to AMPK-dependent miscarriage and lesser developmental pathogenesis. AMPK activity is a homeostatic mechanism that when optimized can return stressed embryos a developmental trajectory that emulates those of the “quiet embryo” [86, 87, 88]. However suboptimal manipulation of AMPK activity can create an artifactual “noise” that is not desired. Whether CoQ10 has greater potential to return stressed embryos and oocytes to a developmental trajectory with less noise than AMPK agonism needs further testing. The emphasis has been on modeling the capabilities of the response of embryos and their stem cells in vitro, and these hypotheses need to be tested in vivo where indirect maternal effects and accessibility to embryos or their stem cells may be limiting.

Acknowledgements We thank Dr. Todd Leff, Dr. Sudipta Dutta, and Dr. Gloria Perez for comments on the manuscript.

Funding This study received funding from DAR and EEP from the Office of the Vice President for Research at Wayne State University and an R03 to DAR 1R03HD061431 and an R41 to DAR 1R41ES028991-01 from the REI fellows’ fund (AB) and from the funding of the Mary Iacobell and Kamran Moghissi Endowed Chairs.

References

1. Puscheck EE, Awonuga AO, Yang Y, Jiang Z, Rappolee DA. Molecular biology of the stress response in the early embryo and its stem cells. *Adv Exp Med Biol*. 2015;843:77–128.
2. Vazquez-Martin A, Corominas-Faja B, Cufi S, Vellon L, Oliveras-Ferraro C, Menendez OJ, et al. The mitochondrial H(+)-ATP synthase and the lipogenic switch: new core components of metabolic reprogramming in induced pluripotent stem (iPS) cells. *Cell Cycle*. 2013;12:207–18.
3. Vazquez-Martin A, Vellon L, Quiros PM, Cufi S, Ruiz de Galarreta E, Oliveras-Ferraro C, et al. Activation of AMP-activated protein kinase (AMPK) provides a metabolic barrier to reprogramming somatic cells into stem cells. *Cell Cycle*. 2012;11:974–89.
4. Abdulhasan MK, Li Q, Dai J, Abu-Soud HM, Puscheck EE, Rappolee DA. CoQ10 increases mitochondrial mass and polarization, ATP and Oct4 potency levels, and bovine oocyte MII during IVM while decreasing AMPK activity and oocyte death. *J Assist Reprod Genet*. 2017;34:1595–607.
5. Bolnick A, Abdulhasan M, Kilburn B, Xie Y, Howard M, Andresen P, et al. Two-cell embryos are more sensitive than blastocysts to AMPK-dependent suppression of anabolism and stemness by commonly used fertility drugs, a diet supplement, and stress. *J Assist Reprod Genet*. 2017;34:1609–17.
6. Bolnick A, Abdulhasan M, Kilburn B, Xie Y, Howard M, Andresen P, et al. Commonly used fertility drugs, a diet supplement, and stress force AMPK-dependent block of stemness and development in cultured mammalian embryos. *J Assist Reprod Genet*. 2016;33:1027–39.
7. Xie Y, Awonuga A, Liu J, Rings E, Puscheck EE, Rappolee DA. Stress induces AMPK-dependent loss of potency factors Id2 and Cdx2 in early embryos and stem cells. *Stem Cells Dev*. 2013;22:1564–75.
8. Calder MD, Edwards NA, Betts DH, Watson AJ. Treatment with AICAR inhibits blastocyst development, trophectoderm differentiation and tight junction formation and function in mice. *Mol Hum Reprod*. 2017;23:771–85.
9. Li Q, Yang Y, Loudon E, Puscheck E, Rappolee D. High throughput screens for embryonic stem cells: stress-forced potency-stemness loss enables toxicological assays. In: Faqi A, ed. *Methods in toxicology and pharmacology*: Springer, 2016.
10. Pikiou O, Vasilaki A, Leondaritis G, Vamvakopoulos N, Messinis IE. Effects of metformin on fertilisation of bovine oocytes and early embryo development: possible involvement of AMPK3-mediated TSC2 activation. *Zygote*. 2015;23:58–67.
11. Li Q, Gomez-Lopez N, Drewlo S, Sanchez-Rodriguez E, Dai J, Puscheck EE, et al. Development and validation of a Rex1-RFP potency activity reporter assay that quantifies stress-forced potency loss in mouse embryonic stem cells. *Stem Cells Dev*. 2016;25:320–8.
12. Bertoldo MJ, Faure M, Dupont J, Froment P. AMPK: a master energy regulator for gonadal function. *Front Neurosci*. 2015;9:235.
13. Bertoldo MJ, Guibert E, Faure M, Rame C, Foretz M, Viollet B, et al. Specific deletion of AMP-activated protein kinase (alpha1AMPK) in murine oocytes alters junctional protein expression and mitochondrial physiology. *PLoS One*. 2015;10:e0119680.
14. Bertoldo MJ, Faure M, Dupont J, Froment P. Impact of metformin on reproductive tissues: an overview from gametogenesis to gestation. *Ann Transl Med*. 2014;2:55.
15. LaRosa C, Downs SM. Stress stimulates AMP-activated protein kinase and meiotic resumption in mouse oocytes. *Biol Reprod*. 2006;74:585–92.
16. Downs SM, Chen J. Induction of meiotic maturation in mouse oocytes by adenosine analogs. *Mol Reprod Dev*. 2006;73:1159–68.
17. Chen J, Hudson E, Chi MM, Chang AS, Moley KH, Hardie DG, et al. AMPK regulation of mouse oocyte meiotic resumption in vitro. *Dev Biol*. 2006;291:227–38.
18. Loudon ED, Luzzo KM, Jimenez PT, Chi T, Chi M, Moley KH. TallyHO obese female mice experience poor reproductive outcomes and abnormal blastocyst metabolism that is reversed by metformin. *Reprod Fertil Dev*. 2014;27:31–9.
19. Ratchford AM, Chang AS, Chi MM, Sheridan R, Moley KH. Maternal diabetes adversely affects AMP-activated protein kinase activity and cellular metabolism in murine oocytes. *Am J Physiol Endocrinol Metab*. 2007;293:E1198–206.
20. Eng GS, Sheridan RA, Wyman A, Chi MM, Bibee KP, Jungheim ES, et al. AMP kinase activation increases glucose uptake, decreases apoptosis, and improves pregnancy outcome in embryos exposed to high IGF-I concentrations. *Diabetes*. 2007;56:2228–34.
21. Dattilo M, Giuseppe D, Ettore C, Menezo Y. Improvement of gamete quality by stimulating and feeding the endogenous antioxidant system: mechanisms, clinical results, insights on gene-environment interactions and the role of diet. *J Assist Reprod Genet*. 2016;33:1633–48.
22. Duranteau L, Lefevre P, Jeandidier N, Simon T, Christin-Maitre S. Should physicians prescribe metformin to women with polycystic ovary syndrome PCOS? *Ann Endocrinol (Paris)*. 2010;71:25–7.
23. Palomba S, Pasquali R, Orio F Jr, Nestler JE. Clomiphene citrate, metformin or both as first-step approach in treating anovulatory infertility in patients with polycystic ovary syndrome (PCOS): a systematic review of head-to-head randomized controlled studies and meta-analysis. *Clin Endocrinol*. 2009;70:311–21.

24. Sinawat S, Buppasiri P, Lumbiganon P, Pattanittum P. Long versus short course treatment with metformin and clomiphene citrate for ovulation induction in women with PCOS. *Cochrane Database Syst Rev*. 2008;CD006226.
25. Tang T, Lord JM, Norman RJ, Yasmin E, Balen AH. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. *Cochrane Database Syst Rev* 2012: CD003053.
26. Lord JM, Flight IH, Norman RJ. Metformin in polycystic ovary syndrome: systematic review and meta-analysis. *BMJ*. 2003;327: 951–3.
27. Rena G, Pearson ER, Sakamoto K. Molecular mechanism of action of metformin: old or new insights? *Diabetologia*. 2013;56:1898–906.
28. Yilmaz M, Biri A, Karakoc A, Toruner F, Bingol B, Cakir N, et al. The effects of rosiglitazone and metformin on insulin resistance and serum androgen levels in obese and lean patients with polycystic ovary syndrome. *J Endocrinol Investig*. 2005;28:1003–8.
29. Vane JR, Botting RM. The mechanism of action of aspirin. *Thromb Res*. 2003;110:255–8.
30. Jamal A, Milani F, Al-Yasin A. Evaluation of the effect of metformin and aspirin on utero placental circulation of pregnant women with PCOS. *Iran J Reprod Med*. 2012;10:265–70.
31. de Oliveira Baraldi C, Lanchote VL, de Jesus Antunes N, de Jesus Ponte Carvalho TM, Dantas Moises EC, Duarte G, et al. Metformin pharmacokinetics in nondiabetic pregnant women with polycystic ovary syndrome. *Eur J Clin Pharmacol*. 2011;67:1027–33.
32. Vause TD, Cheung AP, Sierra S, Claman P, Graham J, Guillemain JA, et al. Ovulation induction in polycystic ovary syndrome. *J Obstet Gynaecol Can*. 2010;32:495–502.
33. Jungheim ES, Odibo AO. Fertility treatment in women with polycystic ovary syndrome: a decision analysis of different oral ovulation induction agents. *Fertil Steril*. 2010;94:2659–64.
34. Genazzani AD, Ricchieri F, Lanzoni C. Use of metformin in the treatment of polycystic ovary syndrome. *Women's Health (Lond Engl)*. 2010;6:577–93.
35. Palomba S, Falbo A, Russo T, Orio F, Tollino A, Zullo F. Role of metformin in patients with polycystic ovary syndrome: the state of the art. *Minerva Ginecol*. 2008;60:77–82.
36. Escobar-Morreale HF. Polycystic ovary syndrome: treatment strategies and management. *Expert Opin Pharmacother*. 2008;9:2995–3008.
37. Moll E, van der Veen F, van Wely M. The role of metformin in polycystic ovary syndrome: a systematic review. *Hum Reprod Update*. 2007;13:527–37.
38. Legro RS, Barnhart HX, Schlaff WD, Carr BR, Diamond MP, Carson SA, et al. Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. *N Engl J Med*. 2007;356:551–66.
39. Cheang KI, Sharma ST, Nestler JE. Is metformin a primary ovulatory agent in patients with polycystic ovary syndrome? *Gynecol Endocrinol*. 2006;22:595–604.
40. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001;108:1167–74.
41. Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevtzoff C, Walker KJ, et al. The ancient drug salicylate directly activates AMP-activated protein kinase. *Science*. 2012;336:918–22.
42. Higdon JV, Delage B, Williams DE, Dashwood RH. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol Res*. 2007;55:224–36.
43. Le HT, Schaldach CM, Firestone GL, Bjeldanes LF. Plant-derived 3,3'-diindolylmethane is a strong androgen antagonist in human prostate cancer cells. *J Biol Chem*. 2003;278:21136–45.
44. Li Y, Li X, Guo B. Chemopreventive agent 3,3'-diindolylmethane selectively induces proteasomal degradation of class I histone deacetylases. *Cancer Res*. 2010;70:646–54.
45. Chen D, Banerjee S, Cui QC, Kong D, Sarkar FH, Dou QP. Activation of AMP-activated protein kinase by 3,3'-diindolylmethane (DIM) is associated with human prostate cancer cell death in vitro and in vivo. *PLoS One*. 2012;7:e47186.
46. Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, et al. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab*. 2010;11: 554–65.
47. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J*. 2000;348(Pt 3):607–14.
48. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature*. 2014;510:542–6.
49. Drahota Z, Palenickova E, Endlicher R, Milerova M, Brejchova J, Vosahlikova M, et al. Biguanides inhibit complex I, II and IV of rat liver mitochondria and modify their functional properties. *Physiol Res*. 2014;63:1–11.
50. Bridges HR, Jones AJ, Pollak MN, Hirst J. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochem J*. 2014;462:475–87.
51. Cao J, Meng S, Chang E, Beckwith-Fickas K, Xiong L, Cole RN, et al. Low concentrations of metformin suppress glucose production in hepatocytes through AMP-activated protein kinase (AMPK). *J Biol Chem*. 2014;289:20435–46.
52. Ben-Meir A, Burstein E, Borrego-Alvarez A, Chong J, Wong E, Yavorska T, et al. Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging. *Aging Cell*. 2015;14:887–95.
53. Rojas J, Chavez-Castillo M, Bermudez V. The role of metformin in metabolic disturbances during pregnancy: polycystic ovary syndrome and gestational diabetes mellitus. *Int J Reprod Med*. 2014;2014(797681):1–14.
54. Kovo M, Kogman N, Ovadia O, Nakash I, Golan A, Hoffman A. Carrier-mediated transport of metformin across the human placenta determined by using the ex vivo perfusion of the placental cotyledon model. *Prenat Diagn*. 2008;28:544–8.
55. Banerjee P, Bhonde RR, Pal R. Diverse roles of metformin during peri-implantation development: revisiting novel molecular mechanisms underlying clinical implications. *Stem Cells Dev*. 2013;22: 2927–34.
56. Wu Y, Viana M, Thirumangalathu S, Loeken MR. AMP-activated protein kinase mediates effects of oxidative stress on embryo gene expression in a mouse model of diabetic embryopathy. *Diabetologia*. 2012;55:245–54.
57. Lautatzis ME, Goulis DG, Vrontakis M. Efficacy and safety of metformin during pregnancy in women with gestational diabetes mellitus or polycystic ovary syndrome: a systematic review. *Metab Clin Exp*. 2013;62:1522–34.
58. Fantus IG. Is metformin ready for prime time in pregnancy? Probably not yet. *Diabetes Metab Res Rev*. 2015;31:36–8.
59. Caenepeel S, Charyczak G, Sudarsanam S, Hunter T, Manning G. The mouse kinome: discovery and comparative genomics of all mouse protein kinases. *Proc Natl Acad Sci U S A*. 2004;101: 11707–12.
60. Grimwood J, Gordon LA, Olsen A, Terry A, Schmutz J, Lamerdin J, et al. The DNA sequence and biology of human chromosome 19. *Nature*. 2004;428:529–35.
61. Hardie DG. AMP-activated protein kinase: maintaining energy homeostasis at the cellular and whole-body levels. *Annu Rev Nutr*. 2014;34:31–55.

62. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol*. 2012;13:251–62.
63. Dupont J, Reverchon M, Bertoldo MJ, Froment P. Nutritional signals and reproduction. *Mol Cell Endocrinol*. 2014;382:527–37.
64. Bolnick A, Awonuga AO, Yang Y, Abdulhasan M, Xie Y, Zhou S, et al. Using stem cell oxygen physiology to optimize blastocyst culture while minimizing hypoxic stress. *J Assist Reprod Genet*. 2017;34:1251–9.
65. Rich PR. The molecular machinery of Keilin's respiratory chain. *Biochem Soc Trans*. 2003;31:1095–105.
66. Secor E, Froment P, Loudon E, Bolnick A, Yang Y, Abdulhasan M et al. AMPK agonists in diet supplements and Pharma mediate wide-ranging maternal and reproductive effects. *eCAM* 2018;Submitted.
67. Lissa D, Senovilla L, Rello-Varona S, Vitale I, Michaud M, Pietrocola F, et al. Resveratrol and aspirin eliminate tetraploid cells for anticancer chemoprevention. *Proc Natl Acad Sci U S A*. 2014;111:3020–5.
68. O'Brien AJ, Villani LA, Broadfield LA, Houde VP, Galic S, Blandino G, et al. Salicylate activates AMPK and synergizes with metformin to reduce the survival of prostate and lung cancer cells ex vivo through inhibition of de novo lipogenesis. *Biochem J*. 2015;469:177–87.
69. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009;324:1029–33.
70. Yang Y, Bolnick A, Shamir A, Abdulhasan M, Li Q, Parker GC, et al. Blastocyst-derived stem cell populations under stress: impact of nutrition and metabolism on stem cell potency loss and miscarriage. *Stem Cell Rev*. 2017;13:454–64.
71. Christianson MS, Wu H, Zhao Y, Yemini M, Leong M, Shoham Z. Metformin use in patients undergoing in vitro fertilization treatment: results of a worldwide web-based survey. *J Assist Reprod Genet*. 2015;32:401–6.
72. Pfeiffer T, Schuster S, Bonhoeffer S. Cooperation and competition in the evolution of ATP-producing pathways. *Science*. 2001;292:504–7.
73. Martin KL, Leese HJ. Role of glucose in mouse preimplantation embryo development. *Mol Reprod Dev*. 1995;40:436–43.
74. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell*. 2012;21:297–308.
75. Zhang CS, Hawley SA, Zong Y, Li M, Wang Z, Gray A, et al. Fructose-1,6-bisphosphate and aldolase mediate glucose sensing by AMPK. *Nature*. 2017;548:112–6.
76. Lin SC, Hardie DG. AMPK: sensing glucose as well as cellular energy status. *Cell Metab*. 2018;27:299–313.
77. Conaghan J, Handyside AH, Winston RM, Leese HJ. Effects of pyruvate and glucose on the development of human preimplantation embryos in vitro. *J Reprod Fertil*. 1993;99:87–95.
78. Javed MH, Wright RW Jr. Determination of pentose phosphate and Embden-Meyerhof pathway activities in bovine embryos. *Theriogenology*. 1991;35:1029–37.
79. O'Fallon JV, Wright RW Jr. Quantitative determination of the pentose phosphate pathway in preimplantation mouse embryos. *Biol Reprod*. 1986;34:58–64.
80. Slater JA, Zhou S, Puscheck EE, Rappolee DA. Stress-induced enzyme activation primes murine embryonic stem cells to differentiate toward the first extraembryonic lineage. *Stem Cells Dev*. 2014;23:3049–64.
81. Zhong W, Xie Y, Abdallah M, Awonuga AO, Slater JA, Sipahi L, et al. Cellular stress causes reversible, PRKAA1/2-, and proteasome-dependent ID2 protein loss in trophoblast stem cells. *Reproduction*. 2010;140:921–30.
82. Chae HD, Lee MR, Broxmeyer HE. 5-Aminoimidazole-4-carboxamide ribonucleoside induces G(1)/S arrest and Nanog downregulation via p53 and enhances erythroid differentiation. *Stem Cells*. 2012;30:140–9.
83. Mansouri L, Xie Y, Rappolee DA. Adaptive and pathogenic responses to stress by stem cells during development. *Cell*. 2012;1:1197–224.
84. Ford RJ, Fullerton MD, Pinkosky SL, Day EA, Scott JW, Oakhill JS, et al. Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. *Biochem J*. 2015;468:125–32.
85. Kohonen P, Benfenati E, Bower D, Ceder R, Crump M, Cross K, et al. The ToxBank data warehouse: supporting the replacement of in vivo repeated dose systemic toxicity testing. *Mol Inform*. 2013;32:47–63.
86. Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *BioEssays*. 2002;24(9):845–9.
87. Baumann CG, Morris DG, Sreenan JM, Leese HJ. The quiet embryo hypothesis: molecular characteristics favoring viability. *Mol Reprod Dev*. 2007;74:1345–53.
88. Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. Metabolism of the viable mammalian embryo: quietness revisited. *Mol Hum Reprod*. 2008;14:667–72.