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NEW RESEARCH HORIZON

Permanent embryo arrest: molecular and cellular concepts

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Developmental arrest is one of the mechanisms responsible for the elevated levels of embryo demise during the first week of *in vitro* development. Approximately 10–15% of IVF embryos permanently arrest in mitosis at the 2- to 4-cell cleavage stage showing no indication of apoptosis. Reactive oxygen species (ROS) are implicated in this process and must be controlled in order to optimize embryo production. A stress sensor that can provide a key understanding of permanent cell cycle arrest and link ROS with cellular signaling pathway(s) is p66Shc, an adaptor protein for apoptotic-response to oxidative stress. Deletion of the p66Shc gene in mice results in extended lifespan, which is linked to their enhanced resistance to oxidative stress and reduced levels of apoptosis. p66Shc has been shown to generate mitochondrial H_2O_2 to trigger apoptosis, but may also serve as an integration point for many signaling pathways that affect mitochondrial function. We have detected elevated levels of p66Shc and ROS within arrested embryos and believe that p66Shc plays a central role in regulating permanent embryo arrest. In this paper, we review the cellular and molecular aspects of permanent embryo arrest and speculate on the mechanism(s) and etiology of this method of embryo demise.

Keywords: anti-apoptosis; p66Shc; ROS; telomere; mitochondria

Introduction

One of the features of *in vitro*-produced (IVP) mammalian embryos is the high frequency of early developmental failure thought to be brought on by sub-optimal culture environments (Johnson and Nasr-Esfahani, 1994; Betts and King, 2001; Favetta *et al*., 2004a,b). Fewer than 50% of all *in vitro* fertilized (IVF) embryos reach the blastocyst stage of development (Xu *et al*., 1992) with many of these unable to sustain development following embryo transfer (Farin *et al*., 2001). Data generated from the European registers by European Society of Human Reproduction and Embryology (ESHRE) indicate that the clinical pregnancy rates per aspiration and per embryo transfer were 26.6 and 30.1%, respectively, for all human IVF cycles in 2004 (Andersen *et al*., 2008). The reasons for this high rate of embryo demise remains unclear, but it has been proposed as a protective mechanism for preventing further development of abnormal, poor-quality embryos. Almost half of all arrested human embryos display chromosomal abnormalities (Almeida and Bolton, 1998), and significantly more chromosomal aberrations are observed, alongside delayed development, for IVP bovine embryos compared to their *in vivo*-derived counterparts (Kawarsky *et al*., 1996; Viuff *et al*., 1999). Blastomeres with characteristic features of apoptosis, including nuclear and cytoplasmic fragmentation, have been detected in both *in vitro*- and *in vivo*-derived embryos, indicating that high levels of apoptosis might play a role in early embryo death (Hardy, 1997; Kamjoo *et al*., 2002). The *in situ* terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which detects fragmented DNA, has identified a greater incidence of apoptotic nuclei in cultured bovine blastocysts compared with those derived *in vivo* (Gjorret *et al*., 2003). Interestingly, no morphological or biochemical signs of apoptosis has been observed during the early cleavage stages of embryogenesis (Byrne *et al*., 1999; Matwee *et al*., 2000, 2001; Betts and King, 2001; Hardy *et al*., 2001; Gjorret *et al*., 2003). It is at this early developmental stage that ~15% of all IVP bovine embryos are permanently arrested in a senescence-like state (Fig. 1). Our preliminary results have revealed that roughly 10% of all human embryos produced by IVF or intracytoplasmic sperm injection permanently arrest at the early cleavage stages in culture and that 40% of patients exhibit at least one arrested embryo per treatment cycle indicating that this is a common phenomenon in humans as well (D. H. Betts, unpublished results). In this review we discuss what permanent early embryo arrest is, the role of reactive oxygen species (ROS) and the possible molecular mechanism(s) involved in mediating this event, and speculate on the causative role of telomere integrity in this context.

Permanent embryo arrest is a non-apoptotic event

Programmed cell death, or apoptosis, is a feature of both IVP and *in vivo*-derived preimplantation/preattachment stage embryos (Hardy, 1997; Matwee *et al*., 2000; Kamjoo *et al*., 2002). Apoptosis, a mechanism to rid the early embryo of unwanted or damaged cells, if extensive, could also lead to developmental failure. In spite of this effective program, the ability of an early embryo to undergo apoptosis appears to be developmentally regulated (Betts and King, 2001;

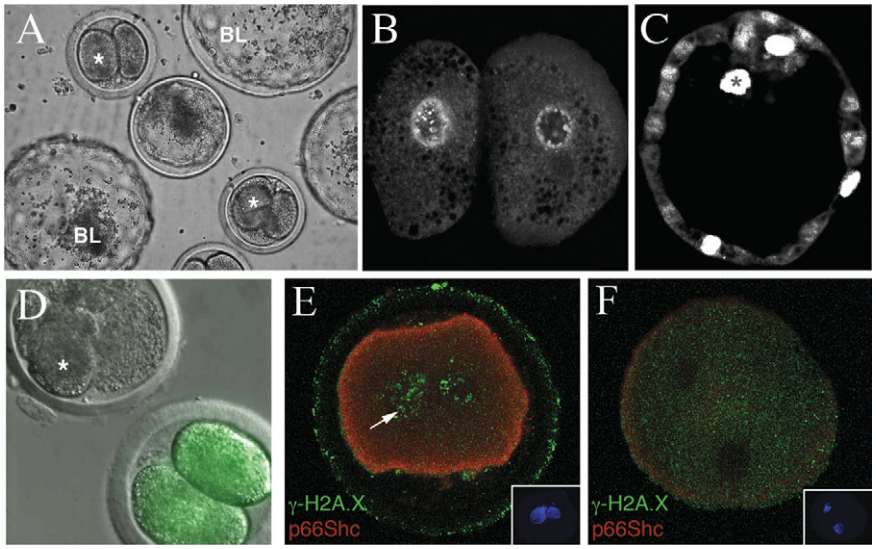

Figure 1: Permanently arrested 2- to 4-cell embryos are metabolically active, non-apoptotic and display high levels of p66Shc and phosphorylated histone γ-H2A.X foci.
(**A**) On Day 8 of *in vitro* bovine embryo culture, blastocyst (BL) development is typical but there also exists arrested (*) 2- to 4-cell embryos displaying no morphological signs of apoptosis. (**B** and **C**) Immunofluorescent detection of fragmented DNA in bovine 2-cell embryos and BLs by TUNEL™ assay. No DNA fragmentation (a hallmark of apoptosis) was ever observed in early 2- to 4-cell cleavage stage embryos (B) but is a normal occurrence (*) in later stage embryos (i.e. 8- to 16-cells, morulae and BLs) (C). (**D**) Two- to four-cell arrested embryos (even on the 6–8 days of culture) stain positive for Calcein (green fluorescence) indicating that, like their senescent somatic cell counterparts, arrested embryos are still metabolically active whereas fragmenting embryos are not (*). (**E** and **F**) The association of elevated p66Shc (red staining) with phosphorylated histone γ-H2A.X foci (green foci, arrow) is apparent in arrested 2-cell embryos (E) compared with low p66Shc and no nuclear γ-H2A.X staining in proliferating 2-cell embryos (F). Green and red colors in each representative photomicrograph indicate positive staining for phosphorylated γ-H2A.X (Alexa Flour™ 488) and p66Shc (Alexa Flour™ 596), respectively. Corresponding nuclei (insets) of each embryo are stained with DAPI (blue).

Hardy *et al*., 2001). No morphological, biochemical or molecular indication of apoptosis has been observed before the 8-cell stage of bovine and human embryos (Byrne *et al*., 1999; Hardy, 1999; Matwee *et al*., 2000; Gjorret *et al*., 2003). Conversely, exposure of 2- to 4-cell embryos to agents that either depolarize mitochondria (carbonyl cyanide *m*-chlorophenylhydrazone, CCCP) or inhibit protein kinases (staurosporine) can partially activate the apoptotic pathway, with embryos exhibiting some caspase activation and limited DNA fragmentation (Matwee *et al*., 2000; Brad *et al*., 2007; Gjorret *et al*., 2007). These observations suggest that all the components of the apoptotic machinery are at hand, but that 2- to 4-cell embryos cannot normally elicit this process because they either have immature (undifferentiated) mitochondria (Plante and King, 1994; Van Blerkom, 2004) and/or that they have to overcome some inhibition of the cell death pathway by the way of non-ascertained factor(s) (Weil *et al*., 1996; Brad *et al*., 2007). Interestingly, embryos derived from somatic cell nuclear transfer (SCNT) display some characteristics of apoptosis at the 2- to 4-cell cleavage stage (Gjorret *et al*., 2005), probably because nuclear reprogramming of the differentiated somatic cell genome (which does has the ability to trigger apoptosis) occurs gradually over the first 2- or 3-cell cycles (King *et al*., 1996). It would appear, therefore, that a unique mode of embryo demise is in operation at the 2- to 4-cell cleavage stage where they enter a permanent cell cycle arrest state (Favetta *et al*., 2004a,b), are still metabolically active (D. H. Betts, unpublished data; Fig. 1D) and exhibit high levels of intracellular ROS (Favetta *et al*., 2007b). These characteristics of permanent embryo arrest are reminiscent of the well-known and delineated phenomenon-cellular senescence.

Permanent embryo arrest = cellular senescence?

Replicative senescence is an *in vitro* event, described first by Leonard Hayflick who proposed that most proliferating somatic cell types permanently stop dividing after a limited number of population doublings (Hayflick and Moorhead, 1961). Senescent cells display distinct morphological and gene expression profiles of 'aged' cells that accumulate *in vivo* in tissues with increasing age (Herbig *et al*., 2006). *In vivo* senescence may contribute to organismal aging as a consequence of its proposed role as a potent tumor suppressor mechanism (Di Micco *et al*., 2007). Cellular senescence is thought to be a DNA damage-response to oncogene-induced DNA replication stress and/or to telomere disruption, both converging on the p53 DNA damage signaling pathway (Bartek *et al*., 2007). We have previously shown that permanent replication arrest of cultured bovine somatic cells is associated with telomere shortening (Betts *et al*., 2008), increased levels of serine 20-p53 phosphorylation, and elevated levels of oxidative damage (Favetta *et al*., 2004a). Since cellular senescence can occur prematurely under conditions of elevated oxidative stress (Toussaint *et al*., 2000; von Zglinicki, 2000) and can be activated by disruption of the telomere structure itself, even at maximally long telomere lengths (Li *et al*., 2003; Stewart *et al*., 2003), we hypothesize that arrested mammalian embryos permanently stop dividing at the 2- to 4-cell stage of early development by a similar stress signaling pathway.

Permanent embryo arrest is dependent on the time of first cleavage, with ~15% of embryos arrested at the 2- to 4-cell stage if their first cleavage event was at 32 hpi, while only ~1% of embryos were arrested among those cleaved at 26–28 hpi (Favetta *et al*., 2004b). Embryos cultured in 20% oxygen conditions display significantly elevated levels of intracellular ROS and higher frequencies of permanent embryo arrest compared with the embryos produced under 5% oxygen atmospheres (Favetta *et al*., 2007b). Unlike senescent fibroblasts (Favetta *et al*., 2004a), no significant differences in mRNA and protein levels of the tumor suppressor p53 are observed in both arrested and late cleaving embryos compared with their early cleaving counterparts (Favetta *et al*., 2004b, 2007a,b). However, like senescent fibroblasts (Favetta

et al., 2004a), significantly higher levels of the oxidative stress adaptor protein p66Shc have been detected in arrested embryos (Favetta *et al*., 2004b, 2007a,b). These results further substantiate the view that permanent embryo arrest may be mediated by the production of intracellular ROS.

The involvement of oxygen tension and ROS in permanent embryo arrest

ROS are implicated in the induction of apoptosis (Stone and Yang, 2006) and cellular senescence (Passos *et al*., 2007). Specificity could be determined by the intensity of the pro-oxidant challenge since the treatment of different primary cells with increasing doses of exogenous hydrogen peroxide (H_2O_2) induces proliferation, senescence or apoptosis, respectively (Chen *et al*., 2000). Somatic cell proliferation is inhibited by high-oxygen culture environments (Balin *et al*., 2002) as well as by treatment with H_2O_2 (Chen and Ames, 1994). Conversely, low-oxygen conditions and antioxidant treatments prolong the proliferative lifespan of cell cultures (Packer and Fuehr, 1977; Poot, 1991).

Among the various culture conditions and exogenous factors that lead to elevated production of ROS in embryos, including the traces of metallic cations, visible light and amino oxidases, the *in vitro* oxygen tension is the most studied and the easiest to control (Guerin *et al*., 2001). Earlier studies have demonstrated the detrimental effects of culturing embryos of various species under atmospheric (~20%) oxygen concentrations (Pabon *et al*., 1989; Nagao *et al*., 1994; Dumoulin *et al*., 1999) and the beneficial effects of reducing the oxygen tension (Dumoulin *et al*., 1999; Hashimoto *et al*., 2000; Orsi and Leese, 2001) and co-culturing with somatic cells to provide an oxidant 'buffering' system (Xu *et al*., 1992; Nagao *et al*., 1994). Reducing the oxygen concentration of embryo culture from 20 to 5% enhances embryo development to the blastocyst stage and increases their total cell counts (Batt *et al*., 1991; Nagao *et al*., 1994; Gardner and Lane, 1996), probably by reducing the proportion of cells undergoing apoptosis (Van Soom *et al*., 2002). Enhanced embryo development under lower oxygen conditions is thought to be attributable to improved embryo metabolism (Khurana and Wales, 1989; Du and Wales, 1993) and decreased ROS production leading to reduced oxidative stress (Guerin *et al*., 2001).

A direct relationship between increased H_2O_2 production and elevated embryo fragmentation has also been documented, suggesting that ROS may induce apoptosis in embryos (Yang *et al*., 1998). In addition, it has been noted that embryos cultured in 20% oxygen conditions sustain a 10-fold increase in intracellular H_2O_2 levels and a 2-fold increase in the frequency of permanent embryo arrest at the 2- to 4-cell stage, compared with embryos cultured in 5% oxygen tensions (Favetta *et al*., 2007b).

More recently, we have observed a dose-dependent increase in permanent embryo arrest after exposure of 2- to 4-cell embryos to H_2O_2 and that this oxidant-induced embryo arrest can be abrogated by exposure to an antioxidant, PEG-Catalase (D. H. Betts, unpublished results). Although oxidative stress appears to signal a permanent senescence-like state in 2- to 4-cell bovine embryos, exposure of oocytes and late-cleavage stage embryos (8- to 16-cells, morulae and blastocysts) to exogenous H_2O_2 imposes a dose-dependent increase in apoptosis (D. H. Betts, unpublished results), suggesting that ROS-mediated events are developmentally regulated. Intracellular ROS levels are significantly more abundant in 2- to 4-cell arrested embryos (D. H. Betts, unpublished results) and in an embryo population that exhibit higher frequencies of permanent embryo arrest (Favetta *et al*., 2007b). Concentration of non-toxic ROS, via alterations in the reduction-oxidation (redox) state, is considered to be an important mechanism that regulates cellular functions including energy production and proliferation in early embryos by way of redox-sensitive transcription factors (Harvey *et al*., 2002). Interestingly, oxygen tension has recently been shown to alter gene expression in blastocysts (Harvey *et al*., 2004, 2007) possibly through epigenetic mechanisms (Islam and Mendelson, 2006). It is intriguing to speculate that permanent embryo arrest may be activated directly by ROS/oxygen-mediated gene expression. It is well established that the elevated ROS levels generate damage to cells/embryos through increased lipid peroxidation (Nasr-Esfahani *et al*., 1990; Nasr-Esfahani and Johnson, 1992), and protein oxidation, and induce DNA strand breaks (Guerin *et al*., 2001; Orsi and Leese, 2001), including telomeric DNA (Petersen *et al*., 1998). All of these are also observed features of senescent somatic cells.

Does telomere dysfunction play a role in embryo arrest?

The signaling of permanent cell cycle arrest has long been attributed to the critical shortening of telomere(s), the repetitive DNA sequences $(TTAGGG)^n$ and associated proteins located at the ends of mammalian chromosomes (Harley *et al*., 1990, 1992; Allsopp *et al*., 1992, 1995; Vaziri, 1997; Vaziri and Benchimol, 1998). Telomere shortening can be overcome by the *de novo* synthesis of telomeric DNA by telomerase (Greider and Blackburn, 1985), a multi-subunit reverse transcriptase that uses its RNA component (TERC) to align itself to the chromosomal ends and as a template for the synthesis of telomeric sequences (Collins and Greider, 1995; Collins *et al*., 1995). High levels of telomerase activity have been detected in germ line tissues, cells of renewal tissues, cancer cells and immortalized cell lines but not in most somatic tissues (Kim *et al*., 1994; Harle-Bachor and Boukamp, 1996; Wright *et al*., 1996; Betts and King, 1999). Ectopic expression of the telomerase catalytic subunit (*TERT*) extends replicative lifespan while preserving long telomere lengths and normal karyotypes in diploid somatic cells (Bodnar *et al*., 1998; Thomas *et al*., 2000). Conversely, late generation mice lacking the telomerase RNA (mTERC−/−) component display shortened telomeres, chromosome abnormalities and exhibit infertility, increased apoptosis and a decreased cell proliferation in the testis, bone marrow and spleen (Blasco *et al*., 1997; Lee *et al*., 1998). Together, these results demonstrate that telomeres provide chromosomal stability and regulate the proliferative capacity of cells *in vivo* and *in vitro*.

We have previously shown that the permanent cell growth arrest of cultured bovine somatic cells is associated with telomere shortening (Betts *et al*., 2008), increased levels of serine 20-p53 phosphorylation, and elevated levels of oxidative damage (Favetta *et al*., 2004a). Cellular senescence occurs prematurely under the conditions of elevated oxidative stress (von Zglinicki, 2000, 2002) and can be activated by disruption of the telomere structure itself, even at maximally long telomere lengths (Li *et al*., 2003; Stewart *et al*., 2003). Mild hyperoxia and even normoxic (20% oxygen) culture environments inhibit the proliferation of human fibroblasts and increase telomere shortening/damage by causing single-stranded breaks specifically within telomeric DNA (von Zglinicki *et al*., 1995; Petersen *et al*., 1998; Sitte *et al*., 1998). ROS damage to the telomeres accumulates due to cellular deficiencies in the repair of such damage (Petersen *et al*., 1998). Single-stranded telomeric DNA contributes significantly to telomere shortening (Makarov *et al*., 1997; Petersen *et al*., 1998), but also acts as a trigger of p53-dependent cell cycle arrest and cell death (von Zglinicki, 1998; Saretzki *et al*., 1999). Telomeres therefore act as sentinels that signal cell cycle arrests by the accumulation of single-stranded telomeric DNA above a certain threshold in length and/or amount that abrogates a functional telomere structure. Indeed,

experimental disruption of the telomerase catalytic subunit (hTERT) in proliferating normal diploid somatic cells disrupts telomere structure (telomere uncapping) and subsequently activates an early senescence phenotype (Masutomi *et al*., 2003). ROS-mediated disruption of the telomere structure may explain the high rates of developmental arrest of IVP embryos and the stochastic variation in division capacity within somatic cells cultures (Sozou and Kirkwood, 2001). Conversely, under high-oxidative stress conditions, *TERT* is reversibly excluded from the nucleus, where it co-localizes with mitochondria, possibly providing protection through improved mitochondria metabolism and reduced ROS-generation (Ahmed *et al*., 2008). Interestingly, we have localized *TERT* in proliferating 2-cell bovine embryos as large punctate foci reminiscent of the mitochondria staining patterns observed in early cleavage-stage embryos (D. H. Betts, unpublished results). Also, our preliminary results from pharmacologically treating bovine embryos with telomerase inhibitors have shown an increased incidence of permanent embryo arrest (D. H. Betts, unpublished results). There is now supporting evidence that telomere-dysfunction induced senescence/apoptosis is triggered by the production of mitochondrial ROS (Liu *et al*., 2002a,b,c; Liu *et al*., 2003; Passos *et al*., 2007). Co-localization of phosphorylated histone γ-H2A.X fluorescence, a marker of DNA damage, to the telomeres indicates telomere dysfunction-induced foci (Herbig *et al*., 2004, 2006). We have observed γ-H2A.X foci in the arrested 2-cell embryos but no staining in their proliferating counterparts (D. H. Betts, unpublished results; Fig. 1E and F). Telomere and/or mitochondrial dysfunction could explain the 'pre-mature' arrest states that arise *in vivo* or *in vitro* after exposure to oxidative stresses for cells and embryos that possess relatively long telomere lengths (Betts and King, 2001; Favetta *et al*., 2004a,b; Kurz *et al*., 2004).

The role of the stress adaptor protein p66Shc in embryo arrest

At the molecular level, the response of somatic cells to oxidative stress appears to involve the tumor suppressor protein p53, which recognizes free radical-induced DNA damage (Migliaccio *et al*., 1999; Sharpless and DePinho, 2002). p53 leads cells to either die through apoptosis, or to arrest in response to a variety of cellular stresses, such as DNA damage, hypoxia, oxidative stress, excessive mitogenic stimuli or denuded telomeres (Donehower, 2002; Sharpless and DePinho, 2002). At low concentrations, ROS can modulate p53, but are also suggested to be downstream mediators of p53 in p53-dependent apoptosis (Johnson *et al*., 1996). Although we have detected significantly elevated levels of p53 in senescent bovine fibroblasts (Favetta *et al*., 2004a), our studies have also shown that p53 might not play a significant role during early embryo development (Matwee *et al*., 2000; Favetta *et al*., 2004b). This was further confirmed by more recent studies (Velez-Pardo *et al*., 2007). It would be interesting to examine the isoforms of p53 (p63 and p73) as possible inducers of early embryo arrest.

A stress sensor that can link intracellular ROS levels with permanent arrest of replication in cultured cells and embryos is p66Shc, a newly identified protein belonging to the Shc family of adaptors for signal transduction in mitogenic and apoptotic-responses (Pinton and Rizzuto, 2008). p66Shc is a splice variant of p52Shc/p46Shc, a cytoplasmic signal transducer Shc family involved in mitigating proliferation signals from activated receptors to Ras (Pelicci *et al*., 1992). Deletion of p66Shc in mice results in approximately a 30% increase in lifespan because of a greater resistance to oxidative stress and reduction in p53-mediated apoptosis (Migliaccio *et al*., 1999). The observations that p66Shc is required for early mitochondrial responses to oxidative challenge including mitochondrial fragmentation and suppression of Ca^{2+} signal propagation (Pinton *et al*., 2007) and that forkhead/FOXO activity is regulated by intracellular ROS in a p66Shc-dependent, mitochondrial and extra-mitochondrial manner (Fig. 2) suggest that intracellular H_2O_2/ROS might also provide specific signaling functions in cellular senescence (Nemoto and Finkel, 2002). Upon serine-36 phosphorylation, p66Shc is translocated into the mitochondrial intermembrane space where it interacts with reduced cytochrome *c* to produce H_2O_2 and to open the permeability transition pores which allow the generation and release of ROS into the cytosol (Orsini *et al*., 2004; Giorgio *et al*., 2005; Pinton *et al*., 2007). This p66Shc-mediated intracellular ROS production may facilitate permanent replication arrest in cells/embryos at modest ROS levels and induce apoptosis at high ROS doses (Fig. 2). The activation of permeability pores in a subpopulation of mitochondria may provide the means for the embryo to regulate mitochondrial metabolism and/or remove impaired mitochondria by triggering their autophagic degradation (Elmore *et al*., 2001; Hajnoczky and Hoek, 2007). Morphologically good-quality embryos have mitochondria localized at the nuclear periphery; however, they are also distributed at the outside edges of cytoplasm in granular and clumped aggregates (Neganova *et al*., 2000; Wilding *et al*., 2001). In contrast, in slow developing or blocked embryos, the distribution pattern is more uniform with dense mitochondria accumulated around the nuclei while the marginalized cytoplasmic clusters of mitochondria and mitochondrial activity remain strikingly reduced (Neganova *et al*., 2000; Wilding *et al*., 2001). These observations point to a possible connection between cytoskeletal organization and the activity of motor proteins involved in mitochondrial transport (Neganova *et al*., 2000). The mitochondrial aggregation patterns closely parallel the p66Shc distribution pattern observed in proliferating cells and arrested 2- to 4-cell embryos (Favetta *et al*., 2004b, 2007a,b). It would be interesting to perform co-localization studies of activated p66Shc with MitoTracker® staining of mitochondria in embryos to correlate p66Shc staining intensities with *trans*-membrane potentials. It would be equally interesting to test whether or not the serine–threonine kinase mTOR is present in arrested embryos since it inhibits autophagy (Meijer and Codogno, 2006) and augments mitochondrial metabolism and ROS generation (Nemoto *et al*., 2006). This mTOR-regulation of mitochondrial autophagy/metabolism may also be under the control of the extra-mitochondrial p66Shc pathway (Fig. 2). This line of events has been linked to the activation of Akt, which leads to the phosphorylation of Forkhead (FOXO) transcription factors (Nemoto and Finkel, 2002). Subsequent reduction in forkhead-dependent transcriptional activity (Kops *et al*., 1999; Takaishi *et al*., 1999; Nemoto and Finkel, 2002) provides for cell survival and resistance to apoptosis (Kops *et al*., 2002; Nemoto and Finkel, 2002), which are requirements for permanent cell cycle arrest.

Although low levels of p66Shc are detected in post-mitotic cells (Conti *et al*., 1997, 2001), we have demonstrated significantly elevated levels of p66Shc mRNA and protein in senescent bovine fibroblasts and permanently arrested embryos in the absence of apoptosis (Favetta *et al*., 2004a,b). These elevated levels of p66Shc are associated with more extensive oxidative damage and the production of intracellular ROS (Favetta *et al*., 2004a,b, 2007a,b). It is interesting that early cleavage-stage embryos, which are more or less transcriptionally quiescent, have rising p66Shc mRNA levels as the duration of arrest increases (Favetta *et al*., 2004b). The high levels of p66Shc in arrested embryos may be a result of faulty degradation of maternal mRNAs. A recent report from Richard Schultz's group has revealed through global gene expression profiling that many maternal transcripts are not properly degraded during maturation of aged (low quality) oocytes (Pan *et al*., 2008). Gene products such as p66Shc may be present at sufficient quantities in low-quality cleavage-stage

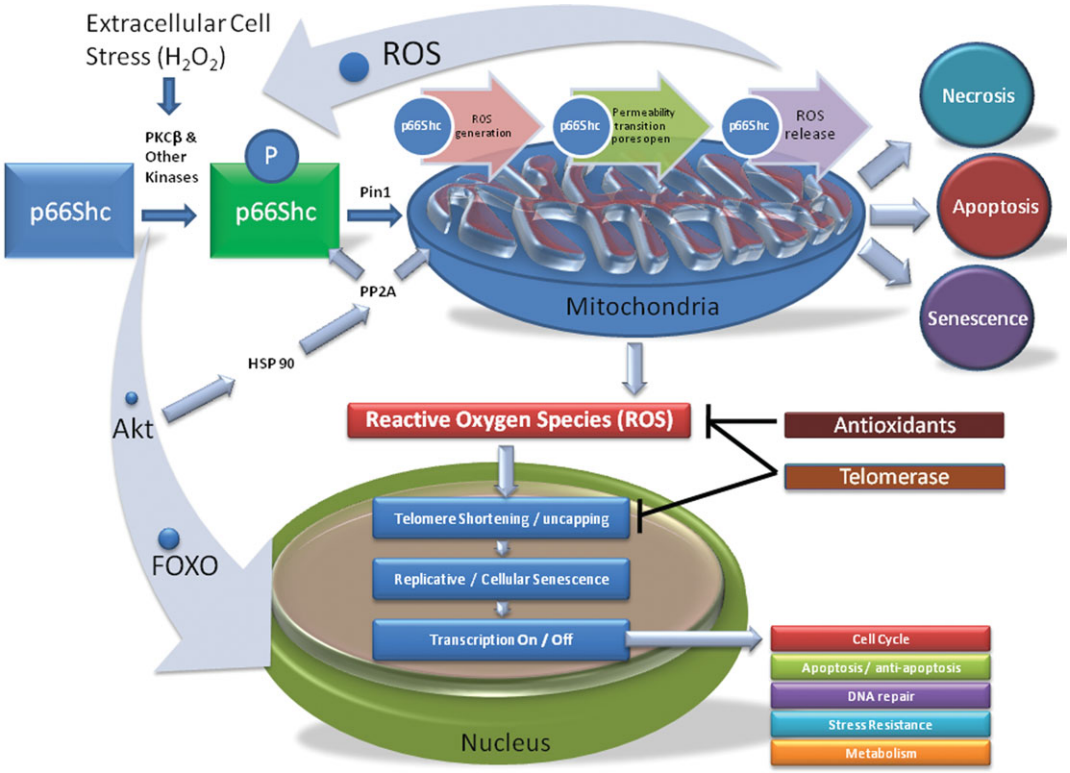

Figure 2: p66Shc is proposed to regulate a ROS-mediated, telomere dysfunction pathway that signals permanent embryo arrest.
Extracellular stressors such as H_2O_2 or intracellular mitochondrial ROS production can activate various kinases that subsequently activate p66Shc (serine-36 phosphorylation) leading to its mitochondrial translocation and p66Shc-mediated ROS production and release from the mitochondria that can be partially detoxified by antioxidants. Oxidative stress can also activate the p66Shc-Akt-FOXO pathway, which leads to the activation/inactivation of the forkhead family (FOXO) of transcription factors by post-translational modifications. The effects of acetylation and deacetylation of FOXO appear to be promoter specific, altering (up- or down-regulation) the expression of various genes that will promote permanent cell cycle arrest. Although high levels of intracellular ROS can lead to necrosis or apoptosis, moderate levels of ROS can accelerate telomere shortening and/or cause telomere-uncapping leading to a DNA damage response that activates permanent cell cycle arrest. This cyclic pattern of ROS-mediated activation of p66Shc leads towards continual intracellular ROS production and mitochondrial dysfunction, allowing for a cellular environment favoring mitochondria autophagy or senescence-activation (anti-apoptosis) via a retrograde response and/or other Ca^{2+}-dependent signaling pathways (not shown). HSP90, Heat shock protein 90; PKCβ, protein kinase C β; PP2A, protein phosphatase 2A; Pin 1, peptidyl-prolyl *cis/trans* isomerase; AKt, protein kinase β.

embryos prior to embryonic genome activation to elicit various cellular processes including permanent cell cycle arrest. The detection of increased levels of the cyclin-dependent kinase inhibitor p27 in arrested cleavage-stage human embryos (Civico *et al*., 2002) further supports this assumption.

Likewise, increasing levels of p66Shc have been detected in aging human diploid fibroblasts and exposure to oxidative stress has been shown to induce greater levels of p66Shc in cells from aged individuals relative to their younger counterparts (Pandolfi *et al*., 2005). Elevated quantities of the cell cycle regulator $p21^{waf1/cip1}$ have been detected upon p66Shc activation in epidermal growth factor-stimulated A431 cells (Sato *et al*., 2002). Oxidant or antioxidant treatment of embryos at different embryonic stages modulates the occurrence of permanent embryo arrest or apoptosis depending on the stage of development at which the embryos were treated (D. H. Betts, unpublished data). These observations suggest that ROS-induced embryo arrest is developmentally regulated. Furthermore, our recent observation that RNA interference knockdown of p66Shc in bovine embryos significantly diminishes the occurrence of permanent embryo arrest (Favetta *et al*., 2007a) supports the hypothesis that p66Shc regulates the senescence-signaling pathway in cells and embryos. Moderate production of ROS/H_2O_2 (oxidative stress) by mitochondrial p66Shc may mediate global changes in gene expression/activation and the induction of oxidative telomeric damage resulting in permanent arrest of growth in cells and blastomeres (Fig. 2).

Why embryo arrest?

The question that still comes up is: why do early embryos, equipped with such a potent apoptotic mechanism to rid itself of damaged or unwanted cells, have this alternative permanent-arrest state? The early cleavage divisions are under maternal control (Braude *et al*., 1988), using transcripts accumulated during oogenesis. Depending on the level of specific maternal stores and the typically low gene expression profiles of most genes, early cleavage embryos, which are sensitive to their micro-environment, including suboptimal culture conditions, may signal permanent embryo arrest at this stage of development as a means to prevent further development of low-quality (abnormal/damaged) embryos (Betts and King, 2001). Embryo arrest may be another mechanism to prevent further development of certain chromosomally abnormal embryos (Almeida and Bolton, 1998), and/or embryos that fail to activate embryonic genomes (Artley *et al*., 1992). This is especially relevant since early cleavage embryos, which display a high proportion of undifferentiated mitochondria (Sathananthan and Trounson, 2000), are not subject to the scrutiny of cell cycle checkpoints (Hartwell and Weinert, 1989). Alternatively, p66Shc-mediated embryo arrest may represent a failed attempt to regulate mitochondrial *trans*-membrane potential to compensate for metabolic over-reactivity resulting in the overproduction of ROS.

Germ cells show high levels of telomerase (Betts and King, 1999) and it has long been held that the telomere length is reset in gametes

(Kozik *et al*., 1998; Baird *et al*., 2006) to ensure that each generation begins life with its complement of telomeres intact. Surprisingly, recent studies have shown that there is considerable telomere lengthening in early post-fertilization stages of embryo development (Schaetzlein *et al*., 2004; Liu *et al*., 2007). Telomeres of mouse oocytes are shorter compared with their somatic cells counterparts; but, they are predominantly elongated in the early cleavage stages presumably by telomere sister-chromatid exchanges (Liu *et al*., 2007). Permanent embryo arrest could be the result of a checkpoint mechanism that evaluates the ability of an embryo to establish the correct telomere length and structure (telomere integrity) at the outset of development since proper telomere length and structure have long-lasting implications for health and reproduction (Liu *et al*., 2002a,b,c; Epel *et al*., 2004, 2006; Aydos *et al*., 2005; Keefe *et al*., 2005, 2006).

Future perspectives

Entry into a permanently arrested state is likely determined by an embryos' ability to protect and lengthen its telomeres (*TERT* and other telomere-associated proteins), its potential to combat ROS (antioxidants) and its capacity to regulate the p66Shc pathway. Oxidative stress activation of p66Shc, which induces H_2O_2/ROS generation and mitochondrial dysfunction, may be a key player in the positive feedback signaling pathway required for permanent cell cycle arrest. Other investigators have suggested that p66Shc polymorphism may be linked to longevity (Ventura *et al*., 2002; Mooijaart *et al*., 2004) and may even be associated with fertility in humans. P66Shc appears to regulate mitochondrial metabolism in a way that modulates the quantity of ROS released into the cytosol. Depending on when p66Shc is activated during preimplantation development and on the amount of ROS generated, the oxidative stress-induced telomere uncapping may lead to permanent embryo arrest or apoptosis. Telomere length has been recently used to predict developmental competence of human embryos (Keefe *et al*., 2005). Therefore, genetic or pharmacological modification of the p66Shc pathway by promoting stress resistance could have direct implications for various age-related diseases and infertility in humans (Napoli *et al*., 2003; Francia *et al*., 2004; Graiani *et al*., 2005; Berry *et al*., 2006; Tothova *et al*., 2007). The observation that ~50% of human embryos arrested at the 2- to 4-cell stage display a normal karyotype (Almeida and Bolton, 1998) indicates that technical means to combat permanent cell cycle arrest could alleviate some cases of age-related infertility and that these embryos, originally deemed to be non-viable biological waste, could serve as a less 'controversial' source for embryonic stem cells for use in regenerative medicine or in the very least additional means to study human embryonic stem cell biology.

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