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P66Shc, a key regulator of metabolism and mitochondrial ROS production, is dysregulated by mouse embryo culture
P66Shc, a key regulator of metabolism and mitochondrial ROS production, is dysregulated by mouse embryo culture

Running title: p66Shc in preimplantation mouse embryos

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Abstract

**Study hypothesis:** High oxygen tension and high medium glucose concentrations dysregulate p66Shc expression and function during mouse preimplantation embryo culture.

**Study finding:** P66Shc expression abnormally increases and oxidative phosphorylation metabolism is impaired in blastocysts produced in culture.

**What is known already:** Growth in culture adversely impacts preimplantation embryo development and alters the expression levels of the oxidative stress adaptor protein p66Shc, but it is not known if p66Shc expression differences are linked to metabolic changes observed in cultured embryos.

**Study design, samples/materials, methods:** We used a standard wild type CD1 mouse model of preimplantation embryo development and embryo culture to modulate atmospheric oxygen tension and glucose media concentrations. Changes to p66Shc expression in mouse blastocysts were measured using RT-qPCR, immunoblotting, and immunofluorescence with confocal microscopy. Changes to oxidative phosphorylation metabolism were measured by total ATP content and superoxide production. Statistical analyses were performed on a minimum of three experimental replicates using Students’ t-test or one-way ANOVA.

**Main results and the role of chance:** P66Shc is basally expressed during *in vivo* mouse preimplantation development. Within *in vivo* blastocysts, p66Shc is primarily localized to the cell periphery of the trophectoderm. Blastocysts cultured under atmospheric oxygen levels have significantly increased p66Shc transcript and protein abundances compared to *in vivo* controls (p < 0.05). However, phosphorylated serine 36 (S36) p66Shc to total p66Shc ratio decreased under culture regardless of O₂ atmosphere used, supporting a shift in the mitochondrial fraction of p66Shc. Total p66Shc localized to the cell periphery of the blastocyst trophectoderm and

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phosphorylated S36 p66Shc displayed nuclear and cytoplasmic immunoreactivity, suggesting distinct compartmentalization of phosphorylated S36 p66Shc and the remaining p66Shc fraction.

Glucose medium concentration did not significantly affect p66Shc expression or its localization. Blastocysts cultured under low or high oxygen conditions exhibited significantly decreased cellular ATP and increased superoxide production compared to *in vivo* derived embryos (p < 0.05).

**Limitations, reasons for caution:** This study associates embryonic p66Shc expression levels with metabolic abnormalities but does not directly implicate p66Shc in metabolic changes.

**Wider implications of the findings:** This is the first study to show distinct immunolocalization of p66Shc to the trophectoderm of blastocysts and that its levels are abnormally increased in embryos exposed to culture conditions. Changes to p66Shc expression and/or localization could serve as a molecular marker of embryo viability for clinical applications. The outcomes provide insight into the potential metabolic role of p66Shc. Metabolic anomalies are induced even under current best culture conditions, which could negatively impact trophectoderm and placental development.

**Large scale data:** Not applicable.

**Study funding and competing interest(s):** Canadian Institutes of Health Research (CIHR) operating funds, Ontario Graduate Scholarship. There are no competing interests.

**Key words:** blastocyst; embryo culture; metabolism; mitochondria; p66Shc; preimplantation embryo; ROS, stress adaptor
Introduction

In assisted reproductive technologies (ARTs), embryo culture routinely follows *in vitro* fertilization (IVF) to permit growth to the blastocyst stage. Despite improvements in culture medium formulations and the use of physiological oxygen environments, the rate of successful pregnancy after embryo culture remains considerably low. In 2014, the average live birth rate per IVF cycle for women in Canada was 23% (CFAS, 2015). One possible reason for this low success rate is the preimplantation embryo may be exposed to stress not normally encountered *in vivo* as a result of adverse culturing conditions (Feuer and Rinaudo, 2012; Wale and Gardner, 2015). The mammalian preimplantation embryo may adapt to these adverse culture conditions, however, these stress induced responses can result in major changes to gene expression, epigenetic modifications, and cellular metabolism (Rinaudo and Schultz, 2004; Wale and Gardner, 2012; de Waal et al., 2014). These changes are currently undetectable by morphological non-invasive assessment methods, and thus embryos selected by morphology for transfer may still not be the most developmentally competent. This is a particular concern in current efforts to reduce multiple pregnancies by single embryo transfer (Grady et al., 2012).

To further advance embryo culture and optimize culture parameters, it is important to understand the biological mechanisms of the preimplantation embryo and its interactions with the maternal and *in vitro* environment. Metabolism has emerged as an important research avenue in efforts to understand how culture conditions affect the developmental competence of early embryos (Gardner et al., 2001; Seli et al., 2010; Wale and Gardner, 2013). Modulating oxygen tension during embryo culture alters glucose metabolism, demonstrating that the culture atmosphere can dramatically influence embryo metabolism and subsequent viability (Wale and Gardner, 2012). This may affect the later stages of development in particular, as the
trophectoderm must generate ATP to power the Na\(^+\)/K\(^-\) ATPases and form the blastocoele cavity (Betts et al., 1998; Houghton et al., 2003). The adaptor protein p66Shc is responsive to oxygen tension and is involved in the bovine embryo’s oxidative stress response by promoting permanent embryo arrest and apoptosis under adverse environmental conditions (Favetta et al., 2007a; Betts et al., 2014). P66Shc is a member of the Shc1 family of proteins with functions in growth factor receptor signaling, reactive oxygen species (ROS) production, and oxidative phosphorylation metabolism (Migliaccio et al., 1997, 1999; Nemoto et al., 2006; Acin-Perez et al., 2010). Loss-of-function studies in mouse embryonic fibroblasts (MEFs) and more recently in HeLa cells provide evidence that p66Shc is involved in ATP production by oxidative phosphorylation (Nemoto et al., 2006; Soliman et al., 2014). Dysregulated p66Shc function in the mammalian embryo may therefore not only negatively impact development through high ROS production inducing embryo arrest or apoptosis (Favetta et al., 2004, 2007b; Betts et al., 2014), but may also affect cellular metabolism (Favetta et al., 2007a).

To define a new metabolic route in which preimplantation embryo culture may affect early embryonic development, the objective of our study sought to determine if p66Shc expression changes in cultured embryos compared to in vivo derived embryos, and if altered p66Shc expression is a marker of altered embryo metabolism. In the following study, we use a well-defined preimplantation mouse embryo culture model to modulate atmospheric conditions (oxygen) and culture media (glucose concentration) to determine their effects on p66Shc expression and readouts of oxidative phosphorylation metabolism. Our outcomes demonstrate preimplantation developmental variations in p66Shc expression that are further exacerbated by culture and correlate with aberrant mitochondrial ATP and ROS production.
Materials and Methods

Animal Source and Ethical Approval

Experimental protocols were approved by the Canadian Council of Animal Care and the University of Western Ontario Animal Care and Veterinary Services (Watson #2010-021).

Female and male CD1 mice were obtained from Charles River Canada (St-Constant, Quebec, Canada). Mice were conventionally housed with a 12h light/dark cycle and had access to food and water ad libitum. For all experiments, mice were euthanized by CO\textsubscript{2} asphyxiation.

Embryo Collection and Culture

Three-to-four week old female mice were injected intraperitoneal with 7.5 IU pregnant mare serum gonadotropin (Merck Animal Health, Canada) followed by injection of 7.5 IU human chorionic gonadotropin (Merck Animal Health, Canada) 48 hours later. Female mice were then placed with males for mating. Confirmation of mating was determined by checking for the presence of a vaginal plug the next morning; presence of a vaginal plug indicated embryonic day 0.5 (E0.5). Embryos were flushed with M2 medium (Sigma Aldrich, Canada) from the oviducts and/or uteri of female mice according to the time post injection (hpi): zygotes (18 hpi), 2-cell embryos (44 hpi), 8-cell embryos (68 hpi) and blastocysts (90 hpi). Zygotes were briefly incubated in M2 medium containing 1% hyaluronidase (Sigma Aldrich, Canada) to remove cumulus cells. Embryos were washed twice in M2, then transferred to Extraction Buffer or radioimmunoprecipitation assay buffer (RIPA buffer, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris) until analysis, or to pre-equilibrated KSOMaa Evolve medium supplemented with 1% bovine serum albumin (Zenith Biotech, USA). Embryos were cultured under low (5% O\textsubscript{2}) or high (in air) oxygen tensions in a 5% CO\textsubscript{2}, 37°C incubator.
For glucose experiments, D- or L-glucose (Sigma Aldrich, Canada) was added to KSOMaa Evolve to the desired concentration and embryos were cultured under low oxygen. For transcriptional inhibition experiments, 10 mg/ml α-amanitin (Sigma Aldrich, Canada) in water was diluted to 10 µg/ml in KSOMaa Evolve.

**Real time RT-qPCR**

Pools of twenty embryos collected from 1-3 mice were stored in Extraction Buffer (Life Technologies, USA) at -80°C until use. Total RNA was extracted using the PicoPure RNA isolation kit (Life Technologies, USA) according to the manufacturer’s guidelines. For glucose treatment experiments, 0.5 pg of exogenous luciferase mRNA (Promega, USA) was added to the extract prior to ethanol precipitation. Eluted RNA was reverse transcribed to cDNA using SuperScript III (Life Technologies, USA) according to manufacturer’s instructions, with final concentrations of 150 ng random hexamers (Life Technologies, USA) and 2 pmol p66Shc-specific reverse primer (Table I). Real time qPCR was performed in a CFX384 thermocycler (BioRad, Canada) with each reaction containing 7 µl PerfeCTa SYBR Green 2X SuperMix (Quanta BioSciences, USA), 200 nM of forward and reverse primers (see Table I for all primer sequences) and 4 µl cDNA (equivalent to 0.25 embryo per reaction). PCR conditions are as follows: 95°C for 3 minutes, followed by 45 cycles of 95°C for 15 seconds, 59°C for 15 seconds, and 72°C for 30 seconds. Relative transcript abundance was determined using the delta-delta CT method using expression of *Ppia* and *H2afz*, or luciferase, for normalization (Mamo *et al.*, 2007).

To determine amplification specificity, PCR products after qPCR amplification of p66Shc in blastocyst cDNA were purified using the PureLink Quick Gel Extraction and PCR Purification Kit (Life Technologies, USA) according to manufacturer’s instructions. PCR products were
sequenced by the Robarts Research Institute DNA Sequencing Facility (London, Ontario, Canada). Amplified p66Shc PCR products displayed 96% sequence identity to *Mus musculus* src homology 2 domain-containing transforming protein C1 (Shc1), transcript variant 1 (NM_001113331.2) after BLAST analysis (NCBI database), indicating specific amplification of the p66Shc isoform.

Western Blot Analysis

Pools of 30-50 embryos collected from 2-4 mice were stored in RIPA buffer containing protease and phosphatase inhibitor cocktails (Millipore, USA) at -80°C until use. Total protein lysates were resolved on a 4-12% Bis-Tris gel (Life Technologies) and transferred to a PVDF membrane (Millipore, USA). Membranes were blocked in 5% skim milk or 5% bovine serum albumin in PBS with 0.1% Tween-20 (PBST, Sigma Aldrich) for 1 hour at room temperature, followed by overnight incubation in primary antibody at the indicated concentration at 4°C. Primary antibodies used: anti NT-Shc (Acris Antibodies, USA, 1:100), anti-(phospho S36) p66Shc (Abcam, USA, 1:100), anti-(phospho Y239/Y240) p66Shc (Cell Signaling Technologies, USA, 1:500), and HRP-conjugated anti β-actin (Sigma Aldrich, Canada, 1:20,000). Membranes were then incubated in HRP-conjugated secondary antibody (Jackson Laboratories, USA). Membranes were visualized by detection of Forte ECL (Millipore, USA). Densitometry analysis was performed in Image Lab 4.0 (BioRad, USA).

HT-22 culture and transfection

The HT-22 cell line (immortalized mouse hippocampal cells) and human p66Shc-HA expression plasmid were obtained from Dr. Robert Cumming (University of Western Ontario,
London, Canada). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, USA), at 37°C and 5% CO₂ in air. Cells were transfected with the p66Shc-HA expression plasmid using Lipofectamine 3000 according to the manufacturer’s protocol (Life Technologies, USA), fixed in 4% paraformaldehyde in PBS and processed for immunofluorescence and confocal microscopy.

**Immunofluorescence and Confocal Microscopy**

Embryos were fixed in 2% paraformaldehyde in PBS and permeabilized in 0.1% Triton X-100 in PBS (Sigma Aldrich, Canada) for 30 minutes. Fixed cells were blocked in 5% normal goat serum (Sigma Aldrich, Canada) for one hour at room temperature, followed by overnight incubation in primary antibody at the indicated concentration at 4°C. Primary antibodies used: anti NT-Shc (Acris Antibodies, 1:100), anti phospho-S36-p66Shc (Abcam, 1:100), anti CDX2 (Abcam, 1:100), anti HA-Alexa 647 (Santa Cruz, USA, 1:50). Embryos were incubated in rabbit-anti-mouse Alexa 488 (Life Technologies) for 30 minutes, followed by incubation in goat-anti-rabbit Alexa 488 (Life Technologies) for signal amplification. For CDX2 immunoreactivity, embryos were incubated in goat-anti-rabbit Alexa 547 (Life Technologies). Cells were counterstained with 0.5 µg/ml DAPI (Sigma Aldrich, Canada) and mounted on a glass microscope slide in VectaShield antifade medium (Vector Laboratories, USA). Cells were imaged with a laser scanning confocal microscope (Zeiss LSM510). Laser settings were unchanged when detecting the same primary antibody.

**ATP content assay**
Pools of 5 blastocysts collected from individual mice after treatment under each oxygen tension group were transferred to 96-well plates containing KSOMaa Evolve. ATP content was measured using the Luminescent ATP Detection Assay Kit (Abcam, USA) according to manufacturer’s guidelines. Luminescence was quantified using an eight-point ATP standard curve (0.78 pmol to 100 pmol) and normalized to blastocyst cell number.

**MitoSOX superoxide staining**

Blastocysts from each oxygen tension group were transferred to KSOMaa Evolve containing 5 µM MitoSOX red mitochondrial superoxide indicator (Life Technologies, USA) and incubated for 1 hour at 37°C, 5% CO₂, 5% O₂ (in vivo and low oxygen groups) or in air (high oxygen groups). Blastocysts were transferred to a drop of PBS covered by embryo culture grade mineral oil (Zenith Biotech, USA) for imaging. Blastocysts were imaged using laser scanning confocal microscopy (Zeiss LSM510). Relative fluorescence was quantified by measuring the mean gray value in Image J (NIH). Only blastocyst images with visible inner cell mass were quantified for fluorescence and compared between groups.

**Blastocyst Cell Counts**

Blastocysts were fixed in 4% paraformaldehyde in PBS, permeabilized in 0.2% Triton X-100 in PBS, and stained with DAPI for 1 hour at room temperature. Stained blastocysts were imaged using laser scanning confocal microscopy, with three z-stacks taken per embryo. DAPI-positive nuclei from three stacks were counted using ImageJ.

**Statistical Analyses**
Experiments were performed a minimum of three times using independent replicates with the indicated sample sizes. Statistical analyses were performed in Graph Pad Prism (6.0) for Student’s t-test (unpaired, two-tailed, equal variance) or one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) test to correct for multiple comparisons. Values presented in figures are the mean ± the standard error of the mean (SEM). Probability values less than 0.05 (p < 0.05) were considered statistically significant.
Results

**P66Shc expression increases in blastocysts during mouse preimplantation development**

P66Shc expression has been previously detected in bovine (Favetta et al., 2004) and murine embryos (Ren et al., 2014), but an analysis of expression during the progression of mouse preimplantation development *in vivo* has not been carried out. To determine the expression profile of p66Shc during preimplantation development, we performed real time RT-qPCR and immunoblotting on pools of embryos from four developmental stages. P66Shc transcript and protein were detectable in all stages observed. We observed a significant increase in both transcript and protein abundance from the 8-cell to blastocyst stages (Figure 1A, B). To determine the cellular localization of p66Shc during preimplantation development, we performed whole mount immunofluorescence followed by confocal microscopy using a p66Shc-specific antibody on embryos from six developmental stages. We observed p66Shc immunoreactivity throughout the cytoplasm of pre-compaction stage embryos (Figure 2A-D), with restriction to the apical cell periphery of compacted 16 cell morulae (Figure 2E). To determine if p66Shc localization is restricted to the trophectoderm lineage, we co-stained blastocysts with CDX2. Of all blastocysts observed, p66Shc showed detectable cell periphery localization in only CDX2 positive cells. P66Shc immunoreactivity was undetectable in CDX2 negative cells (Figure 2F). These results indicate that p66Shc expression is normally upregulated in the blastocyst and may be restricted primarily to the trophectoderm of *in vivo* produced blastocysts.

**Validation of NT-Shc antibody specificity**

To verify that the antibodies used to detect p66Shc and phosphorylated (S36) p66Shc only recognized the 66-kDa Shc isoform by immunofluorescence confocal microscopy, we
cultured mature neurons known to have undetectable basal p66Shc expression (Ventura et al., 2002). We performed immunofluorescence using both antibodies on the mouse HT-22 hippocampal cell line. HT-22 cells transfected with a HA-tagged p66Shc DNA construct showed p66Shc and HA immunoreactivity, while non-transfected cells showed no detectable p66Shc or HA immunoreactivity (Supplemental Figure 1A). Transfected HT-22 cells also displayed phosphorylated S36 p66Shc and HA immunoreactivity compared to undetectable levels in non-transfected cells (Supplemental Figure 1B). These results validate the use of these antibodies for immunofluorescent detection of p66Shc and S36-phosphorylated p66Shc cell localization in mouse preimplantation embryos.

P66Shc expression is sensitive to oxygen tension, but not glucose concentration, during embryo culture

Under *in vivo* conditions, p66Shc expression levels may be fine-tuned to prevent adverse developmental events. Given our observations within *in vivo* derived mouse embryos, we then aimed to determine whether certain embryo culture conditions induce aberrant changes in embryonic p66Shc expression levels. Mouse zygotes were cultured to three developmental stages under low oxygen tension (5% O₂) or high oxygen tension (21% O₂). Real time RT-qPCR was performed on pools of embryos to determine changes in p66Shc transcript abundance. Blastocysts examined after 96 hours of culture showed increasing p66Shc transcript abundance with increasing oxygen tension (Figure 3A). This increase was dependent on *de novo* transcription of p66Shc, as the increase in p66Shc abundance was abolished in blastocysts cultured at high oxygen tension in the presence of the transcriptional inhibitor α-amanitin (Figure 3B). It is interesting to note that some p66Shc transcripts were still detectable in treated
blastocysts, suggesting that maternally stored p66Shc may still be present at the blastocyst stage (Figure 3B). Overall, these observations suggest that p66Shc is actively transcribed by the embryo under atmospheric oxygen conditions.

We next aimed to determine if p66Shc protein abundance also increased under culture and high oxygen. Immunoblotting for total p66Shc on pools of embryos showed a significant increase in p66Shc protein abundance in cultured blastocysts (Figure 4A). This induction of p66Shc expression was unique to the blastocyst stage, as p66Shc transcript abundance decreased and protein abundance was unchanged in cultured 2-cell and 8-cell embryos (Supplemental Figure 2A and B). We then saw that increasing oxygen tension significantly decreased the phosphorylated S36 p66Shc to total p66Shc ratio in blastocysts, suggesting a possible change in the mitochondrial fraction of p66Shc in cultured blastocysts (Figure 4B). Oxygen tension did not alter the phosphorylated Y239/Y240 p66Shc to total p66Shc ratio (Figure 4C). These two residues on Shc1 proteins that are known to be phosphorylated after interaction with receptor tyrosine kinases (Gotoh et al., 1997). This result suggests that the shift in the 66-kDa band seen in cultured blastocysts may be due to an alternative (e.g. Ser138, Y317) or novel post-translational modification induced by culture.

To determine if p66Shc cellular localization changed with embryo culture, cultured blastocysts were stained for p66Shc immunoreactivity and were compared to freshly flushed, in vivo derived blastocysts. Blastocysts cultured in high oxygen conditions showed an increase in p66Shc fluorescence intensity and detectable diffuse p66Shc staining in putative ICM cells, compared to in vivo and low oxygen cultured blastocysts (Figure 5A-C). To determine the localization of phosphorylated S36 p66Shc, cultured blastocysts were stained for phosphorylated S36 p66Shc immunoreactivity and compared to in vivo controls. Consistent with the
immunoblotting results, neither the fluorescence levels of phosphorylated S36 p66Shc nor its localization appeared to change between treatment groups. However, phosphorylated S36 p66Shc did show a distinct cellular localization pattern compared to total p66Shc, showing cytoplasmic and nuclear immunoreactivity in the outer and inner cells of the blastocyst (Figure 6ATC). In addition, phosphorylated S36 p66Shc was also detectable in inner cells of the in vivo produced blastocyst while total p66Shc was not, indicating that there may be differences in sensitivity between the two p66Shc antibodies (Figures 5A and 6A). The localization pattern suggests that the phosphorylated S36 p66Shc fraction in blastocysts produced in vivo or in culture may be localized to a distinct compartment in the cytoplasm or nucleus compared to non-phosphorylated, or p66Shc phosphorylated at a different residue.

In addition to its role in mediating the oxidative stress response, several studies have implicated p66Shc in regulating cellular glucose uptake through growth factor receptor signaling, actin cytoskeleton regulation, or modulation of anaerobic respiration (Natalicchio et al., 2009; Soliman et al., 2014). Thus, we next aimed to determine if p66Shc expression is sensitive to medium glucose concentration, another component modified in embryo culture to simulate in vivo microenvironmental conditions. We cultured 8-cell stage embryos for 24 hours in KSOM varying in glucose concentrations under low oxygen tension: 0.2 mM (standard KSOM), 3.4 mM (equivalent to normal mouse oviductal glucose levels, (Gardner and Leese, 1990)), 30 mM D-glucose (hyperglycemia, (Moley et al., 1998)) and 30 mM L-glucose to control for increased osmolarity. We observed that embryos cultured in 30 mM D-glucose have decreased rates of blastocyst cavitation (Figure 7A). The embryos did not fail to cavitate due to glucose toxicity, as 18 hours culture in 0.2 mM D-glucose rescued cavitation (Figure 7B). Furthermore, cell number
in non-cavitated embryos did not significantly change with high glucose culture compared to control, suggesting that these embryos were not developing slower than the controls (Figure 7C).

To determine if p66Shc expression changed during culture in high glucose, we performed RT-qPCR and immunoblotting for p66Shc in pools of blastocysts cultured in the four glucose concentrations. Neither transcript levels nor protein abundance significantly changed in embryos cultured in varying glucose conditions (Figure 8A-B), suggesting that p66Shc expression levels are not sensitive to increased glucose in embryo culture media. To determine if p66Shc cellular localization changed with glucose concentration, embryos cultured in 30 mM D-glucose were stained for p66Shc immunoreactivity and compared to embryos cultured in KSOM. We saw comparable peripheral and cytoplasmic p66Shc immunoreactivity in non-cavitated embryos after high glucose culture compared to controls, suggesting that p66Shc cellular localization is not impacted by media glucose concentrations (Figure 8C).

**Changes to p66Shc expression in culture correlate with altered embryo metabolism**

To determine if increased p66Shc expression levels in cultured embryos could be a marker of altered embryo metabolism, we performed two metabolic assays on blastocysts derived *in vivo* and after culture under low and high oxygen. We first assessed total ATP content of blastocysts from each group, and observed that ATP levels per cell significantly decreased in blastocysts cultured under low oxygen compared to *in vivo* blastocysts (Figure 9A). As oxidative phosphorylation in the trophectoderm is the major source of cellular ATP in the blastocyst (Houghton, 2006), we then assayed for production of superoxide in the same treatment groups. Superoxide is a free radical produced as a by-product of oxidative phosphorylation that is normally present at low levels and is readily scavenged by superoxide dismutase. Blastocysts
were incubated in MitoSOX red superoxide indicator and imaged using confocal microscopy. We observed that blastocysts cultured under low and high oxygen showed significantly higher MitoSOX fluorescence compared to in vivo controls, suggesting increased superoxide production or decreased antioxidant scavenging in these culture conditions (Figure 9B). Our results suggest that even under low oxygen conditions, cultured blastocysts contain less ATP and increased superoxide levels, correlating with increased mRNA and protein abundance of p66Shc.
Discussion

Here we demonstrate that p66Shc is basally expressed in mouse preimplantation embryos and its expression is altered by embryo culture. We also show that dysregulated p66Shc expression coincides with metabolic changes in culture that may negatively affect embryo developmental viability. Our results suggest that p66Shc is an oogenetic-stored transcript that is degraded during the maternal-to-embryonic transition, later upregulated by the blastocyst stage, and predominately located at the cell periphery of trophectoderm cells. Blastocysts grown in vitro show increasing p66Shc expression with increasing oxygen tension, coupled with alterations to phosphorylated residues that have implications in the protein’s cellular compartmentalization and function. These changes appear to be oxygen-sensitive, while changing media glucose concentrations did not significantly affect p66Shc expression levels in the blastocyst. Lastly, we are the first to correlate these changes in culture and high oxygen tension to dysregulated ATP and superoxide production within in vitro produced blastocysts.

Our expression analysis of p66Shc during in vivo blastocyst development suggests that p66Shc is normally upregulated during the eight-cell embryo to blastocyst transition. This basal level of expression during in vivo development implies that despite promoting apoptosis, p66Shc expression maybe necessary for survival and prevent blastocysts from being selected against during development. One possible biological function of p66Shc during preimplantation development may be the promotion of oxidative phosphorylation. Basal oxygen consumption in 66Shc-null MEFs decreases by 30-50% with no change in mitochondrial or cytochrome c content, with a compensatory increase in ATP production by anaerobic respiration (Nemoto et al., 2006). There is also evidence suggesting that in MEFs, p66Shc forms a complex with cytochrome c in the inner mitochondrial membrane to regulate pyruvate dehydrogenase kinase...
(PDK), ultimately regulating the activity of pyruvate dehydrogenase (PDH) depending on the redox state of cytochrome c (Acin-Perez et al., 2010). In the mouse blastocyst, the trophoderm produces ATP through oxidative phosphorylation to support development, but the ICM is relatively metabolically quiescent (Houghton, 2006). Metabolic differences between the two embryonic lineages could account for our immunolocalization results, as p66Shc appears to localize predominately to the trophoderm in vivo and under low oxygen conditions, suggesting that p66Shc could be involved in trophoderm metabolism. Although our study did not directly test the role of p66Shc in oxidative phosphorylation, we have correlated increasing p66Shc transcript and protein abundances after embryo culture with alterations to ATP and superoxide production, suggesting that dysregulated p66Shc levels in the embryo may have a negative impact on embryo metabolism.

Studies of p66Shc in mammalian embryos have thus far focused primarily on the apoptosis- and senescence-promoting functions of p66Shc, basally or in stress-inducing culture conditions. In bovine preimplantation embryos, siRNA-mediated knockdown of p66Shc reduces levels of intracellular ROS, DNA damage, and apoptosis in untreated and oxidant-treated culture conditions (Betts et al., 2014). Bovine preimplantation embryos exhibit high levels of developmental arrest (>50%) in culture (Leidenfrost et al., 2011), likely due to suboptimal culture conditions, which could result in increased p66Shc transcript levels, leading to senescence (permanent embryo arrest) and apoptosis. Due to species-specific differences in early development, or better optimized conditions, mouse preimplantation embryos from inbred strains exhibit high developmental rates with >75% of zygotes reaching the blastocyst stage in optimized media and low oxygen conditions (Karagenc et al., 2004). It is possible that p66Shc
expression is carefully regulated during preimplantation development, such that both abnormally
high and low p66Shc expression levels are detrimental to the embryo.

Consistent with our findings in our mouse embryo culture model, there is strong evidence
associating p66Shc induction under adverse embryo culture conditions. Bovine embryos under
oviductal epithelial cell co-culture growth conditions show significantly increased p66Shc
transcript abundance compared to culture under chemically defined synthetic oviductal fluid
media at lower oxygen tension. This increase was associated with increased markers of oxidative
stress (intracellular ROS, DNA damage) and embryo arrest (Favetta et al., 2007b). Mouse
embryos treated with arsenic show increasing p66Shc immunofluorescence intensity, suggesting
that p66Shc may mediate a stress response to arsenic (Zhang et al., 2010). Preimplantation
development under both cases improved when p66Shc was knocked down by RNA interference
(Favetta et al., 2007a; Betts et al., 2014; Ren et al., 2014). Previous RNA-interference
experiments may have normalized an adverse environment-induced “spike” in p66Shc
expression, but not completely deplete the embryo of maternal- or zygotic-derived p66Shc, thus
masking any loss-of-function phenotype. Maternally-derived p66Shc function may be important
to preimplantation development, as embryo cleavage and blastocyst development is impaired
when p66Shc is knocked down in immature bovine oocytes (Favetta et al., 2007a). We are the
first to show that p66Shc transcript and protein expression is upregulated at the blastocyst stage
during mouse in vivo development, indicating that p66Shc may also have an important
physiological function other than promoting apoptosis and embryo arrest.

The induction of p66Shc transcription in cultured blastocysts appears to be specific to
oxygen, as increasing media glucose concentrations did not significantly change p66Shc
transcript abundance compared to controls. Oxygen-sensitive induction in our results is
consistent with findings that p66Shc transcription can be regulated by the Nrf2-antioxidant response element (ARE) pathway under stress-inducing conditions. Chromatin immunoprecipitation assays performed in hemin-treated human erythroleukemic cells demonstrated that Nrf2 binds to an ARE enhancer upstream of the transcriptional start site of p66Shc and that Nrf2 induction of expression is isoform-specific (Miyazawa and Tsuji, 2014). This could be the upstream mechanism in our model of p66Shc transcriptional upregulation in blastocysts cultured under high oxygen. High media glucose concentrations did not significantly change p66Shc expression in blastocysts, but did affect cavitation. This is consistent with previous reports of hyperglycemic conditions negatively affecting blastocyst development (Fraser et al., 2007). Thus it is unlikely that the cell’s response to high glucose regulates the transcription of p66Shc, but instead may affect other genes known to be involved in cavitation (e.g. Atp1b1, Aqp3, Aqp9, Cdh1). Furthermore, it is not known whether p66Shc is important for the regulation of glucose uptake in preimplantation embryos or if this function is dependent on mTOR or growth factor receptor signaling pathways (Natalicchio et al., 2009; Soliman et al., 2014). It is possible that p66Shc could mediate a response to high glucose levels in embryos independent of an increase in its transcript or protein abundance, through phosphorylation of certain residues.

It is possible that culture conditions increase p66Shc expression to promote its apoptotic functions, removing it from its metabolic function in the mitochondria. Our results suggest that culture-mediated changes to phosphorylated residues on p66Shc may impact its cellular compartmentalization and may ultimately be a key factor in its cellular function. Subcellular fractionation of untreated MEF lysates showed that p66Shc is detectable in the soluble, mitochondrial, and endoplasmic reticulum fractions (Orsini et al., 2004). Phosphorylation of the
serine-36 residue, which is unique to the 66 kDa isoform of the Shc1 family, has been implicated in its cellular localization. Serine-36 phosphorylation of p66Shc under oxidizing conditions increases its association with the prolyl isomerase Pin-1, ultimately resulting in p66Shc translocation to the mitochondria. Fibroblasts lacking Pin-1 have a decreased mitochondrial fraction of p66Shc after H$_2$O$_2$ treatment compared to wild type fibroblasts, linking the modification of this residue to the protein’s mitochondrial localization (Pinton et al., 2007). In our study, blastocysts cultured under low or high oxygen conditions show decreased phosphorylated 36 to total p66Shc ratios, suggesting that these conditions may decrease the mitochondrial fraction of p66Shc despite an increase in total p66Shc protein abundance. This alteration in cellular localization may affect p66Shc’s functions in the mitochondria, which our results of altered embryo metabolism may reflect.

Despite using optimal culture conditions, both p66Shc expression and the metabolic parameters measured were significantly altered in blastocysts grown under low oxygen tension. No significant difference between increased superoxide production in blastocysts after culture in low or high oxygen tension suggests that oxidative phosphorylation metabolism may be adversely affected regardless of oxygen tension, or that there is another parameter in the microenvironment that must be further optimized to limit metabolic alterations in cultured embryos. Levels of p66Shc may therefore be an indicator of altered blastocyst metabolism, particularly of the trophectoderm, which is responsible for generating nearly all of the blastocyst’s ATP content (Houghton, 2006). Altered expression levels and/or p66Shc function in culture may lead to adverse trophectoderm development through increases in ROS-mediated apoptosis or decreases in ATP production, which may impact implantation and placentation. Our study did not follow up on peri- and post-implantation stage embryos and p66Shc expression
levels, but we suspect that p66Shc expression is likely altered in the trophoblast or post-implantation trophoblast-derived tissues after embryo culture. Supporting this is evidence that p66Shc CpG promoter methylation is decreased in human placental tissue of intrauterine growth restricted neonates compared to neonates appropriate and small for gestational age (Tzschoppe et al., 2013). This is also consistent with the finding most culture-induced embryo abnormalities affect the trophoblast and placenta, and to a lesser extent the fetal tissues (Fauque et al., 2010; de Waal et al., 2014). For clinical applications, using increased p66Shc expression as a molecular marker of altered metabolism may impact which blastocyst may be the most developmentally competent for embryo transfer.

Acknowledgements

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Authors’ Roles

Study conception and design: NAE, AJW, DHB. Performed the experiments: NAE. Data analysis: NAE, AJW, DHB. Drafted and proofread the manuscript: NAE, AJW, DHB.

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Conflict of Interest

None to declare.
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Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2015;22:dmv034–.

Zhang C, Liu C, Li D, Yao N, Yuan X, Yu A, Lu C, Ma X. Intracellular redox imbalance and extracellular amino acid metabolic abnormality contribute to arsenic-induced developmental
Figure Legends

Figure 1. p66Shc expression increases during mouse preimplantation development in vivo. (A) RT-qPCR for p66Shc relative transcript abundance was performed on three replicates of pools of 20 embryos per stage. P66Shc relative transcript abundance significantly increases from eight cell to blastocyst-stage embryos (n=3, mean ± SEM, p=0.0476 1W-ANOVA). (B) Immunoblotting for total p66Shc protein abundance was performed on three replicates of pools of 30-50 embryos per stage. P66Shc relative protein abundance increases from eight cell to blastocyst-stage embryos (n=3, mean ± SEM, p=0.0331 1W-ANOVA). A representative blot is shown.

Figure 2. p66Shc progressively localizes to the cell periphery during mouse preimplantation development. Immunofluorescence and confocal microscopy for p66Shc was performed on 10-20 embryos per stage. Representative confocal images are shown: (A) Zygote (B) 2-cell embryo (C) 4-cell embryo (D) 8-cell non-compact embryo (E) 8-16 cell compacted morula (F) Blastocyst, counterstained for CDX2 (G) Primary antibody omitted. Green = p66Shc, Red = CDX2, Blue = DAPI. Scale bar = 20 μm.

Figure 3. Culture and high oxygen tension increases the relative p66Shc mRNA abundance in blastocysts. (A) RT-qPCR for p66She was performed on four replicates of pools of 20 blastocysts. There is a significant increase in p66Shc mRNA abundance in blastocysts cultured at high oxygen tension compared to in vivo controls (n=4, mean ± SEM, p=0.0305 1W-ANOVA). (B) Blastocysts cultured for 24h in 10 μg/ml α-amanitin showed significantly decreased p66Shc transcript abundance compared to controls (n=3, mean ± SEM, p=0.0477 Student’s t-test).
Figure 4. Culture and high oxygen tension increases the relative p66Shc protein abundance in blastocysts. (A) Immunoblotting for p66Shc was performed on four replicates of pools of 50 blastocysts. P66Shc protein abundance significantly increases in blastocysts cultured at low oxygen tension compared to in vivo controls (n=4, mean ± SEM, p=0.0306 1W-ANOVA). A representative blot is shown. (B) Immunoblotting for phosphorylated p66Shc on serine 36 (S36) and total p66Shc was performed on three replicates of pools of 40-50 blastocysts. The ratio of phospho (S36)-p66Shc:total p66Shc significantly decreases in cultured blastocysts compared to controls (n=3, mean ± SEM, p=0.0057 for low O₂; p=0.0219 for high O₂ 1W-ANOVA). A representative blot is shown. (C) Immunoblotting for phosphorylated Y239/Y240-p66Shc and total p66Shc was performed on three replicates of pools of 20-30 blastocysts. The ratio of phosphor Y239/Y240-p66Shc:total p66Shc does not significantly in cultured blastocysts compared to controls (n=3, mean ± SEM, p=0.5043, 1W-ANOVA). A representative blot is shown.

Figure 5. Total p66Shc becomes detectable in the inner cells of blastocysts cultured under atmospheric oxygen tension. Representative immunofluorescence and confocal microscopy images for total p66Shc in pools of 10-15 blastocysts per treatment group. (A) In vivo flushed blastocysts. (B) Blastocysts after 96 h culture under low oxygen tension. (C) Blastocysts after 96 h culture under high oxygen tension. Green = p66Shc, Blue = DAPI. Scale bar = 20 µm.

Figure 6. Phosphorylated S36 p66Shc localization does not change in cultured blastocysts. Representative immunofluorescence and confocal microscopy images for phosphorylated (S36)
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**Figure 7.** High glucose media concentrations reversibly inhibit embryo cavitation. (A) Percent cavitation of blastocysts after 24 h culture in each treatment group, indicated by the formation of any cavity in the embryo (n=4, mean ± SEM, p=0.0052 1W-ANOVA). (B) Bright field microscopy images of embryos after 24 h treatment in 30 mM D-glucose, followed by recovery in low glucose KSOM for 18 hours. Arrows in the left panel indicate examples of embryos classified as non-cavitated. Thirteen of sixteen non-cavitated embryos after high glucose treatment cavitated after 18 hours of recovery. (C) Blastocyst cell number after 24 h culture in each treatment group (n=19-21 per group, mean ± SEM, p=0.5099 1W-ANOVA).

**Figure 8.** High glucose media concentrations do not significantly change relative p66Shc mRNA and protein abundance in blastocysts. (A) qRT-PCR was performed on pools of 10 blastocysts for relative p66Shc transcript abundance, normalized to levels of exogenously added luciferase (n=3, mean ± SEM, p=0.3783 1W-ANOVA). (B) Immunoblotting was performed on pools of 30 blastocysts per treatment group for total p66Shc protein abundance, normalized to levels of β-actin. A representative blot is shown (n=3, mean ± SEM, p=0.5549 1W-ANOVA). (C) Representative immunofluorescence and confocal microscopy images of blastocysts cultured in 30 mM D-glucose (right panel) and KSOM only (left panel) for total p66Shc reactivity. Green = p66Shc, Blue = DNA. Scale bar = 20 µm.
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(B) MitoSOX relative fluorescence was quantified in blastocysts in each treatment group. MitoSOX fluorescence significantly increases in blastocysts cultured under low or high oxygen compared to in vivo controls (in vivo n=28, low oxygen n=26, high oxygen n=23, mean ± SEM, p<0.0001 1W-ANOVA). Representative images of MitoSOX staining are shown in the three panels.

Supplemental Figure 1. NT-Shc and phosphorylated S36 p66Shc antibody validation for immunofluorescence and confocal microscopy. (A) Immunofluorescence and confocal microscopy images of p66Shc-HA transfected HT-22 cells (left) and non-transfected cells (right). Green = total p66Shc, Red = HA, Blue = DAPI. Scale bar = 50 µm. (B) Images of p66Shc-HA transfected HT-22 cells (left) and non-transfected cells (right). Green = pSer36-p66Shc, Red = HA, Blue = DAPI. Scale bar = 50 µm.

Supplemental Figure 2. Relative p66Shc mRNA and protein abundance in cultured 2-cell and 8-cell embryos. (A) qRT-PCR was performed on pools of 20 2-cell embryos for p66Shc relative transcript abundance. P66Shc transcript abundance significantly decreases with culture and increasing oxygen tension (n=4, mean ± SEM, p=0.0310 1W-ANOVA). Immunoblotting was
performed on pools of 50 2-cell embryos for p66Shc relative protein abundance. A representative blot is shown (n=3, mean ± SEM, p=0.7256 1W-ANOVA). (B) qRT-PCR was performed on pools of 20 8-cell embryos for p66Shc relative transcript abundance, which significantly decreases with culture and increasing oxygen tension (n=4, mean ± SEM, p=0.0004 1W-ANOVA). Immunoblotting was performed on pools of 50 8-cell embryos for p66Shc relative protein abundance. A representative blot is shown (n=4, mean ± SEM, p=0.8375 1W-ANOVA).
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<tr>
<td>p66Shc R (reverse transcription)</td>
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<td>p66Shc (qPCR)</td>
<td>F: 5’-CCGACTACCTGTGTTCTTT-3’</td>
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<td>Luciferase</td>
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<tr>
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<td>R: 5’-TTCCGTACTTCCAGCTCACA-3’</td>
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215x219mm (300 x 300 DPI)