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The induction of tumour suppressor protein P53 limits the entry of cells into the pluripotent inner cell mass lineage in the mouse embryo

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ABSTRACT

The early preimplantation embryo is susceptible to a range of exogenous stresses which result in their reduced long-term developmental potential. The P53 tumour suppressor protein is normally held at low levels in the preimplantation embryo and we show that culture stress induces the expression of a range of canonical P53-response genes (*Mdm2, Bax* and *Cdkn1a*). Culture stress caused a *P53*-dependent loss of cells from resulting blastocysts, and this was most evident within the inner cell mass population. Culture stress increased the proportion of cells expressing active caspase-3 and undergoing apoptosis, while inhibition of caspase-3 increased the number of cells within the inner cell mass. The *P53*-dependent loss of cells from the inner cell mass was accompanied by a loss of NANOG-positive epiblast progenitors. Pharmacological activation of P53 by the MDM2 inhibitor, Nutlin-3, also caused increased P53-dependent transcription and the loss of cells from the inner cell mass. This loss of cells could be ameliorated by simultaneous treatment with the P53 inhibitor, Pifithrin-α. Culture stress causes reduced signalling via the phosphatidylinositol-3-kinase signalling pathway, and blocking this pathway caused P53-dependent loss of cells from the inner cell mass. These results point to P53 acting to limit the accumulation and survival of cells within the pluripotent lineage of the blastocyst and provide a molecular framework for the further investigation of the factors determining the effects of stressors on the embryo's developmental potential.

1. Introduction

The preimplantation embryo forms by a series of reductive mitoses initiated by fertilisation. The cells generated by these mitoses have an indeterminate fate until the formation of the morula stage when some cells take interior positions and others form an outer layer. Although there is plasticity in the relationship [1], the interior cells are destined to differentiate into the pluripotent inner cell mass (ICM) stem cell population and the outer cells into the multipotent trophectoderm stem cells (TE). Following implantation the inner cell mass will form the embryo and embryonic membranes while the trophectoderm will form the embryonic component of the placenta.

Tumour related protein 53 (P53, also known as TRP53 or TP53 in humans) is a tumour suppressor protein that negatively regulates cell survival or cell-cycle progression [2]. The maintenance of P53 in a latent state is essential for the normal development of the early embryo [3–7]. The exposure of the preimplantation embryo to a range of exogenous genotoxic and non-genotoxic stressors breaches this latency and compromises the embryo's developmental potential [5,8,9]. Stress resulting from culture of embryos in a simple defined medium can cause the accumulation of P53 in such embryos [5,9]. The resulting embryos accumulated fewer cells and have a reduced capacity for further development [3–5]. Blastocysts resulting from culture throughout the preimplantation stage had the capacity to undergo implantation after embryo transfer, but there is an increased incidence of early resorption [5]. An in vitro model of implantation (blastocyst outgrowth) showed that preimplantation embryo development in simple media caused a *P53*-dependent failure of normal epiblast development [3]. These studies indicate that the accumulation of P53 caused by culture stress compromises the normal formation of the pluripotent cell lineage in embryos although the mechanisms for this are not yet described.

Embryo culture plays a central role in all forms of assisted reproductive technology and it is therefore important to understand the effects this has on normal embryo function. An understanding of the impacts of this form of embryo stress may also provide a framework for a broader understanding of the factors that determine embryo viability. A detailed molecular description of the causes of the reduced developmental potential that commonly results from the culture of embryos is awaited. Here we show that one effector is the enhanced

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Table 1
Primer sequence for each gene analyzed.

Transcript	Forward	Reverse	Conc. (nM)	Product (bp)
18s rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	50	187
Gapdh	TGACGTGCCGCCTGGAGAAA	AGTGTAGCCCAAGATGCCCTTCAG	250	98
β-Actin	CTAAGGCCAACCGTGAAAAG	GTACGACCAGAGGCATACAG	500	109
Cdkn1a	TCCACAGCGATATCCAGACA	GGACATCACCAGGATTGGAC	100	60
Bax	GTGAGCGGCTGCTTGTCT	GGTCCCGAAGTAGGAGAGGA	250	68
P53	ATGCCCATGCTACAGAGGAG	AGACTGGCCCTTCTTGGTCT	50	74
Mdm2	GAAGGAGCACAGGAAAATATATGCA	GTCTGCTCTCACTCAGCGATGT	100	92

expression of P53-response genes that lead to BAX-mediated apoptosis of cells within the blastocyst. This preferentially affects the accumulation of cells within the ICM, with a resulting diminution of cells destined to the epiblast lineage.

2. Materials and methods

2.1. Animals

The use of animals was in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the institutional animal care and ethics committee. Mice were *P53*$^{+/+}$ and *P53*$^{-/-}$ (B6.129S2-P53^{tm1Tyj} strain was extensively backcrossed with C57BL/6j and maintained in the heterozygous state). The progeny were genotyped at time of weaning before assignment to experiments [10]. In some experiments hybrid (C57BL/6j (B6) X CBA/He and CBA/He X C57BL/6; B6CBF1) mice were used. Five to six week old female mice were superovulated by intraperitoneal injection of 5 IU of equine chorionic gonadotropin (Folligon; Intervet International, Boxmeer, The Netherlands) followed 48 h later by 5 IU of human chorionic gonadotropin (hCG, Chorulon; Intervet). Females were paired with males of proven fertility. *P53*$^{+/+}$ *or P53*$^{-/-}$ embryos were produced by mating males and females of the same genotype. Pregnancy was confirmed by the presence of a copulation plug.

2.2. Embryo collection, culture and treatments

Embryos were collected from the reproductive tracts by flushing with HEPES-HTF [11] medium and cultured in GE-HTF [12]. Zygotes (20–21 h post-hCG) were freed from their surrounding cumulus cells by brief exposure to 300 U of hyaluronidase (Sigma Chemical Co, St. Louis, MO, USA) in HEPES-HTF. They were then assigned to various treatments as required in GE-HTF and cultured as groups of 10 in 10 μl volumes [13] at 37 °C in 5% CO_2 in air for 96 h to the blastocyst stage. GE-HTF medium is a minimal essential medium for the early embryo rather than an optimized growth medium. It is used to allow dissection of the essential growth requirements of the early embryo [12,14]. Embryos were grown in groups because the release and action of autocrine tropic ligands is essential for normal embryo development [14,15]. Differential staining of ICM/TE within blastocysts was achieved by incubating for 10 s in bovine serum albumin (BSA)-free Hepes-modHTF containing 100 μg/ml propidium iodide (PI; Sigma) and 1% Triton X-100, followed by 1 h in 100% ethanol with 25 μg/ml bisbenzimide at 4 °C. The outer TE cells stain with both PI and bisbenzimide, and inner cells stain only with bisbenzimide.

Nutlin-3 ((±)-4-[4,5-bis-(4-chlorophenyl)-2-(2-isopropoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl]-piperazin-2-one; Merck, Darmstadt, Germany), Pifithrin-α (2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-*p*-tolylethanone hydrobromide; Sigma), Z-DEVD-FMK (benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; BD Biosciences, Franklin Lakes, NJ, USA), Deguelin ((7aS,13aS)-13,13a-Dihydro-9,10-dimethoxy-3,3-dimethyl-3H-bis[1]benzopyrano[3,4-b:6′,5′-e]pyran-7(7aH)-one, Sigma) and LY294002 (2-(4-Morpholinyl)–8-phenyl-4H-1-benzopyran-4-one, Calbiochem) were added to media in the concentrations indicated in relevant experiments, to inhibit MDM2, P53, caspase 3, AKT and phosphatidylinositol-3-kinase, respectively. They were prepared as stock solutions in DMSO and then diluted in media to their working concentration where the final concentration of DMSO was below 0.05% (v/v). The control media contained the same concentration of DMSO.

2.3. Quantitative reverse transcriptase PCR

Blastocysts were washed in cold PBS (pH 7.4; Sigma) and transferred to lysis solution consisting of 1× PCR buffer II [final 1x concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 8.3)] and 1 U/μl RNase-inhibitor (Applied Biosystems)] and snap-frozen in liquid nitrogen. The samples were lysed by 3 rounds of thaw, vortex and freeze. DNase-treatment was performed at 37 °C for 15 min in 1x DNase I buffer supplemented with 1 U DNase I (Ambion), followed by 5 mM EDTA (Sigma) addition and incubation at 75 °C for 10 min. Primer annealing to RNA occurred in 1x PCR buffer II, 9 mM $MgCl_2$, 0.5 mM each dNTP (all from Applied Biosystems) and 2.5 μM random decamers (Roche). RNA was denatured at 65 °C for 10 min, supplemented with 1 U/μl of MuLV reverse transcriptase and 0.4 U/μl of RNase inhibitor (both from Applied Biosystems), and incubated at 25 °C for 10 min for primer annealing, and at 42 °C for 40 min for cDNA synthesis. The primer sequence for each target gene is shown in Table 1. PCR was performed in an AB7900HT Real Time PCR system (Applied Biosystems) in 10 μl reactions consisting of Power SYBR Green PCR master mix (Applied Biosystems), cDNA template, and gene-specific primers. A threshold was set where the amplification was close to the reaction's maximum rate and where negative controls were not significant. The relative quantity of the gene of interest in the cDNA sample was determined relative to the standard from ES cells (D3).

2.4. Immunofluorescence

Embryos were washed in Dulbeccos phosphate buffered saline (PBS, pH 7.4) containing 0.2% (w/v) sodium azide, 0.1% (w/v) BSA and 0.1% (v/v) tween-20 (washing solution) and then fixed in freshly prepared 2% (w/v) paraformaldehyde (Sigma) in PBS (pH 7.4) for 30 min at room temperature. They were permeabilized with 0.3% (v/v) tween-20, 0.2% (v/v) triton X-100 (Bio-Rad) and 2% (w/v) paraformaldehyde in PBS for a further 30 min; and blocked in PBS containing 2% (w/v) BSA and 30% (v/v) serum for 3 h. They were stained overnight at 4 °C with primary antibodies [1 μg anti-UTF1 polyclonal antibody/ml (Ab24273); 5 μg anti-active caspase 3 polyclonal antibody/ml (Ab13847)(both from Abcam, Cambridge, UK); 1 μg anti-NANOG polyclonal antibody/ml (Cosmo Bio, Tokyo, Japan); 2 μg anti-POU5F1 monoclonal antibody/ml (Santa Cruz, CA, USA)]; or the equivalent concentration of isotype control immunoglobulin (negative control) in PBS with 2% (w/v) BSA, followed by an incubation in FITC-conjugated secondary antibodies in PBS with 2% (w/v) BSA for 1 h at room temperature. The nuclei were counterstained with 1 μg propidium iodide (PI, Sigma)/ml in PBS. Whole section immunolocalization was performed with mercury lamp UV illumination. Optical sectioning was performed with a Bio-Rad Radiance confocal microscope.