Commonly used fertility drugs, a diet supplement, and stress force AMPK-dependent block of stemness and development in cultured mammalian embryos

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Abstract

Purpose The purpose of the present study is to test whether metformin, aspirin, or diet supplement (DS) BioResponse-3, 3'-Diindolylmethane (BR-DIM) can induce AMP-activated protein kinase (AMPK)-dependent potency loss in cultured embryos and whether metformin (Met)+ Aspirin (Asa) or BR-DIM causes an AMPK-dependent decrease in embryonic development.

Methods The methods used were as follows: culture post-thaw mouse zygotes to the two-cell embryo stage and test effects after 1-h AMPK agonists' (e.g., Met, Asa, BR-DIM, control hyperosmotic stress) exposure on AMPK-dependent loss of Oct4 and/or Rex1 nuclear potency factors, confirm AMPK dependence by reversing potency loss in two-cell-stage embryos with AMPK inhibitor compound C (CC), test whether Met + Asa (i.e., co-added) or DS BR-DIM decreases development of

two-cell to blastocyst stage in an AMPK-dependent (CC-sensitive) manner, and evaluate the level of Rex1 and Oct4 nuclear fluorescence in two-cell-stage embryos and rate of two-cell-stage embryo development to blastocysts.

Result(s) Met, Asa, BR-DIM, or hyperosmotic sorbitol stress induces rapid ~50–85 % Rex1 and/or Oct4 protein loss in two-cell embryos. This loss is ~60–90 % reversible by co-culture with AMPK inhibitor CC. Embryo development from two-cell to blastocyst stage is decreased in culture with either Met + Asa or BR-DIM, and this is either >90 or ~60 % reversible with CC, respectively.

Conclusion These experimental designs here showed that Met-, Asa-, BR-DIM-, or sorbitol stress-induced rapid potency loss in two-cell embryos is AMPK dependent as suggested by inhibition of Rex1 and/or Oct4 protein loss with an AMPK inhibitor. The DS BR-DIM or fertility drugs (e.g., Met + Asa)

Capsule Drugs metformin and aspirin and diet supplement BR-DIM cause AMPK-dependent potency loss and decrease embryonic development from two-cell to blastocyst stage.

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that are used to enhance maternal metabolism to support fertility can also chronically slow embryo growth and block development in an AMPK-dependent manner.

Keywords Embryo culture · Fertility drugs · Diet supplement · Embryo development · Embryo quality

Introduction

Drugs such as metformin (Met) or diet supplements (DSs) such as BioResponse-3,3'-Diindolylmethane (BR-DIM) are used for improving fertility and diabetic metabolism. Aspirin (Asa) is regularly utilized for analgesia and as an anti-inflammatory pharmaceutical. Met is commonly used to support women with ovulatory problems due to polycystic ovarian syndrome (PCOS) [1–3] and may be used by women with type 2 diabetes (T2D), and aspirin (Asa) is used by fertile and infertile women episodically for pain relief [2, 4–13] and recurrent pregnancy loss due to anti-phospholipid syndrome [14]. Met [15] and Asa [16] may have part of their therapeutic activity through the activation of AMP-activated protein kinase (AMPK). BR-DIM is a commonly used diet extract of yellow cruciferous vegetables [17]. BR-DIM is used to treat women with reproductive problems such as cervical dysplasia [18] and can affect pregnancy [19]. Consumption of cruciferous vegetables is associated with lower prostate cancer risk [20], and cruciferous diet extract BR-DIM slows growth and causes human prostate cancer cell death, *in vitro* and *in vivo* in an AMPK-dependent manner [21]. However, BR-DIM slows growth or kills cancer stem cells, but in embryos, this effect on stem cells would be detrimental. Thus, diet supplements (DSs) like BR-DIM, and drugs such as Asa and Met, may have therapeutic effects through AMPK activity, but we hypothesize that high-dose ranges may be toxic for early embryos.

AMPK helps oocyte or blastocyst metabolism that is already compromised and helps IVF that has high levels of stress and has high oocyte or embryo loss rate due to diabetes [22–25]. Met or other AMPK agonists are often studied for their positive effects on maternal metabolism or when pathogenic insults are present. *In vivo* AMPK can reverse 88 % resorption rate caused by dehydroepiandrosterone (DHEA); however, rescue to 45 % resorption does not match 35 % normal resorption rate [26]. Similarly, clomiphene and Met reverse low fecundity of PCOS patients, but there is loss of chemical pregnancy in Met metformin alone compared with clomiphene and metformin [12]. However, in most studies, no metformin-alone control is done to test for possible toxicity in oocytes and embryos not under severe stress. We hypothesize that many AMPK activators occur in diets and drugs, and oocytes and embryos that are not under pathogenic stimuli may have a toxic AMPK activation levels that are too high [22, 23, 27, 28].

Hyperosmotic stress led to the cloning of the first stress enzyme in the yeast model for sporulation forced by starvation [29]. The cloning of the first stress enzymes in mammals also used hyperosmotic stress, and this has been used as the positive control by many labs that study stress enzymes in somatic cells or study stress or stress enzymes in reproductive systems [30–32]. Use of hyperosmotic stress enables comparison of results between stem cells and embryos and is universal stress for all cells.

The AMPK heterotrimer has a catalytic α -subunit, positive regulatory γ -subunit, and negative regulatory β -subunit [33, 34]. Met activates the positive regulatory γ -subunit through increasing AMP and decreasing adenosine triphosphate (ATP) [35] or in an ATP-independent manner [36], and Asa decreases the activity of the negative regulatory β -subunit by binding ser108 [16]. Thus, Met [35] and Asa [16] activate the AMPK heterotrimer in indirect and direct mechanisms through different subunits, and therapeutic mechanisms occur through AMPK. But, many DSs or drugs may have additive effects that supersede the beneficial dose range into the toxic range. Many DSs and drugs may have supramaximal effects in regulation of cancer cells and an impact in the liver [37–39]. Additive effects could be from simultaneous exposure or sequential exposure due to dietary or drug consumption.

AMPK is beneficial to mouse oocytes under stress [22, 27, 28, 40] and oocytes and embryos isolated from diabetic mothers and subject to diabetic hyperglycemia and dysfunctional insulin signaling [24, 25, 28]. But, AMPK activation occurring in normal preimplantation blastocysts and cultured embryonic stem cells (ESCs) and trophoblast stem cells (TSCs), isolated from the blastocyst [41], leads to loss of potency factors and inhibitor of differentiation (Id)2, caudal domain homeobox (Cdx)2 in blastocysts, octamer-binding transcription factor (Oct)4 and nanog in pluripotent ESCs and inducible pluripotent stem cells (iPSCs) [42, 43], and Cdx2 and Id2 in TSCs [41, 44–46] and in two-cell-stage embryos [41, 45]. Oct4 is necessary for the ESC stress response and survival [47–49] and is necessary for metabolic control in the blastocyst [50], and null Oct4 mutants are lethal at the blastocyst stage due to insufficient function of cells of the inner cell mass (ICM)/ESC lineage cells [51]. Of significance is that small variations in Oct4 level, including loss in the levels caused by stress, change the fate of the ESC [52]. Rex1 is expressed in the ICM also and, although not necessary for embryo survival, is lost when ICM cells follow either of its immediate cell fates, extraembryonic primitive endoderm, or primitive ectoderm that produces all tissues at gastrulation [30]. Thus, it is important to move from potency factor loss of TSC potency factors to testing for the loss of ESC potency factors that will mark and control the pluripotency of stem cells in the embryo that ultimately produce the neonate.

However, these previous studies used hyperosmotic, hypoxic, and genotoxic stressor but did not study DSs or drugs

that are known to activate AMPK. If potency loss is associated with negative effects, AMPK-activating drugs and DSs may cause toxic effects in embryos. Thus, we *hypothesize* that AMPK-activating drugs, such as Met and Asa, and DSs, such as BR-DIM, can cause potency loss in two-cell embryos and lead to decreased developmental rates in cultured mouse embryos. This hypothesis is tested here.

Materials and methods

Materials

Sorbitol, Asa (tissue culture-grade acetylsalicylic acid), and Met were from Sigma Chemical Co. (St. Louis, MO). The primary antibodies for total mouse monoclonal anti-Oct4 and rabbit polyclonal Rex1 were from Santa Cruz Biotechnology (Santa Cruz, CA). The AMPK inhibitor compound C (CC) was from Calbiochem (San Diego, CA). BR-DIM was from Dr. Dou, Wayne State University School of Medicine, and was prepared and used similar to protocols for in vitro treatment of human prostate cancer cells [21]. BR-DIM was purchased from BioResponse (BioResponse, Boulder, CO)

Embryo culture and treatment

Commercially available cryopreserved mouse zygotes from superovulated female B6C3F-1 $\times$ male B6D2F-1 mice (Embryotech Laboratories, Inc., Haverhill, MA, USA) were used. Both the test and control embryos were set up in triplicate under oil and cultured in 5 % CO₂ at 37 °C until they were scored for development to expanded blastocysts. The one-cell mouse embryo assay noted and recorded the development from one-cell to two-cell in 24 h and one-cell to expanded blastocyst in 96 h. The two-cell mouse embryo assay only noted/recorded development from two-cell to expanded blastocyst in 72 h. Embryotech Laboratories Inc. (ELI) requires greater than 70 % blastocyst formation from the control group to validate the one-cell assay. Minimum blastocyst rate is 80 % for the two-cell assay. The quality of cryopreserved zygotes used in this study was validated by a very high blastocyst formation rate >90 % in vehicle, potassium Simplex optimized media (KSOM) with amino acids (KSOM^{AA}; Global medium) (Fig. 2a) after 4 days of culture. Standard techniques were used for obtaining mouse embryos [53]. Female B₆C₃F₁ mice (3–4 weeks old, Envigo, Indianapolis, IN) were super-ovulated and mated with male B₆D₂F₁ mice. After superovulation and mating, the female B₆C₃F₁ mice were euthanized and the oviducts containing the one-cell mouse embryos were harvested. The cumulus intact one-cell mouse embryos were removed from the oviduct and placed in hyaluronidase to remove all cumulus cells. The cumulus-free one-cell mouse embryos

were rinsed in M2 medium with HEPES (Sigma® Life Science, Catalog M7167) before being placed into cryoprotectant (ethylene glycol-based cryopreservation medium) and loaded into 0.25-cc straws. Thawing was performed according to the manufacturer's protocol. After thawing, the embryos were incubated at 37 °C and 5 % CO₂ in KSOM^{AA} for 18 h and examined for development. Embryos showing signs of fragmentation and delayed or accelerated development were discarded. In all studies, embryos were equilibrated for at least 1 h in lowest-stress KSOM^{AA} [54] and stressed with the stimulus dose for the time period indicated. KSOM^{AA} was 260–270 mOsmol, increasing 1.7-fold to 498 mOsmol with the addition of 200 mM sorbitol. For inhibitor studies (except where indicated), the inhibitors were preloaded with embryos 3 h before the stress and continued during the stress. In the text, the level of sorbitol (w/v) added is used to produce the given molarity of sorbitol [55, 56]. For inhibitor studies, the inhibitors were incubated with embryos for 2 h before stress was added and during stress. The dose of AMPK inhibitor CC used in this study was 5 μ M. Embryos were preloaded with CC for 2 h to saturate endogenous AMPK prior to experimental agonists as done previously [57], and then, the embryos were treated with 200 mM sorbitol or 1 mM Met and/or 10 μ M acetylsalicylic acid or 20 μ M BR-DIM in the continuous presence of 5 μ M CC for 1 h or for 3 days.

Met was used at 1 mM because it caused little cellular morbidity for 3 days of mouse ESC culture but regulated expression of paired box 3 (Pax3) transcription factor [58, 59]. BR-DIM was used at 20 μ M because this dose was used to slow growth or kill cultured prostate cancer cells [21], and a peak of $\sim$ 7 μ M BR-DIM was reported during in vivo mouse exposure [60]. Asa (salicylate) was used at 10 μ M because this is a standard plasma level after enteric pill [61] and well within the 1–3-mM levels after high-dose Asa treatment of T2D [62], and the 2-mM dose was used to show that major therapeutic levels of Asa require AMPK activity [16]. CC at 5 μ M was validated in dose-response testing in other previous studies from our lab for testing AMPK effects in two-cell embryos and blastocysts and recently optimized for TSCs [41, 45, 63].

Immunofluorescence

Two-cell-stage embryos were fixed, quenched, permeabilized, and stained for Oct4 and Rex1 and counterstained for 4',6-diamidino-2-phenylindole (DAPI) by modifying previous protocols used in mouse ESCs and validated by immunoblot [64, 65]. Briefly, embryos were fixed in 2 % paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 30 min at room temperature, quenched in 0.5 M glycine, and rinsed three times in PBS containing 0.5 % bovine serum albumin (PBS/BSA). After paraformaldehyde fixation, two-cell embryos were permeabilized for 15 min with 0.1 % Triton X-100. Potency factors were dual-labeled simultaneously

overnight at 4 °C using a mouse monoclonal antibody against Oct4 and a rabbit polyclonal antibody against Rex1, and both diluted 1:100 from a 200 µg/ml stock (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Binding of primary antibodies was visualized using a combination of fluorescein isothiocyanate (FITC)-conjugated affinity-purified donkey anti-mouse immunoglobulin (IgG) and Texas Red-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:500 from a 1 mg/ml stock. Embryos were incubated for 60 min at room temperature in the secondary antibody mixture, and nuclei were counterstained with DAPI diluted 1:1000 from a 5 mg/ml stock and mounted on glass slides. Fluorescent antibody labeling was imaged using a Hamamatsu Orca cooled-chip digital camera and a Leica DM IRB microscope with filter sets for DAPI, FITC, and Texas Red. Embryos were imaged at an objective magnification of $\times 20$ and an exposure time of 2.0 s. The FITC or Texas Red stain intensities were quantified using Simple PCI (Hamamatsu) imaging software and formatted using Microsoft Excel and Adobe Photoshop 7.0. Fluorescence intensities (grey levels) were determined for each antibody and nonimmune IgG (background) by circumscribing the nuclei. All micrographs were exposed using the same shutter speed, and all experiments were repeated at least three times.

Statistical analysis

All experiments were performed with at least three replications. Data on immunofluorescence were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's post hoc comparisons. Percentages of embryos reaching developmental milestones were arcsine transformed before analysis. A general linear model was used to examine the main effects of stimulation treatments, days, and stages and their two-way and three-way interactions. The interactions between treatments and days and between days and stages were significant, allowing the subsequent comparisons among treatments on a specific day or at a specific stage using Dunnett's and/or Tukey's post hoc tests. The cell numbers per embryo were analyzed using one-way ANOVA followed by Tukey's post hoc test to examine the difference among stimulation treatments on day 2. Independent *t* test was used to compare the cell numbers per embryo between days 2 and 4 for Met+Asa or BR-DIM. All analyses were performed in Statistical Package for the Social Sciences (SPSS) version 23.0 (SPSS Inc., Chicago, IL).

Results

We previously showed that standard positive control hyperosmotic stress caused AMPK-dependent Cdx2 and Id2 protein loss in two-cell embryos where the function of loss is

not known and in embryos and TSCs where forced loss leads to depletion of stem cells and differentiation to first lineage [41, 45, 46]. Here, we test the hypothesis that clinically relevant drugs and a DSs cause AMPK-dependent potency factor loss and embryo development. The rationale for doses used for sorbitol, Met, Asa, BR-DIM, and CC is analyzed in the "Embryo culture and treatment" section of the "Materials and methods" section. Culture of embryos in KSOM^{AA} sustained nuclear expression of Oct4 and Rex1 (Fig. 1a), and 1-h treatment of two-cell-stage embryos with 1 mM Met or 200 mM sorbitol decreased Oct4 by 64 and 57 %, respectively, and Rex1 by 53 and 52 %, respectively. The hyperosmotic stress- or drug-induced loss of Oct4 and Rex1 was significant ($p < 0.05$) (Fig. 1b). CC pretreatment and co-treatment with sorbitol or Met reversed Rex1 loss by 92 or 97 %, respectively ($p < 0.05$). CC pretreatment and co-treatment with sorbitol or Met reversed Oct4 loss 78 % or 85 %, respectively, and the reversal of hyperosmotic stress- or drug-induced loss of Oct4 and Rex1 were significant ($p < 0.05$). Rex1 loss caused by metformin was reversed by CC to a level that was not significantly different than Rex1 expression in unstressed embryos ($p = 0.26$). Thus, either positive control hyperosmotic sorbitol or Met causes significant loss of two nuclear, potency factor proteins and is AMPK dependent as AMPK antagonist reverses loss to the point where embryos have nearly the potency of unstressed embryos.

KSOM^{AA} was previously shown to be a very low-stress medium; it enabled highest developmental rates, and >90 % of zygotes reached the blastocyst stage by day 4 (Fig. 2a). CC (5 µM) also enabled high growth and development that was not significantly different from KSOM^{AA} alone on days 2 and 3. However, by day 4, 64 % of embryos had reached blastocyst stage. Both Met+Asa (1 mM+10 µM respectively) and 20 µM BR-DIM significantly ($p < 0.05$) slowed embryo development on days 2 and 3, and by day 4, only 3 % of two-cell-stage embryo in the Met+Asa group and 0 % of two-cell-stage embryo on the BR-DIM group had reached the blastocyst stage. However, co-culture with CC significantly ($p < 0.05$) reversed the nearly absolute block of development of 65.6 and 42.3 % of embryos to the blastocyst stage by day 4 after treatment with Met+Asa and BR-DIM, respectively. Although CC had some effects on day 2 for both BR-DIM and Met+Asa, more embryos were in the least developed state. It was not until day 4 that a strong reversal and over half the embryos had progressed to more advance developmental stages. By day 4, CC significantly ($p < 0.05$) reversed Met+Asa from 22.3 to 88.4 % and BR-DIM 0 to 71 %. Taken together, the data suggest that AMPK agonism is associated with slower early development followed by high levels of arrested development by the blastocyst stage

The data suggest that there is an optimal level of AMPK activity that is between addition of antagonist and addition of two agonists. In order to determine the effects of levels of

Fig. 1 AMPK mediates hyperosmotic stress- and Met AMPK agonist-induced loss of nuclear Oct4 and Rex1 potency factors that is largely reversed by CC (**a**). Zygotes were cultured overnight in lowest-stress media, some two-cell embryos were then preloaded with 5 μ M CC for 2 h, and at time 0, embryos were incubated with 200 mM hyperosmotic stress or 1 mM Met for 1 h. Embryos were fixed, quenched, permeabilized, and exposed to polyclonal anti-Rex1 or monoclonal anti-Oct4 antibodies and counterstained with anti-rabbit TxRed or anti-mouse FITC and Hoechst and then micrographed. Embryos were treated with KSOM^{AA} alone (aka unstimulated; A–C), 200 mM sorbitol (D–F), sorbitol with CC (G–I), 1 mM Met (J–L), or 5 μ M CC with 1 mM Met (M–O). The micrographs represent Hoechst (A, D, G, J, M), Oct4 (B, E, H, K, N), and Rex1 (C, F, I, L, O) after 1 h of the indicated treatments after exposures of the same embryos. Biological experiments were done in

triplicate, and quantitative immunofluorescence of nuclei was done using Simple PCI DN module and analyzed for significance using ANOVA and Tukey post hoc test (**b**). AMPK induces loss of nuclear potency factors through hyperosmotic stress or the AMPK agonist Met, and loss is reversed by AMPK antagonist CC. Biological experiments were done in triplicate, and quantitative immunofluorescence of nuclei was done using Simple PCI DN module and analyzed for significance using ANOVA and Tukey post hoc test. ^aShows a significant difference compared with KSOM^{AA} control ($p < 0.05$). ^bShows a significant difference between sorbitol and sorbitol + CC or between Met and Met + CC and a significant difference compared with KSOM^{AA} ($p < 0.05$). ^cShows significant difference compared with Met ($p < 0.05$) but no significant difference compared with KSOM^{AA}

Fig. 2 AMPK antagonist compound C (CC) reverses retarded embryo development caused by AMPK agonist drugs (Met+Asa) and diet supplement (BR-DIM). Embryos were cultured overnight from day 0 to day 1, stimulated +/- CC day 1 at the two-cell stage, and micrographed daily to assay effects on embryo development. From *top to bottom*, development was assayed after stimulation by **a** KSOM^{AA} alone, **b** KSOM^{AA} + CC, **c** Met+Asa, **d** Met+Asa+CC, **e** BR-DIM, and **f** BR-DIM+CC. On days 2, 3, and 4 embryos were categorized for development from the two-cell stage, compaction at eight-cell stage through morula. On days 3 and 4, the embryos were also categorized as blastocysts. Biological experiments were done in triplicate, and quantitative immunofluorescence of nuclei was done using Simple PCI DN module and analyzed for significance using ANOVA and Tukey post hoc test. ^aShows that BR-DIM or Met+Asa is different than KSOM^{AA} control for time- and developmental stage-matched embryos ($p < 0.05$). ^bShows that BR-DIM+CC or Met+Asa+CC is significantly different compared to time- and developmental stage-matched embryos BR-DIM or Met+Asa, respectively ($p < 0.05$)

AMPK modulation on development of embryos cultured from two-cell to blastocyst, we compared the micrographs and the fractions of embryos developed to blastocyst in increasing levels of AMPK modulation; single antagonist, no modulation, two agonists with one antagonist, one agonist, and two agonists. The data show that embryos with antagonist CC develop to blastocyst stage ($64.6 \pm 17.6\%$) (Fig. 3) but are not of apparent high quality and would not typically be reimplanted in an IVF clinic. A higher rate of untreated embryos develops to blastocyst ($90 \pm 5.9\%$) than embryos treated with two agonists and

CC ($65.6 \pm 8.8\%$ blastocysts), and the untreated embryos have a larger apparent ICM and would be judged the best for reimplantation. Single agonists (BR-DIM) or double agonists (Met+Asa) produced less than 4 % blastocysts. Clearly, the reversal by CC of poor development caused by Met+Asa is apparent morphologically as the CC-reversed group is of generally high quality. Although, all stimulants have off-target effects; taken together, the data suggest an optimization of embryonic development of AMPK levels whereby medium alone is optimal and antagonism or agonism is suboptimal.

The severity of effects of AMPK activity modulation is related to culture with AMPK agonists or antagonists and affects cell number. We next tested whether embryos showed immediate, morphological signs of a response to AMPK activity modulators after 1 day of culture. All treatment groups were translucent, except embryos in the CC which were slightly opaque (Fig. 4a). Embryos at the initiation of treatment at day 1 were at the two-cell stage, and cell number at day 2 suggests that the two agonists most severely limited cell proliferation and that the antagonist slightly increased proliferation compared with control or the group where antagonist was added to two agonists (Met+Asa+CC). This suggests that AMPK agonism may slow growth and AMPK antagonism speed growth. Importantly, the embryos were translucent after 1 day of treatment on day 2, and thus, DS Asa and Met did not have AMPK agonist or other moieties that immediately caused necrosis or morbidity. But, by day 4, the cells/embryo of two agonist treatment groups was not significantly different from those with the same treatment at day 2 ($p > 0.05$) (Fig. 4b), and these embryos were mostly dead by day 4. In contrast, the other three groups had developed higher cell number and had progressed to over 60 % blastocysts in all of these three groups. However, the CC-treated group was not highly cavitated and remained opaque. The Met+Asa+CC-treated embryos were highly cavitated but had smaller ICMs than the control group cultured in KSOM^{AA} alone. It should be noted that the x-axis assignment of severity of effect was multifactorial and based on outcomes from days 2 and 4 and from morphological criteria and cell number. For example, CC had the highest cell number/embryo at day 2 but was also opaque, and opacity is a known predictor of low embryo quality in IVF [66]. Additionally, although progression and size of cavitation were similar at day 4 for KSOM^{AA} and CC alone, CC alone had a smaller ICM, and these would be considered of lower quality and of lesser fitness for reimplantation in IVF [67].

The block of development by Met+Asa or BR-DIM suggested that Asa and BR-DIM should also be tested for AMPK-dependent potency loss at the two-cell stage. Using the same culture and assay strategy as in Fig. 1, we found that both BR-DIM and Asa cause a significant decrease in Oct4 nuclear protein in two-cell embryos ($p < 0.05$) (Fig. 5a). Similarly to Met alone, Asa- and BR-DIM-induced loss was significantly reversed by CC ($p < 0.05$) (Fig. 5b). In the case of Asa,

Fig. 3 Regulation of AMPK activity by AMPK agonists and antagonists is critical for embryo development and suggests an optimal AMPK activity (**a**). All zygotes were cultured overnight in lowest-stress media KSOM^{AA} to adapt to culture. Prior to treatment (time 0), two-cell embryos were pretreated with vehicle or 5 μ M CC for 2 h followed by treatment with vehicle, or AMPK agonists, 10 μ M Asa, and 1 mM Met or with 20 μ M BR-DIM alone until day 4 when final development was assayed from micrographs (one experiment shown above). All experiments were done in triplicate, and embryo development was assessed twice daily. Micrographs

were taken and number of blastocysts was formed from initial two-cell-stage embryo recorded. Micrograph images show treatment with AMPK antagonist CC and AMPK antagonists Met + Asa, or BR-DIM has putative effects on embryo development (**b**). Biological experiments were done in triplicate, and quantitative immunofluorescence of nuclei was done using Simple PCI DN module and analyzed for significance using ANOVA and Tukey post hoc test. ^aShows significant difference compared to KSOM^{AA} no stimulus control ($p < 0.05$). ^bShows significant difference compared with CC, KSOM^{AA}, and Met + Asa + CC ($p < 0.05$)

reversal was to an Oct4 level not significantly different than unstressed two-cell embryos, suggesting a strong AMPK component in Asa effects. Interestingly, CC significantly increased Oct4 ($p < 0.05$) (Fig. 5a, b), suggesting that culture stress causes AMPK-dependent decrease in Oct4.

Discussion

For the first time, we show here that AMPK agonist drugs, Asa and Met, and DS BR-DIM can have negative effects on stem cell potency, cell growth, and embryo development in early mammalian development. Cultured embryos are translucent and not immediately morbid after 1 day of culture with the two AMPK agonist treatments Met + Asa or BR-DIM. But, cell growth is retarded and rapidly arrested and cell accumulation is highly decreased compared with media control. The AMPK antagonist CC improves slowed cell growth and early retardation of embryo development by AMPK agonists in the first day of treatment (e.g., day 2). But, full effects on reversal of retardation are not apparent until the third and last day of treatment in culture (e.g., day 4). It is clear that CC reverses the effects of Met + Asa or BR-DIM, but it is likely that some of the agonists or the antagonists are having their effects solely through AMPK.

We sought to test the hypothesis that many stimuli cause AMPK-dependent ESC potency factor loss in two-cell

embryo, as had been shown for TSCs, blastocysts, and TSC potency factors in two-cell embryos. For the first time, we arrested development by the blastocyst stage arrested development by the blastocyst stage arrested development by the blastocyst stage cause AMPK-dependent Oct4 and Rex1 potency factor loss in two-cell embryos as Met-, Asa-, or BR-DIM-induced loss is largely reversed by the AMPK antagonist CC. Interestingly, the rapid potency loss at 1 h occurs in two-cell embryos and Met + Asa or BR-DIM delays or stops embryonic development at the two-cell stage or soon after. Taken together, these data suggest that rapid potency factor loss could be part of the mechanism of embryo delay. But, AMPK also is known to mediate anabolic to catabolic metabolism shifts in oocytes, embryos, and stem cells from the embryos [22, 24, 41], and this hypothetically would mediate delay. An important aspect of future studies is to test for AMPK-dependent effects on decreasing anabolism in cultured embryos.

AMPK agonists have been reported to enable cultured oocytes stressed by four different stressors to mature [22], or oocytes or blastocysts derived from metabolically stressed diabetic mothers [23, 25], to develop more normally than occurs in culture with media alone. Met improves maternal metabolism [6, 68–76] and ovulation [68, 77] and is good for oocytes and embryos derived from females under obese and diabetic conditions [23–25, 78], and thus, AMPK can improve compromised oocytes and embryos. CC alone increases Oct4, suggesting that

Fig. 4 After 1 day, embryos in all stimuli are translucent, but development is delayed in agonists BR-DIM or Met + Asa, but by 4 days, the two agonist treatment groups have arrested with similar cell number as after 1 day (**a**). Embryos were stimulated on day 1 and micrographed on days 2 and 4, and cell counts were performed on day 2 embryos (white inset numbers). The severity of outcomes at day 2 (measured by cell number and opacity) and day 4 (measured by morbidity, arrest, cavitation, and ICM density) (**b**). Cell counts $\pm$ SEM are shown for all six stimulus groups on day 2 and for the two AMPK agonist-only stimuli (BR-DIM and Met + Asa), where nearly all embryo remained in cell countable cleavage stages, cell counts are also shown for day 4. Biological experiments were done in triplicate, and quantitative immunofluorescence of nuclei was done using Simple PCI DN module and analyzed for significance using ANOVA and Tukey post hoc test. ^aShows a significant difference compared to KSOM^{AA} ($p < 0.05$). ^bShows no significant difference between day 2 and day 4 for BR-DIM and Met + Asa, but significant difference compared with KSOM^{AA} ($p < 0.05$). ^cShows no significance compared with KSOM^{AA}

there is some stress during culture in optimal KSOM media and high clinical doses of single and paired AMPK agonists have much larger effect on potency loss on near-normal embryos. The results here do not contradict the reports of positive functions for AMPK in gametogenesis and embryogenesis on specimens derived from or in conditions of stress. Our data suggest that potency is lost and morbidity increases when levels of AMPK activity are above those in *normal*, low-stress embryos cultured in low-stress media, or when metabolically stressed embryos are treated by increasing abnormally low AMPK [22, 25] activity back to an optimum level.

We used hyperosmotic sorbitol at 200 mM because this dose is not toxic to embryos, TSCs, or ESCs [41, 55, 56, 65, 79] but slows their proliferation and has significant effects in causing AMPK-dependent Cdx2 and Id2 loss [41, 45], PL1 increase in TSCs [80], Oct4 and Rex1 loss and first lineage Dab2 and LRP2 markers in ESCs [65, 81, 82], and significant AMPK-dependent Id2 loss in blastocysts [41]. We had previously shown that 200 mM sorbitol causes Cdx2 and Id2 loss in

Fig. 5 AMPK mediates BR-DIM- and Asa-induced loss of nuclear Oct4 potency factor proteins that is largely reversed by CC (**a**). Zygotes were cultured overnight in lowest-stress media, some two-cell embryos were then preloaded with 5 μ M CC for 2 h, and at time 0, embryos were incubated with 20 μ M BR-DIM or 10 μ M Asa $\pm$ CC or continued with CC alone for 1 h. Embryos were fixed, quenched, permeabilized, and exposed to monoclonal anti-Oct4 antibodies and counterstained with anti-mouse FITC and Hoechst and then micrographed. Embryos were treated with KSOM^{AA} alone (A, B), 5 μ M CC alone (C, D), 20 μ M BR-DIM alone (E, F), BR-DIM + CC (G, H), 10 μ M Asa (I, J), and Asa + CC (K, L). The micrographs represent Hoechst (A, C, E, G, I, K) and Oct4 (B, D, F, H, J, L) after 1 h of the indicated treatments after exposures of the same embryos (**b**). AMPK induces loss of nuclear potency factors through BR-DIM or Asa, and loss is reversed by AMPK antagonist CC. Biological experiments were done in triplicate, and quantitative immunofluorescence of nuclei was done using Simple PCI DN module and analyzed for significance using ANOVA and Tukey post hoc test. ^aShows a significant difference compared with KSOM^{AA} control ($p < 0.05$). ^bShows a significant difference between CC and KSOM^{AA}, BR-DIM, and BR-DIM + CC or between Asa and Asa + CC and a significant difference compared with KSOM^{AA} ($p < 0.05$). ^cShows significant difference between Asa + CC compared with Asa ($p < 0.05$) but no significant difference compared with KSOM^{AA}

two-cell embryos in an AMPK-dependent manner [45], and this report adds AMPK-dependent Rex1 and Oct4 loss to make up a group of four AMPK-regulated potency factors expressed in both ESC and TSC lineages by the blastocyst stage. When taken together with BR-DIM, Asa, or Met, *all* purative AMPK agonist stimuli tested to date mediate AMPK-dependent potency loss in early embryos. The single AMPK antagonist tested, CC, increased potency.

Although potency factors may begin to prime cells in the four-cell embryo [83] to allocate later to the two stem cell lineages at the blastocyst stage, AMPK-mediated loss of both ESC and TSC lineage potency factors does not yet indicate

that AMPK is a regulating potency that favors either lineage at the two- to four-cell stage.

Do Asa and other drugs get to the uterine fluids? Early studies of radioactive drugs suggest that Asa is at the same level in uterine fluids as plasma [84, 85]. Although Asa was not studied, many drugs such as barbital, caffeine, and nicotine are taken up by the gestationally exposed blastocysts [86]. A cocktail of small compounds, including salicylate, led to gestational exposure and ensuing intrauterine growth restriction (IUGR) [87], although individually, only nicotine and DDT caused IUGR. Asa is associated with miscarriage after early pregnancy exposure [88].

The 10 μM Asa dose used here was well within normal usage where normal to medium doses produce C_{max} plasma doses of $\sim 2\text{--}200\text{ }\mu\text{M}$ [61, 89]. Interestingly, it was previously reported that Asa blocks development of cultured embryos to the blastocyst stage and reduces the number of cells/embryo [90, 91]. Although an Asa-only test for embryo development blockage was not done here, the Met + Asa and BR-DIM treatments are consistent with this and, for the first time, show an AMPK dependence of the arrest.

These Asa preimplantation studies of the 1950s and 1960s were done before Met was studied, but Met is a small molecule like Asa and is likely to enter follicular and luminal fluids. Recent studies have shown that Met is significantly higher in umbilical arterial and venous plasma than in maternal plasma [92], suggesting that Met crosses the placenta and accesses the embryo and its stem cells after implantation. The earliest post-implantation human embryo in the trophoblast plate stage and lacunar stage has direct access to maternal capillary plasma, which surrounds the embryo [93, 94].

The Met dose used here was 1000 μM , the same one used to study non-morbid regulation of the transcription factor Pax3 during a 3-day culture of ESCs [58]. Here, this dose caused Oct4 and Rex1 loss that was reversed largely by CC, suggesting AMPK dependence. Using a high dose such as 1 mM, when reversed by CC, suggests strongly that Asa effects are through AMPK and not off-target effects, which would be highest at high doses. With the addition of 10 μM Asa, metformin also blocked embryo development, soon after addition of the two drugs at the two-cell stage. Ultimately, this caused an 95 % block of development to the blastocyst stage. Interestingly, it was previously shown that a 780 μM metformin caused a 93 % block of blastocyst development from the two-cell stage [95], similar to the 95 % block by 1000 μM Met and 10 μM Asa here. In these previous studies, the normal plasma dose of 39 μM had no effect on blocking development blastocyst from two-cell embryos during culture. Our next hypothesis to be tested in future studies is that combined exposure to normal plasma levels of 39 μM Met and 10 μM Asa will block embryo development in vitro and in vivo as Met and Asa are known to synergize in activating AMPK on rates and deactivating AMPK off rates, respectively [39].

The 20- μM dose for BR-DIM used here is above the 2.5- μM therapeutic plasma dose range for treatments of cervical dysplasia [18] and dose range used for anti-cancer treatments that slow growth and can kill prostate cancer cells [96]. However, different diet formulations can increase BR-DIM to 7 μM in plasma, and the anti-cancer uses of BR-DIM have led to new formulations and dietary combinations (e.g., delivery with cod liver oil) that will improve absorption and enable a 20- μM plasma dose. Here, 20 μM BR-DIM caused highest loss of nuclear Oct4 potency factor protein compared with all other stimuli. This loss was reversed by CC, suggesting AMPK-dependent loss. The same BR-DIM dose quickly arrests all two-cell embryos after 24-h exposure, although these initially appear healthy. By the blastocyst stage, 2 days later, the BR-DIM-treated embryos had not significantly increased cell number but were opaque and dead. Co-addition of CC with BR-DIM increased the fraction of embryos reaching blastocyst stage from 60 % compared with 0 % blastocysts for BR-DIM alone.

These data clearly show that BR-DIM is the most powerful blocker of Oct4, causing nearly 85 % loss, and of development of culture cell embryos to blastocyst stage, causing 100 % block. CC significantly but not totally reverses both effects. This suggests that BR-DIM can act powerfully on the early embryo and may do so through AMPK-dependent and AMPK-independent mechanisms.

It is likely that Met, Asa, and BR-DIM reach plasma and uterine fluid levels that would stimulate AMPK-dependent responses in vivo as well as in vitro. However, Met [97], Asa [98], and BR-DIM [60, 99] reach pharmacokinetic peaks within 30–144 min of ingestion but are cleared within 12 h. It will be important to expose gestational females to these drugs and remove embryos at the timing of the peak to test for potency factor loss. We previously showed that shear stress by mouth pipetting causes activation of stress-activated protein kinase (SAPK) to levels as high as highest activation dose of hyperosmotic sorbitol, about a 10-fold increase [30]. However, this dose has no effect of mouth pipetting for 15 min on cell number 24 h later [100]. Thus, transient high molecular responses may not lead to biological changes. In addition, three of four ESC potency factors that were decreased from 1 to 4 h of 200 mM sorbitol rebounded to normal levels at 24 h despite continuing hyperosmotic stress [65]. These data suggest that in vitro effects may be higher due to lack of clearance and that ESCs in the early embryo may regain potency after prolonged exposures. Either hypothesis requires testing in vivo.

If results here are corroborated in vivo, then some caution should be used when prescribing Met for PCOS and diabetic women, especially after potential sensitive periods of ovulation, fertilization, and rapid growth and differentiation starting at the blastocyst stage that produces anti-luteolytic hormones. Some embryo loss was noted in women treated with Met alone after detection of chemical pregnancy and before clinical detection by ultrasound [12]. But, in general, the use of Met improves

pregnancy compared with untreated women with lessened infertility where the oocytes and blastocysts are compromised and many causes of infertility lessen AMPK activity [11, 101, 102]. However, the possibility of additive or synergistic exposure of drugs and DSs with AMPK agonist activity deserves further study in vitro and in vivo. This is especially true since AMPK agonists can both activate the enzyme through the positive regulatory γ -subunit and by inactivating the negative regulatory β -subunit, causing supramaximal effects [38, 39, 103].

Similar to past reports for two-cell embryos [45], blastocysts [41, 45], TSCs [41, 45, 46, 63, 104, 105], ESCs [41, 64, 65], and induced pluripotent stem cells (iPSCs) [43], AMPK is the master regulatory enzyme for stress-induced potency loss of Oct4, Rex1, Cdx1, Id2, [41, 43–46, 106], and Nanog [42]. Oct4 and Rex1 and other potency factor loss in two-cell-stage embryos, blastocysts, TSCs, and ESCs occur within 0.5–2 h, suggesting that the stemness program change may be an important strategy requiring rapid, molecular action.

However, it is not clear what the stress-induced, AMPK-dependent Id2, Cdx2, Oct4, or Rex1 loss effects are in early embryos. For TSCs, it is clear that dominant negative Id2 loss is needed to enable stem cell differentiation to produce anti-luteolytic placental lactogen (PL)1 in rodents [41, 80]. In the two-cell-stage embryo, the role of Oct4 and other potency factors is not established. Oct4 may be needed for zygotic genome activation in zebrafish [107, 108], and the authors suggest that conservation of binding sites suggests similar roles in mammals. Oct4 and Cdx2 are necessary at the blastocyst stage to allocate ESC and TSC lineages, respectively [109], and knockouts for either of these are lethal at the blastocyst stage [51, 110]. But, Oct4 also regulates stem cell metabolism [50] and is necessary for stress preparation and response [48, 49]. But, in mouse and human ESCs, Oct4 actively silences hundreds of promoters [111, 112] that would mediate differentiation. After knockdown of maternal and zygotic Cdx2, the most highly upregulated mRNA in mouse blastocysts is Hand1 [113], a marker of first differentiated lineage of TSC [32]. This suggests that like Oct4, Cdx2 function may also contribute to silencing possible differentiated lineages. There are many candidate mechanisms that may be dysregulated soon after addition at the two-cell-stage embryo, when AMPK agonists mediate loss of these potency factors. Although death is delayed, the early arrest soon after the two-cell stage suggests that AMPK-regulated molecular mechanisms at this stage are the best candidates for pathogenicity.

Many food groups and DSs are agonists for AMPK. The following food groups and food extracts have high AMPK-activating capacity: resveratrol (*red grape skin extract*) [114–118], nootkatone (*grapefruit extract*) [119], ajoene (*garlic extract*) [120], BDIM (*cruciferous vegetable extract*) [19, 121], and many others [122]. Medicines activate AMPK including *Asa* [16], *Met* [35], *phenformin* [123, 124], rosiglitazone, and phenobarbital [125, 126], and there may be others.

Taken together, the data reported here suggest the need for further studies testing for effects in vivo for stimuli reported here. Further studies using in vitro high-throughput screening methods for stress-induced potency loss [64] are needed to test for single and additive AMPK agonists from diet and drug sources that cause potency loss and diminish stem cell growth. Since no single hypothetical mechanism for AMPK-mediated pathogenicity stands out alone, studies of changes in the global transcriptome, epigenome, and metabolome of cultured embryos are needed to help sharpen hypotheses of mechanisms.

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Compliance with ethical standards

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