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Mitochondrial ROS direct the differentiation of murine pluripotent P19 cells

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ABSTRACT

ROS are frequently associated with deleterious effects caused by oxidative stress. Despite the harmful effects of non-specific oxidation, ROS also function as signal transduction molecules that regulate various biological processes, including stem cell proliferation and differentiation. Here we show that mitochondrial ROS level determines cell fate during differentiation of the pluripotent stem cell line P19. As stem cells in general, P19 cells are characterized by a low respiration activity, accompanied by a low level of ROS formation. Nevertheless, we found that P19 cells contain fully assembled mitochondrial electron transport chain supercomplexes (respirasomes), suggesting that low respiration activity may serve as a protective mechanism against ROS. Upon elevated mitochondrial ROS formation, the proliferative potential of P19 cells is decreased due to longer S phase of the cell cycle. Our data show that besides being harmful, mitochondrial ROS production regulates the differentiation potential of P19 cells: elevated mitochondrial ROS level favours trophoblast differentiation, whereas preventing neuron differentiation. Therefore, our results suggest that mitochondrial ROS level serves as an important factor that directs differentiation towards certain cell types while preventing others.

Key resource table

Reagent or resource	Source	Identifier
Antibodies		
Anti-CK8	Abcam	EP1628Y
Anti-Oct4	Santa Cruz Biotech	sc-5279
Anti-β-III-Tubulin	Santa Cruz Biotech	sc-80005
Anti-β-Actin	Sigma-Aldrich	A5441
Anti-Sod2	Abcam	ab13533
Native WB Antibody Cocktail (1:250) that contains antibodies against CI-NDUFA9 (ab14713), CII-70 kDa subunit (ab14715), CIII-core protein 2 (ab14745), CIV-subunit IV (ab14744) and CV-a subunit (ab14748).	Abcam	ab110412
Mouse HRP Linked	GE Healthcare	NXA931
Rabbit HRP Linked	Agrisera	AS09602
Mouse/Rabbit Alexa Fluor 488 conjugated	Thermofisher Scientific	A-11029/A-11034
Experimental Models: Cell Lines		
P19 mouse embryonic carcinoma cell line	European Collection of Authenticated Cell Cultures	ECACC 95102107
Chemicals		
Rotenone	Sigma Aldrich	R8875
Potassium cyanide	Millipore	1.04965 EMD

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Antimycin	Sigma Aldrich	A8674
Myxothiazol	Sigma Aldrich	T5580
Paraquat	Sigma Aldrich	856177
RIPA buffer	Abcam	ab156034
NucBlue Fixed Cell ReadyProbes Reagent	Thermofisher Scientific	R37606
RNase Cocktail	Thermofisher Scientific	AM2286
Critical Commercial Assays		
ADP/ATP Ratio Assay Kit	Sigma Aldrich	MAK135
Mitochondria Isolation Kit for Cultured Cells	Abcam	ab110170
Rnase-free DNase set	Qiagen	79254
RNeasy mini kit	Qiagen	74104
DC protein assay kit	BioRad	500111
ECLPlus Detection Kit	GE Healthcare	RPN2133
A High Molecular Weight Calibration kit	GE Healthcare	17-0445-01
A High Molecular Weight Calibration kit Mouse 8x60K chip	Agilent Genomics	074809
Deposited Data		
Raw microarray data	This paper	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107414

1. Introduction

Reactive oxygen species (ROS), products of one-electron reduction of molecular oxygen, possess a high oxidative potential and can cause severe damage in living cells. A high ROS level is frequently associated with oxidative stress. ROS-dependent oxidation of lipids, DNA, and proteins leads to the accumulation of damaged molecules with impaired functions (Magder, 2006) eventually resulting in pathologies such as neurodegenerative diseases (Lin and Beal, 2006), cancer (Liou and Storz, 2010), and aging (Hekimi et al., 2011).

ROS generation in cells is tightly connected with the oxidative phosphorylation metabolic pathway (cellular respiration). During this process electrons flow through four mitochondrial electron transport chain (mETC) enzymatic complexes and two mobile carriers to the final acceptor – oxygen. This process releases energy that is used to form the proton gradient across the mitochondrial inner membrane to produce ATP (Mitchell and Yochim, 1968). mETC complexes are not freely floating in the inner membrane, but associated with each other forming supercomplex structures (Vartak et al., 2013), which provide the optimal conditions for electron flow (Gómez et al., 2009). However, electrons can leak from complex I or complex III, resulting in premature one-electron oxygen reduction and therefore ROS formation (Murphy, 2009). Thus, mETC is not only the most efficient energy production source but also one of the main sources of ROS (Adam-Vizi and Chinopoulos, 2006; Bleier and Dröse, 2013).

Many studies demonstrate that mitochondrial oxidative metabolism plays a critical role in stem cell maintenance and differentiation. Both mouse and human pluripotent stem cells are characterized by a relatively low mitochondrial mass, low respiration rate and high glycolytic activity (Xu et al., 2013). During differentiation, the mitochondrial oxidative metabolism is activated, resulting in increased cellular respiration and ROS production (Chen et al., 2008; Chung et al., 2007; Lonergan et al., 2006). Previous studies have indicated that this metabolic switch is not just an adaptation for new functions of differentiated cells, but mETC activity is an important prerequisite for successful differentiation (Rochard et al., 2000; Schieke et al., 2008; Wagatsuma and Sakuma, 2013; Zhang et al., 2013).

Despite the harmful effects caused by non-specific oxidation, ROS function as signal transduction molecules in various cell types, including stem cells (Holmström and Finkel, 2014). Some studies suggest that ROS signaling regulates stem cell proliferation and differentiation. For example, increased ROS levels due to deletion of AMT kinase (Ito et al., 2004) or FOXO transcriptional factors (Miyamoto et al., 2007; Tothova et al., 2007) impair hematopoietic stem cells proliferation. While these results indicate that increased ROS level impairs stem cell proliferation, a decreased ROS level also has negative effects. For example, neural stem cells (Le Belle et al., 2011), as well as spermatogonia stem cells (Morimoto et al., 2013) require physiological ROS level for self-renewal. Moreover, ROS production is critical for at least some types of differentiation. For instance, mitochondrial ROS production is necessary for adipocyte (Tormos et al., 2012) and muscle (Lee et al., 2011) differentiation.

Here we show that increased mitochondrial ROS production influences both proliferation and differentiation of pluripotent P19 cells. High mitochondrial ROS level reduces the cell proliferation rate due to the S phase elongation and also influences cell fate during differentiation: it favours trophoblast differentiation but prevents neuron differentiation. Our findings suggest that mitochondrial ROS level serves as an important factor that directs differentiation towards certain cell types, whereas preventing others.

2. Materials and methods

2.1. Cell culture and differentiation

Cells were cultured in DMEM medium supplied with 10% FBS at 37 °C, 5% CO_2. Cells were passaged every 48 h and plated at a density of $2*10^4$ cells/cm^2. The medium was changed every day. mETC inhibitors were added to the medium in the following concentrations: 0.1 μM rotenone, 0.25 μM antimycin A, 5 nM myxothiazol, and 25 μM potassium cyanide. Paraquat was added in a final concentration of 100 μM. To induce trophoblast differentiation, P19 cells were plated at a density $0.52*10^4$ cells/cm^2 and grown as a monolayer with 1 μM retinoic acid (RA) for 4 days. To induce neuron differentiation, $8.32*10^6$ cells were plated in 10 cm culture dish covered with 1% agarose. Cells grown in suspension with 1 μM RA formed embryoid bodies (EBs). The EBs were grown for 4 days, trypsinized to disaggregate cells, and grown as a cell monolayer at a density $3.6*10^4$ cells/cm^2 with 1 μM RA for 4 days.

2.2. Immunocytochemistry

Cells were fixed in 10% Neutral Buffered Formalin (NBF) solution for 30 min at room temperature (RT) and permeabilized for 10 min in 0.25% Triton X-100. Then samples were blocked in 10% normal goat serum for 1 h at RT. After blocking, samples were incubated with primary antibodies against CK8 (1:150), Oct4 (1:100), or β-III-tub (1:100)