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 (respirosomes), 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 | AS441 |
| Anti-Sod2           | Abcam  | ab13533    |
| Native WB Antibody Cocktail (1:250) that contains antibodies against CI-NDUF9 (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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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 (Ikeuchi 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., 2006; Schieke et al., 2008; Wagtsum 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 (Miymamoto 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% CO₂. Cells were passaged every 48 h and plated at a density of 2*10⁴ cells/cm². The medium was changed every day. mETC inhibitors were added to the medium in the following concentrations: 0.1 μM antimycin A, 0.25 μM 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*10⁴ cells/cm² and grown as a monolayer with 1 μM retinoic acid (RA) for 4 days. To induce neuron differentiation, 8.32*10⁶ 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*10⁶ cells/cm² 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).
in 1.5% normal goat serum overnight at 4 °C and then with secondary Alexa Fluor 488 conjugated antibodies (1:500) 30 min at RT. Nuclei were stained with 1 μg/ml Hoechst 33342 for 20 min. Cells were imaged with the fluorescent microscope (Axio Observer Z1, Zeiss). Each experiment was repeated at least three times.

To analyse the number of CK8+ in the differentiated cell population, 3×10^6 cells were fixed in 4% NBF for 10 min and permeabilized for 30 min with ice-cold methanol. Then samples were incubated with CK8 antibodies (1:100) in 0.5% BSA in PBS for 1 h at RT and with secondary antibodies (1:500) for 1 h at RT. Cell suspensions were used for flow cytometry analysis (CyFlow Class 1, Partec). CK8+ was gated using the Flow Max software (Partec) and the mean proportions of CK8+ cells in differentiated populations from three independent experiments were compared using the unpaired two-tailed t-test.

2.3. Oxygen consumption measurements

Oxygen consumption rate was measured polarographically using a Clark electrode (Strathkelvin Instruments) as previously described (Barrientos et al., 2009). The medium was refreshed 4 h before measurements. Cells were collected, counted and resuspended in respiration buffer (0.3 M mannitol, 10 mM KCl, 5 mM MgCl2, and 10 mM K2PO4, pH 7.4). The 782 Oxygen System software (Strathkelvin Instruments) was used to calculate oxygen consumption rates, which were normalized to the number of cells used for the analysis. Mean oxygen consumption rates from three independent experiments were compared using the unpaired two-tailed t-test.

2.4. MitoSox staining

Cells were plated and grown for 24 h. Then cells were loaded with 1 μM MitoSox for 15 min and afterwards grown for 12 h with mETC inhibitors or paraquat; the MitoSox fluorescence signal was analysed by flow cytometry (Flow Max CyFlow Class 1, Partec). The mean fluorescent intensity (MFI) of the treated cell population was normalized to MFI of untreated cells. Mean MFIs from four independent experiments were compared using the one sample t-test.

2.5. ATP/ADP level measurement

Trophoblast differentiated cells were incubated with mETC inhibitors for 24 h. After that, ATP and ADP concentrations were measured with ATP/ADP ratio bioluminescent assay kit according to the manufacturer’s manual. Mean ATP/ADP ratios from four independent experiments were compared using the unpaired two-tailed t-test.

2.6. Western blot analysis

Cells were lysed in RIPA buffer and then centrifuged at 11,000 ×g for 20 min at 4 °C. 50 μg of total protein lysate was loaded, separated and blotted. For immunostaining, membranes were blocked in 5% milk in TBS-T for 1 h at RT and then were incubated overnight at 4 °C with 5% milk in TBS-T containing primary antibodies CK8 (1:20000), β-III-tub (1:1000), β-act (1: 5000), Oct4 (1:1000), or Sod2 (1:5000) and afterwards secondary anti-mouse (1:5000) or anti-rabbit (1:10000) antibodies for 30 min at RT. The HRP signal was detected with the G:Box (Syngene). To quantify Sod2 level the signal was evaluated by the software GeneTools (Syngene) as an optical density (OD) using the rolling disk method for background subtraction and correction. The Sod2 signal was corrected to the β-act and quantified as a fold change of the OD in treated cells to the control. Each experiment was repeated at least three times.

2.7. BN-page

BN-PAGE was performed as previously described (Schaegger, 2001).

In brief, 200 μg of mitochondria isolated and lysed in buffer containing digitonin to a protein ratio 4:1 as detergent and loaded to 3–13% polyacrylamide gradient gels.

To visualise active CI, gels were equilibrated in buffer I (2 mM Tris/ HCl pH 7.4) for 1 h at RT and incubated in buffer I with 0.1 mg/ml NADH and 2.5 mg/ml nitro blue tetrazolium until purple bands appeared. For CIV in-gel staining, gels were equilibrated in buffer IV-1 (270 mM glycine/tris; 14 mM MgCl2; pH 8.4) for at least 2 h at RT and then revealed in buffer IV-2 (50 mM KPi; 75 mg/ml sucrose; 1 mg/ml diaminobenzidine; 0.5 mg/ml reduced cytochrome c; pH 7.4).

For the second dimension SDS-PAGE, gel lanes from BN gels were placed on top of a 12% separating polyacrylamide gel containing 0.25% SDS. After separation and blotting, membranes were probed with the total OXPHOS Blue Native WB Antibody Cocktail (1:250).

2.8. Cell population doubling time analysis

19.2×10^4 cells were plated in 6-well plates. Cells were grown with mETC inhibitors or paraquat for 16–18 h. Then, based on the number of cells collected from plates, cell doubling time was calculated. Mean cell population doubling times from four independent experiments were compared using the unpaired two-tailed t-test.

2.9. Cell cycle analysis

Cell cycle profile was analysed based on the DNA content. Cells were grown for 12 h with mETC inhibitors or paraquat. Then 10^6 cells were fixed with ice-cold ethanol for 2 h at 4 °C, washed with PBS and resuspended in the staining solution (0.1% v/v Triton X-100, 10 μg/ml propidium iodide, 5 μl/ml RNase Cocktail in PBS). Cells were incubated for 30 min at 37 °C and then propidium iodide fluorescence was measured with flow cytometry (BD LSR II, BD Bioscience). Cell cycle profiles were analysed using the FlowJo software (FlowJo, LLC). The mean proportion of cells in G1, S and G2/M phases from five independent experiments were compared using the unpaired two-tailed t-test.

2.10. Microarray analysis

mRNA was extracted from cells incubated for 12 h with myxothiazol (three independent samples) as well as from control cells (four independent samples), transcribed to cDNA, labelled and hybridized on a Mouse 8x60K chip. Microarray data were analysed with the GeneSpring software (Agilent Genomics). To reduce false-positive expression changes, background correction with 20th percentile cut-off was performed. Expression rates in myxothiazol treated samples were compared to the control samples to reveal fold-changes of gene expression using the unpaired t-test with Benjamini-Hochberg corrections. All statistically significant expression changes that were lower 1.5 were considered biologically not significant and thus cut off. The gene expression profile of myxothiazol treated cells was analysed with the Ingenuity Pathway Analysis (IPA, Qiagen) software core analysis tool. The microarray data are available at the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107414).

2.11. Statistical analysis

All results are presented in the text as mean ± standard error of the mean. On plots data are presented as median, 25th and 75th and percentile (box) and minimum/maximum values (whiskers). Data were analysed using Student’s t-test. A P-value of < 0.05 was considered statistically significant.
2.12. Key resources table

The Key Resources Table provides the essential data about antibodies and commercial assays used in the work.

3. Results

3.1. Cellular respiration is activated during the differentiation of P19 cells

P19 is a murine embryonal carcinoma pluripotent cell line that possesses main criteria of embryonic stem cells (ESCs) (McBurney and
P19 cells are round-shaped with a high nuclear to cytoplasm ratio that express the main pluripotency markers Oct4, Sox2, and Nanog (Vega-Naredo et al., 2014). We used Oct4 as a marker of pluripotent state of cells. (Fig. 1 a, d). Like ESCs, P19 cells differentiate in response to retinoic acid (RA) yielding various types of differentiated cells depending on growth conditions. Upon addition of RA to a cell monolayer cells differentiated to trophoblast-like cells that express cytotokeratin 8 (CK8) (Fig. 1 b, d) (Jones-Villeneuve et al., 1982; Vega-Naredo et al., 2014). Moreover, some differentiated cells expressed a higher amount of β-III-tubulin that is commonly used as an early neuron marker (Fig. 1 b, d). The respective cells, however, did not demonstrate a neuron morphology (Fig. 1 b). Under a slightly modified differentiation protocol, P19 cells give rise to neuron-like cells (Jones-Villeneuve et al., 1983). To induce this neuron differentiation, P19 cells in suspension were grown with RA to form 3D embryoid bodies (Fig. 1 c). Upon plating as a monolayer, the cells gained the neuron-like morphology and expressed the neuron differentiation marker β-III-tubulin (Fig. 1 c, d). The resemblance to ESCs and the robust differentiation make P19 cells a good model to study stem cell differentiation (Bain et al., 1994).

As stem cells in general, P19 cells rely on glycolysis for energy production (Vega-Naredo et al., 2014). To test whether P19 cells show an activation of the oxidative metabolism during differentiation, we followed the cellular oxygen consumption rate during trophoblast and neuron differentiation. Both trophoblast and neuron differentiated cells were characterized by an increased oxygen consumption (3.8 ± 0.35 nM and 3.7 ± 0.24 nM O2/1 min*10⁶ cells, respectively) compared to undifferentiated cells (1.17 ± 0.08 nM O2/1 min*10⁶ cells) (Fig. 1 e). Increased respiration is frequently accompanied by increased ROS production due to electron leakage from mETC. To test mitochondrial ROS level during differentiation we used the mitochondrial ROS-sensitive fluorescent dye MitoSox (see Supplementary Fig. 1). Both trophoblast and neuron differentiated cells showed a significant increase in the mean intensity of MitoSox fluorescence (1.75 ± 0.17 and 1.46 ± 0.11-fold increase, respectively) (Fig. 1 f). Increased oxygen consumption and ROS production show the activation of mETC activity during differentiation of P19 cells.

### 3.2. P19 cells contain a mature mETC machinery that is activated during differentiation

mETC activity in P19 cells is lower than in their differentiated derivatives. The low respiration rate could be explained by inefficient mETC machinery assembly in pluripotent cells. To analyse the mETC composition, mitochondria from undifferentiated P19 cells, embryoid bodies, trophoblast, and neuron differentiated cells were separated on a Blue Native (BN) PAGE. Part of the gel was stained for complex I (CI) and IV (CIV) activity (Fig. 2 a), while the remaining gel was used for a subsequent SDS-PAGE to reveal the subunit composition of mETC (Fig. 2 b).

The in-gel staining showed no changes in CI and CIV activity upon differentiation (Fig. 2 a). The active CI is visible as a band at a molecular weight of around 1 MDa in all samples. Besides a monomeric form of CI, there are also bands of higher molecular weight corresponding to the active CI in supercomplex structures (C, D, E). The active CIV is visible as a band with the molecular weight of around 440 kDa (Fig. 2 a) corresponding to the dimeric form of CIV (CIV₂) (Krause et al., 2005). Additional bands of higher molecular weight correspond to CIV included in supercomplex structures (A, B, E).

Under the reducing and denaturing conditions of 2D-SDS-PAGE, the complexes and supercomplexes were dissociated into their subunits and analysed by immunostaining with an antibody cocktail containing antibodies against one core subunit for each of mETC complexes. In general, the mETC structures are very similar in undifferentiated P19 cells and differentiated derivatives. CI was present in its monomeric form and in supercomplexes C, D, and E (Fig. 2 b). CIV was detected as a dimer (CIV₂) and as part of A, B, and E supercomplexes. ATP-synthase was detected as two signals, reflecting its monomeric form and a dimeric form. Complex II (CII) was slightly different in differentiated cells compared to P19 cells: in addition to a monomeric form undifferentiated P19 cells contained a CII signal in a lower molecular weight range probably corresponding to only partially assembled CII. The structure of Complex III (CIII) also differed between P19 cells and differentiated derivatives. Although CIII was equally present in supercomplexes (A, B, C, D, and E) in differentiated and undifferentiated cells, the CIII dimer (CIII₂) could only be detected in P19 cells, but not in differentiated cells (Fig. 2 b, red arrow). The exclusive detection of the dimeric form of CIII in P19 cells suggests that assembly of supercomplexes in P19 cells may be less efficient than in the differentiated derivatives.

Supercomplexes’ compositions (Fig. 2 c), based on in-gel activity stainings, were identical to the previously described active supercomplexes extracted from bovine heart (Schäfer et al., 2006). Importantly, supercomplexes D and E correspond to active respirasome (Vartak et al., 2013), indicating that P19 cells despite their low respiration (Fig. 1 e) contain a functional mETC machinery. Therefore, the regulation of mETC activity and ROS production may influence stem cell maintenance and/or differentiation potential.

### 3.3. mETC inhibitors and paraquat induce ROS production in P19 cells

Low activity of mitochondrial oxidative metabolism in stem cells may serve as a mechanism that protects cells against ROS. To test the impact of mitochondrial ROS produced by mETC on stem cells, we used chemical inhibitors of CI (rotenone) and CIII (antimycin A and myxothiazol) that enhance ROS production specifically in mitochondria. These mETC inhibitors prevent electron flow and increase the rate of electron leakage from mETC, resulting in increased ROS production (Fig. 3 a) (Brand, 2010; Kramer et al., 2003; Starkov and Fiskum, 2001). Rotenone, antimycin A, and myxothiazol are structurally unrelated pharmacological agents and therefore unlikely cause the same side effects in cells. Potassium cyanide, that blocks the electron flow at the level of CIV when the electron energy is too low to induce ROS production, was used as a negative control (Murphy, 2009). We also used paraquat as a ROS inducing agent. It accumulates in mitochondria (Castello et al., 2007), easily accepts electrons from different sources and transfers them to oxygen resulting in superoxide formation (Krall et al., 1988).

As mETC activity can influence ATP level, we investigated how mETC inhibitors alter the energetic state of cells. Due to the low level of respiration in undifferentiated P19 cells (Fig. 1 e, f), we used trophoblast differentiated cells with active respiration for this experiment. We measured the ATP/ADP ratio in differentiated cells exposed to mETC inhibitors for 24 h. Due to the low mETC inhibitors’ concentrations used in experiments, none of them led to any significant change of the ATP/ADP ratio (Fig. 3 b).

We next monitored how mETC inhibitors and paraquat influence ROS production of P19 cells. To visualise ROS in mitochondria specifically we used the MitoSox fluorescent probe. As expected, rotenone, antimycin A, myxothiazol, and paraquat increased the mean fluorescent intensity of the MitoSox signal compared to the control (Fig. 3 c), whereas cyanide had no significant effect. As an additional indirect evidence for increased ROS production, we detected mitochondrial superoxide dismutase (Sod2) protein level – one of the main enzymes that protect cells from oxidative stress (Van Remmen et al., 2004). All mETC inhibitors as well as paraquat increased Sod2 protein level, with antimycin A and myxothiazol showing the strongest effect (Fig. 3 d). The increased level of Sod2 caused by potassium cyanide probably is not associated with ROS level, but caused by side effects of cyanides.

### 3.4. Mitochondrial ROS level influences cell cycle progression

Previous studies, as well as our results, suggest that ROS level is...
important for stem cell maintenance. To test this hypothesis, we analysed how increased ROS level influences cell cycle profile of P19.

Firstly, we measured the cell proliferation rate by calculating cell population doubling time. P19 cells divided every 8.5 h (Fig. 4 a), corresponding to the division rate of murine ESCs (Abdelalim, 2013). Rotenone and potassium cyanide slightly slowed down cell division (up to 10.7 h and 9.5 h respectively), while antimycin A and myxothiazol significantly decreased cell duplication time (14.4 h and 21 h respectively). Paraquat also caused an effect that was comparable to CIII inhibitors, but weaker (13.7 h).

Secondly, we analysed the cell cycle profile by nuclear DNA staining with the stoichiometric fluorescent dye propidium iodide. The proportion of cells with single (2n), intermediate or double (4n) DNA set reflects the duration of G1, S, and G2/M phases, respectively. Undifferentiated P19 cells demonstrated a cell cycle profile typical for murine ESCs (Abdelalim, 2013) with a long S phase and short G1 and

\[
\text{Tab. 2.} \quad \begin{array}{c|c}
\text{SC} & \text{Composition} \\
\hline
A & \text{III}_{2}+\text{IV} \\
B & \text{III}_{2}+\text{IV}_{2} \\
C & \text{I}+\text{III} \\
D & \text{I}+\text{III}_{2} \\
E & \text{I}+\text{III}_{2}+\text{IV} \\
\end{array}
\]

Fig. 2. P19 cells contain mature respirasomes. a. In-gel activity staining of CI and CIV separated by BN-PAGE. mETC complexes extracted from not differentiated P19 cells (ND), embryoid bodies (EB), trophoblast (Tr) and neuron (Neu) differentiated cells. Purple bands reveal active CI or CI included in supercomplexes. Brown bands reveal active CIV corresponding to CIV$_{2}$ or CIV included in supercomplexes. CB staining revealed the most abundant proteins. b. 2D-SDS-PAGE that separated mETC structures received from P19 cells and trophoblast differentiated cells. The immunostaining reveals CI, CII, CIII$_{2}$, CIV$_{2}$, CV, and CV$_{2}$ as well as supercomplex structures. The red arrow points to the position where CIII$_{2}$ should be located. c. The table shows the supercomplexes (SC) compositions based on in-gel activity stainings and immunostainings. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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G2/M phases (Fig. 4 b). Although rotenone and potassium cyanide slowed down the cell division rate, they did not change the proportion of cell phases’ duration (Fig. 4 c). Antimycin A, myxothiazol and paraquat, on the other hand, caused a significant elongation of S phase and shortened both G1 and G2/M phases. In summary, elevated ROS decreases the proliferation rate of P19 cells due to elongated S phase.

3.5. Increased ROS level enhances trophoblast and prevents neuron differentiation

Next, we tested the impact of ROS production on the differentiation potential by adding mETC inhibitors or paraquat to the medium (Fig. 5 a) during trophoblast differentiation. Antimycin A, myxothiazol, and paraquat significantly increased the amount of CK8 protein as shown by Western blot analysis; rotenone had a weaker effect on CK8 expression, whereas cyanide did not influence CK8 expression. ROS produced by CI have weaker effects on differentiation most likely due to their localization in mitochondrial matrix. CIII, on the other hand, releases ROS in the intermembrane space, resulting in higher ROS mobility.

The increased amount of CK8 protein could be explained by an increased amount of CK8+ cells upon differentiation. To test this, we performed immunostaining of the cells with further visualisation of the CK8 signal by flow cytometry. The presence of antimycin A or myxothiazol during the differentiation increased the number of CK8+ cells in the cell population (Fig. 5 b); rotenone and paraquat caused a similar, but weaker effect, whereas cyanide did not change the proportion. This result suggests that the elevated mitochondrial ROS level favours differentiation by increasing the proportion of trophoblast cells in the differentiated cell population.

To address the question if ROS level influences exclusively trophoblast differentiation, we induced neuron differentiation in the presence of the same concentrations of mETC inhibitors. Interestingly, paraquat was lethal for cells during neuron differentiation. As the influence of paraquat on ROS level is comparable to CI and CIII inhibitors (Fig. 3 c), the lethality is probably not associated with ROS level, but rather with side effects of paraquat. The efficiency of neuron differentiation was estimated by the early neuron marker β-III-tubulin expression shown by Western blot analysis. Rotenone, antimycin A, and myxothiazol decreased the amount of β-III-tubulin compared to the differentiated control cells (Fig. 5 c). The presence of rotenone significantly decreased the number of neuron-like cells, while antimycin A and myxothiazol almost completely prevented neuron differentiation (Fig. 5 d). The inhibitors, however, did not prevent differentiation in general because they drastically increased the amount of CK8 expression (Fig. 5 c). Apparently, increased mitochondrial ROS level does not generally prevent differentiation, but rather direct it in certain cell types towards trophoblast differentiation.
3.6. Myxothiazol changes expression of genes associated with pluripotency, development and cell cycle progression

Increased ROS level influenced both proliferation and differentiation of P19 cells. To find out how ROS level influences gene expression in P19 cells, we performed a microarray analysis. As myxothiazol showed the strongest effects, we used it to induce mitochondrial ROS production. The microarray analysis showed a change in the expression of 6209 genes by 1.5-fold or more (Fig. 6). Expression profiles of genes that were altered by myxothiazol treatment were analysed with the Ingenuity Pathway Analysis (IPA, Qiagen) software core analysis tool that reveals biological functions and canonical pathways that are most likely relevant to the input gene list based on the annotated records contained in the Ingenuity Pathways Knowledge Base.

IPA canonical pathway analysis revealed the strongest association of myxothiazol with The Transcriptional Regulatory Network in ESCs (Table 1). Myxothiazol changed the expression level of 24 genes that regulate transcription in ESCs and their differentiation potential (see Supplementary Table 1). Firstly, P19 cells exposed to myxothiazol expressed higher level of Eomes (Russ et al., 2000) and Cdx2 (Niwa et al., 2005) genes that regulate the formation of the first differentiated lineage of mammalian embryogenesis – trophectoderm. Eomes and Cdx2 upregulation indicates that P19 cells exposed to myxothiazol are more potent to differentiate in trophectoderm. Secondly, myxothiazol exposure induced the expression of endoderm differentiation regulators Gata3 (Zhang et al., 2007), Gata4 (Soudais et al., 1995), and Gata6 (Morrisey et al., 1998). Finally, myxothiazol probably influenced ectoderm development. For example, the expression of Otx1 (Kurokawa et al., 2004) and Sox2 (Ferri et al., 2004) that are important for forebrain development were downregulated. Moreover, myxothiazol increased the expression of the negative regulator of neurogenesis Isl2 (Thaler et al., 2004). On the other hand, myxothiazol increased the expression of Lhx5 that stimulates proliferation of neuron stem cells (Zhao, 1999) and of L1cam that acts as an anti-apoptotic factor during neuron differentiation (Naus et al., 2004). In summary, myxothiazol changed the expression of genes associated with early ESCs differentiation, such as trophoderm formation and dissociation of germ layers. These results stay in line with our experimental data: the
expression profile of genes involved in the transcriptional regulation of ESCs demonstrated that myxothiazol treatment made P19 cells more potent to differentiate in trophoblast but not to ectoderm.

Fig. 5. mETC inhibitors increase the efficiency of trophoblast differentiation whereas preventing neuron differentiation. a. Western blot analysis of differentiation marker CK8 and β-III-tub expression in cells differentiated with mETC inhibitors or PQ. β-act, PS and CB stainings are used as a loading controls. b. Percentages of CK8 positive (CK8+) cells in the population of differentiated cells exposed to mETC inhibitors or PQ during differentiation, n = 3. Mean cell population proportions were compared using the unpaired two-tailed t-test; * p-value ≤ .05. c. Western blot analysis of the differentiation marker CK8 and β-III-tub expression in cells differentiated with mETC inhibitors. β-act PS and CB stainings are used as a loading controls. d. Fluorescence image of cells differentiated with Rot, Ant or Myx stained with antibodies against β-III tub (green). Nuclei were stained with Hoechst (blue). 10 × magnification, the scale is 100 μm.

Fig. 6. Microarray gene expression analysis. Up- (red) and down-regulated (green) genes in cells exposed to myxothiazol for 12 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression profile of genes involved in the transcriptional regulation of ESCs demonstrated that myxothiazol treatment made P19 cells more potent to differentiate in trophoblast but not to ectoderm.

Table 1
Top list of Ingenuity Canonical Pathways that are associated with myxothiazol treatment.

| Ingenuity canonical pathway                           | p-value     | Overlap |
|-------------------------------------------------------|-------------|---------|
| Transcriptional regulatory network in ESCs            | 9.66*10^{-6} | 24/46   |
| PPAR signaling                                        | 6.73*10^{-4} | 34/90   |
| PCP pathway                                           | 1.22*10^{-3} | 24/59   |
| ErbB2-ErbB3 signaling                                 | 2.48*10^{-3} | 25/65   |

In addition, IPA biological function analysis reveals that myxothiazol treatment was associated with several types of differentiation, including epithelial tissue, neurons, adipocytes, muscles, trophoblasts and endodermal cells, as well with cell proliferation and cell cycle progression (see Supplementary Table 2).
4. Discussion

The presence of highly reactive oxidants such as ROS can be harmful to cells, due to oxidative damage of biomolecules, including lipids, protein, and DNA. DNA damage causes accumulation of mutations that are especially dangerous for long living and fast dividing cell types, such as stem cells. Although high ROS level decreases the proliferation potential of stem cells, ROS cannot be regarded as exclusively harmful for stem cells. Our data show that mitochondrial ROS level directs cell fate during differentiation.

As stem cells in general, P19 cells are characterized by a low respiration activity (Fig. 1 e) and therefore low ROS production (Fig. 1 f). However, it was unclear if the low respiration level is caused by an incomplete mETC. In this work, we show that pluripotent P19 cells contain assembled respirasomes (Fig. 2) - functional units for cellular respiration. In our previous study, we also showed that human mesenchymal stem cells contain assembled supercomplexes, resembling respirasomes (Hofmann et al., 2012). Despite the presence of respirasomes, both P19 cells and human mesenchymal stem cells (Chen et al., 2008) possess a relatively low respiration activity. These results together indicate that stem cells possess a complete respiration machinery which is apparently not active hence preventing ROS generation.

To elucidate how increased mitochondrial ROS level influences cell properties, we used four mETC inhibitors. As expected, the disruption of the electron flow through mETC CI and CIII increase ROS production (Fig. 3) (Brand, 2010; Dröse and Brandt, 2008; Kramer et al., 2003). Effects of mETC inhibitors on ROS level are probably comparable with the physiological ROS induction that happens due to the cellular respiration activation during differentiation. Our results demonstrate that trophoblast differentiation and neuron differentiation increase ROS level by 75% and 51% respectively (Fig. 1 f), while inhibitors by 16–20% (Fig. 3 c). Application of multiple mETC inhibitors provides several advantages: (1) to discriminate the effects of ROS production from side effects of each inhibitor; (2) to provide an appropriate negative control by applying potassium cyanide; (3) to allow monitoring the effects of ROS production both in mitochondrial matrix from CI (Dröse and Brandt, 2008) and intermembrane space from CIII (Bleier and Dröse, 2013). Interestingly, while the CI inhibitor rotenone caused the strongest effect on the MitoSox signal (Fig. 3 c), it had less effect on P19 proliferation (Fig. 4) and differentiation (Fig. 5). This may be explained by the fact that the positively charged MitoSox preferentially accumulates in the mitochondrial matrix (Forkink et al., 2010) and therefore is more sensitive to superoxide produced by CI. We used the expression level of mitochondrial Sod2 as an additional indirect evidence for ROS production level in cells. Indeed, mETC inhibitors increased Sod2 expression (Fig. 3 d), with antimycin A and myxothiazol showing the strongest induction. Potassium cyanide also induced Sod2 production but did not show any influence on MitoSox staining. This result can probably be explained by the fact that cyaniodes block Sod1 activity (Rigo et al., 1975) and cells might compensate reduced Sod1 activity by increased Sod2 expression.

The weaker effect of ROS produced from CI can be also explained by the mechanism of ROS production. The most probable site of electron leakage in CI is located in the peripheral arm, resulting in superoxide formation in the mitochondrial matrix (Dröse and Brandt, 2012). There, superoxide is quickly degraded by highly potent ROS-defending mechanisms (Antunes et al., 2002). That could also explain why the CI inhibitor rotenone doesn’t induce SOD2 expression. CIII, on the other hand, releases superoxide in the more oxidized milieu (Hu et al., 2008) of the intermembrane space (Brand, 2010). Therefore, ROS produced by complex III is stable enough to migrate in the cytoplasm and activate ROS signaling.

High ROS levels induced by CI and CIII inhibitors or paraquat decreased the proliferation potential of P19 cells (Fig. 4). This result stays in line with previous studies (Ito et al., 2004; Miyamoto et al., 2007; Tothova et al., 2007). Moreover, increased mitochondrial ROS generation results in a longer S phase, but a shorter G1 phase (Fig. 4 c). Similar effects of ROS level on G1 phase were previously shown in several publications. The presence of thiol-antioxidant NAC causes G1 cell cycle arrest of mouse fibroblasts (Menon et al., 2003; Sekharam et al., 1998) and rat hepatic stellate cells (Kim et al., 2001). Altogether these results suggest that ROS level regulate the G1/S phase transition: decreased ROS level causes G1 arrest, whereas increased ROS level promotes G1/S transition.

Besides proliferation, ROS level also influences the differentiation potential of P19 cells. Increased ROS production caused by CI and CIII inhibitors prevented neuron differentiation (Fig. 5 a, b). At the same time, increased ROS level enhanced trophoblast differentiation (Fig. 5 c, d). These results also are confirmed by gene expression analysis showing that the expression of main trophoderm regulators Eomes and Cdx2 is enhanced by myxothiazol treatment. Myxothiazol treatment also changed the expression of genes that are involved in neuron differentiation and development of nervous system (see Supplementary Table 2). Our results stay in line with the previously shown association between cell cycle progression and the potential of pluripotent cells to differentiate in trophoblasts: the absence of G1 cyclins D and E in mouse embryonic stem cells causes the cell cycle slow down while enhancing trophoblast differentiation (Liu et al., 2017).

ROS regulation is probably not exclusive for mouse ESCs. Increased ROS level in human ESCs, for instance, enhances the differentiation of cells in mesoderm and endoderm lineages (Ji et al., 2010). Induced pluripotent stem cells (iPSCs) possess mitochondria morphologically and functionally very similar to ESCs (Armstrong et al., 2010; Prigione et al., 2010). ROS level participates in the regulation of differentiation potential of iPSCs. High level of ROS, for example, promotes arterial endothelial cell specification of mouse iPSCs (Kang et al., 2016). Hence it will be very interesting to investigate the influence of ROS in human ESCs and iPSCs in detail in future studies.

Previous studies demonstrated that ROS level could be harmful (Pereira et al., 2013) or beneficial (Ji et al., 2010; Lee et al., 2011; Tormos et al., 2012) for differentiation potential of stem cells. Our work indicates that ROS function is more complex. The ROS level serves as an additional factor that determines the pathway of differentiation, enhancing one, whereas preventing other cell type differentiation. A better understanding of ROS role in stem cell homeostasis could provide a powerful tool for a precise regulation of stem cell differentiation.

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Declarations of interest

None.

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