Oxidative stress and mitochondrial adaptive shift during pituitary tumoral growth


PII: S0891-5849(18)30122-9
DOI: https://doi.org/10.1016/j.freeradbiomed.2018.03.019
Reference: FRB13666

To appear in: Free Radical Biology and Medicine

Received date: 1 November 2017
Revised date: 9 March 2018
Accepted date: 12 March 2018

Cite this article as: Maria Eugenia Sabatino, Ezequiel Grondona, Liliana d.V. Sosa, Bethania Mongi Bragato, Lucia Carreño, Virginia Juarez, Rodrigo A. da Silva, Aline Remor, Lucila de Bortoli, Roberta de Paula Martins, Pablo A. Pérez, Juan Pablo Petiti, Silvina Gutiérrez, Alicia I. Torres, Alexandra Latini and Ana L. De Paul, Oxidative stress and mitochondrial adaptive shift during pituitary tumoral growth, Free Radical Biology and Medicine, https://doi.org/10.1016/j.freeradbiomed.2018.03.019

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Oxidative stress and mitochondrial adaptive shift during pituitary tumoral growth

Sabatino, Maria Eugenia1*; Grondona, Ezequiel1*; Sosa, Liliana d.V.1; Mongi Bragato, Bethania1; Carreño, Lucia1; Juarez, Virginia1; da Silva, Rodrigo A.2; Remor, Aline2; de Bortoli, Lucila2; de Paula Martins, Roberta2; Pérez, Pablo A.1; Petiti, Juan Pablo1; Gutiérrez, Silvina1; Torres, Alicia I.1; Latini, Alexandra2; De Paul, Ana L1#.

1Universidad Nacional de Córdoba, Facultad de Ciencias Médicas, Centro de Microscopía Electrónica. Instituto de Investigaciones en Ciencias de la Salud (INICSA-CONICET), Argentina.

2Laboratório de Bioenergética e Estresse Oxidativo, Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, Brazil.

*Sabatino, Maria Eugenia and Grondona, Ezequiel contributed equally to this work.

# Corresponding Author and person to whom reprint request should be addressed:

Dr. Ana Lucía De Paul.

e-mail: adepaul@cmefcm.uncor.edu

Centro de Microscopía Electrónica, Instituto de Investigaciones en Ciencias de la Salud (INICSA-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba.

Av. Enrique Barros y Enfermera Gordillo, Ciudad Universitaria, 5000 Córdoba, Argentina.

Tel/fax: +54-351-4333021
ABSTRACT

The cellular transformation of normal functional cells to neoplastic ones implies alterations in the cellular metabolism and mitochondrial function in order to provide the bioenergetics and growth requirements for tumour growth progression. Currently, the mitochondrial physiology and dynamic shift during pituitary tumour development are not well understood. Pituitary tumors present endocrine neoplastic benign growth which, in previous reports, we had shown that in addition to increased proliferation, these tumours were also characterized by cellular senescence signs with no indication of apoptosis. Here, we show clear evidence of oxidative stress in pituitary cells, accompanied by bigger and round mitochondria during tumour development, associated with augmented biogenesis and an increased fusion process. An activation of the Nrf2 stress response pathway together with the attenuation of the oxidative damage signs occurring during tumour development were also observed which will probably provide survival advantages to the pituitary cells. These neoplasms also presented a progressive increase in lactate production, suggesting a metabolic shift towards glycolysis metabolism. These findings might imply an oxidative stress state that could impact on the pathogenesis of pituitary tumours. These data may also reflect that pituitary cells can modulate their metabolism to adapt to different energy requirements and signaling events in a pathophysiological situation to obtain protection from damage and enhance their survival chances. Thus, we suggest that mitochondria function, oxidative stress or damage might play a critical role in pituitary tumour progression.
Graphical Abstract

Keywords: Pituitary tumour, Mitochondria, Oxidative stress, Nrf2 pathway, Glycolysis.

HIGHLIGHTS

- Bigger mitochondria with increased fusion evidence were seen in pituitary tumours.
- Pituitary tumours showed increased ROS levels and signs of oxidative damage.
- The Nrf2 pathway was activated in pituitary tumours as an antioxidant response.
- Glycolysis may favour cell growth and survival in experimental pituitary tumours.

Abbreviations

8OH-G: 8-hydroxyguanine, CK: Creatine kinase, CS: citrate synthase, DNMT1-3A-3B: DNA methyltransferase 1, 3A, 3B, DRP1: dynamin-related protein 1, p-DRP1: dynamin-phosphorylated
related protein 1, **ETC**: electron transport chain, **HO-1**: heme oxygenase, **MFF1** and **MFN2**: mitofusin 1 and mitofusin 2, **MMP**: mitochondrial membrane potential, **NRF1** and **NRF2**: nuclear respiratory factor 1 and nuclear respiratory factor 2, **Nrf2**: nuclear factor erythroid- related factor 2, **p-Nrf2**: phosphorilated nuclear factor erythroid- related factor 2, **OGG1**: 8-Oxoguanine DNA glycosylase/AP lyase 1, **OPA1**: Optic atrophy 1 gene protein, **OXPHOS**: oxidative phosphorylation, **PCC**: protein carbonyl group content, **ROS**: reactive oxygen species, **TFAM**: transcription factor A mitochondrial, **γ H2AX**: phosphorilated histone H2AX, **TET1-3**: ten-eleven translocation 1-3.

**Introduction**

Pituitary tumours are trophically stable benign neoplasms that typically show relatively little change in size over many years and may be the main basis of endocrine dysfunction. The current understanding of the molecular pathogenesis of pituitary adenomas is considered as a multifaceted process that involves the participation of both extrinsic and intrinsic factors. Recently, a substantial improvement has been made in the recognition of the mechanisms and agents implicated at the beginning and during the evolution of pituitary tumours. These tumours do not present the typical oncogene mutations often found in nonendocrine neoplasms and the particular aspects that contribute to the initiation and promotion of their development have already been characterized [1, 2]. These factors, such as cell cycle deregulation, growth factor overexpression, hormonal overstimulation, epigenetically silenced tumour suppressor genes, oncogene overexpression, defective signalling pathways and an altered intrapituitary microenvironment, act as mitogenic stimuli that can lead to sustained tumour growth [1, 3-8]. However, an alternative approach based on data derived from the mitochondrial role during pituitary tumorigenesis has not yet been fully considered. Mitochondria influence cell function and adaptation by dynamic fluctuations, and due to their participation in numerous critical outgrowths of cell function, they are important contributors to intracellular signalling and can affect homeostasis in several interconnected manners. Mitochondrial dysfunction can directly provoke several of the adverse cellular outcomes related to chronic stress, including oxidative stress, inflammation, telomere shortening, epigenetic dysregulation, altered gene expression and cellular senescence [9]. In addition, recent advances in molecular biology have provided innovative ways to interpret the role of the cellular metabolism and mitochondrial function in the development and progression of tumours.
Cellular transformation of normal and functional cells to neoplastic ones implies alterations in the cellular metabolism and mitochondrial function in order to provide the bioenergetics and growth requirements for tumour growth progression [10, 11]. Thus, without metabolic transformation, the neoplastic cell will not be able to attain an unregulated growth potential. Therefore, it is critical to establish the role of the mitochondria during pituitary tumorigenesis.

The ultrastructure, composition and function of mitochondria diverge significantly from tumoral to normal cells. For instance, an increased rate of glycolysis (Warburg effect) has been described as an inherent property of tumoral cells [12]. Also, rapidly growing tumours may show fewer and smaller mitochondria with scarcer cristae than those from slowly growing tumours, which tend to present characteristics resembling normal cells [11]. Additional differences between the mitochondria of normal vs. transformed cells have similarly been described with regard to preference for substrates, mitochondrial membrane potential, protein synthesis, organelle turnover and reactive oxygen species (ROS) production [10].

Mitochondria are key components of the stress response owing their role in energy production and their ability to generate signals that promote cellular adaptation [13]. For example, mitochondrial biogenesis can modify the number of organelles to adapt the total cellular energy capacity. Also, mitochondrial network can undergo fission or fusion as a response that could promote survival in the context of cellular stress signals.

Metabolic processes may result in an increase in the ROS production [14]. When production of ROS exceeds the cell antioxidant capacity, oxidative stress and oxidative damage to biomolecules (proteins, DNA and RNA) may take place, and this may have a causative role in the progression of tumorigenesis [15]. Adaptive responses to environmental and endogenous stresses involve the activation of the Keap1-nuclear factor erythroid-related factor 2 (Nrf2) signalling pathway, which regulates the expression of an extended group of cytoprotective genes [16].

Few attempts to elucidate mitochondrial health in pituitary tumours have been made [18-22], with currently little being known about mitochondrial behaviour during endocrine tumour development. The pituitary gland mostly develops benign proliferative injuries with the coexistence of mitotic events together with cellular senescence arrested cells and practically null apoptotic rates [17-26]. As a dynamic and plastic tissue the pituitary gland constantly adapts hormonal and metabolic responses to the changing environments [27]. Thus, we have interpreted
the existence of a pituitary senescent barrier as being a failsafe mechanism that may allow the survival of the gland, complementing its relevant role in organism homeostasis. Here, we focused our attention on how the mitochondrial fitness outcome supports these tumoral features, implying an adaptation of the mitochondrial structure and dynamics, energy production mechanisms and detoxification processes. As little is known about mitochondria behaviour and energy metabolism in pituitary tumours [28-31], in the present work we analysed a wide range of parameters, which were taken into account during the development of in vivo experimental pituitary tumours. This helped us to conceive an overview of the progressive mitochondrial switches needed for the survival of pituitary cells in order to cope with neoplastic injury.
2. Materials and methods

2.1 Animals and experimental models
Three-month-old Wistar strain male rats were maintained at controlled temperature (21±3 °C) and lighting conditions (14 h light: 10 h darkness cycle), with free access to commercial laboratory chow and tap water. Taking into account that exogenous estrogen excess induces pituitary tumours [17, 32], intact animals were treated with estradiol benzoate (Sigma Aldrich, St. Louis, MO, USA) for 10, 20, 40, and 60 days (E10, E20, E40, and E60). Estrogen was implanted subcutaneously in slow releasing silastic brand capsules (Dow Corning, Medical grade, Midland, MI, USA) filled with 10 mg of estrogen crystals and sealed with silastic cement. The control group was implanted with empty capsules. Rats were decapitated within 10 s after being removed from their cage, thus avoiding any stress or external stimuli. The animals were kept in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (1996), and the experiments were approved by the Institutional Animal Care Committee of the School of Medicine, National University of Córdoba.

2.2 Pituitary cell dissociation
The protocol for pituitary cell dissociation has been previously described [33]. Briefly, rat pituitary glands were placed in minimal essential medium, and then minced and digested with 0.4 % w/v trypsin at constant slow agitation at 37°C. The cell suspension was gently dispersed using flame-rounded Pasteur pipettes. Cell viability, tested with Trypan blue exclusion, was always better than 90%. Cell dispersions were transferred to 1.5 ml eppendorf tubes and centrifuged at 400 g for 5 min.

2.3 Cellular fractionation
The subcellular fractionation was performed as described by Mongi Bragato and coworkers (2016). Briefly, pituitary glands from control or estrogenized animals were homogenized in cytoplasmic extract buffer [10 mM Hepes pH 7.5, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM NaVO₄, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 µg/mL Aprotinin, 1 µg/mL Leupeptin, 1 µg/mL pepstatin, 0.075% Nonidet P40 (NP40)], and centrifuged at 2000 xg for 5 min at 4°C to pellet out THE nuclei fraction. Supernatants were centrifuged at 10000 xg for 10 min at 4°C, and the resulting ones were used as cytoplasmic extracts. Nuclear pellets were resuspended in nuclear extract buffer (20 mM Tris HCl pH 8, 420 mM NaCl, 60 mM KCl, 1 mM EDTA, 1 mM NaVO₄, 1mM
PMSF, 25% glycerol, 1 μg/mL Aprotinin, 1 μg/mL Leupeptin, 1 μg/mL pepstatin), and centrifuged at 10000 g for 10 min at 4°C. The resulting supernatants were used as nuclear extracts. Aliquots of each sample were used for total protein quantification according to the Bradford method (Bio-Rad Protein Assay, Bio-Rad).

2.4 Western blotting analysis
Protein (40μg) from the subcellular fractionation was separated by SDS-PAGE and transferred to a nitrocellulose membrane and blocking was performed using bovine serum albumin (BSA) (Sigma Aldrich) 5% in TBS 1X/tween-20 0.1 %. To estimate the corresponding molecular weights, the Precision Plus Protein Standards molecular weight marker was used (Bio-Rad). Membranes were incubated overnight at 4°C with the corresponding primary antibody: goat polyclonal anti-OPA-1 (1/200), rabbit polyclonal anti-MFN-2 (1/250), rabbit polyclonal anti-phosphorilated-DRP-1 serine 637 (1/500; Cell Signaling), mouse monoclonal anti-8-hydroxyguanine (8OH-G) (1/100), mouse polyclonal anti- phosphorylated Histone H2AX (p-γH2AX) (1/2000, Cell Signaling), rabbit monoclonal anti-Nrf-2 (1/500, Abcam) rabbit monoclonal anti-phosphorilated Nrf-2 serine 637 (p-Nrf-2) (1:500, Abcam), mouse monoclonal anti-α tubulin (1:2500; Sigma Aldrich), rabbit anti-Lamin B (Lam B) (1/500; Cell Signaling), rabbit polyclonal anti-Heme Oxygenase 1 (HO-1) (1/200, Santa Cruz Biotechnology), anti-DNA methyltransferase-1 (DNMT-1) (Cell Signaling, 1/1000) or anti-β-Actin (1/4000; Sigma-Aldrich). After washing, blots were incubated with a peroxidase-conjugated (HRP) goat anti-rabbit (1/5000; BioRad), goat anti-mouse (1/3000; Jackson Laboratories) or mouse anti-goat (1/1000, Santa Cruz Biotechnology), diluted in blocking buffer, rinsed in TBS 1X/Tween 0.1 %, and revealed with an ECL detection system (Amershams Biosciences). Emitted light was captured on Hyperfilm (Amersham Pharmacia Biotech). The α-tubulin, β-actin or Lam B antibodies were used as loading controls, and values were expressed as the percentage of the control. Semi-quantitative signals were derived from densitometric analysis using the ImageJ software (1.51o Wayner Rasband National Institute of Health, USA).

2.5 Transmission electron microscopy (TEM)
With the objective of evaluating the mitochondrial ultrastructural features of pituitary cells, normal and estrogen-induced tumoral pituitaries were fixed in a mixture of 4 % formaldehyde and 2 % glutaraldehyde in 0.1 M cacodylate buffer, before being treated with 1% OsO₄ as previously described [34]. Then, the Araldite-embedded pituitary thin sections were cut with a diamond knife
on a JEOL JUM-7 ultramicrotome (Nikon, Tokyo, Japan) and examined using a Zeiss Leo 906 E electron microscope equipped with the digital camera Megaview III (Oberkochen, Germany). For quantitative analysis of the mitochondrial number and morphology, three glands from each experimental condition were used, and fifty random micrographs of the same slide region were acquired at a 10000X magnification (50 micrograph/experimental condition/per animal) and analysed using Image J software (1.51o Wayne Rasband National Institute of Health, USA), to determine the mitochondria number/field, area, circularity \((4\pi \times \text{area}/(\text{perimeter})^2)\) and elongation (mayor axis/minor axis) [35-37].

2.6 Immunohistochemistry
Paraffin-embedded pituitary glands were processed by immunohistochemistry for 8-OHG as described previously [17]. Briefly, an antigen retrieval method using a hot antigen unmasking solution was performed (0.01 M citrate buffer; pH 6), and in order to inhibit endogenous peroxidase, sections were treated with 3 % v/v hydrogen peroxide in methanol for 15 min. Subsequently, these sections were incubated for 30 min in 10 % v/v normal serum (Sigma–Aldrich) to block nonspecific binding, followed by overnight incubation with monoclonal anti-8-OHG antibody (1/200). Next, sections were incubated with biotinylated secondary antibody (1/300; Amersham; Buckinghamshire, England) and ABC complex (Vector Laboratories, Burlingame, CA, USA) with 3,3’-diaminobenzidine tetrahydrochloride being used as a chromogenic substrate (Sigma Aldrich). Finally, sections were rinsed in running water and counterstained with hematoxylin (Sigma Aldrich). Controls were performed by applying the same protocol, but omitting primary antibodies. Slides from three animals of each experimental condition were photographed in randomly chosen fields at 400 X.

2.7 Immunofluorescence
To assess DMNT 1 localization, additional sets of slides from different experimental conditions were processed for fluorescence microscopy. LR White-embedded pituitary glands (London Resin Corporation, Berkshire, UK) were processed as described elsewhere [34]. Briefly, thin sections were cut with a diamond knife on a Porter-Blum MT2 and JEOL JUM-7 ultramicrotome and then incubated for 2 h in glicine (100 mM/Buffer Tris pH 7.4) in order to reduce auto-fluorescence according to Baschong, Suetterlin and Laeng [38]. After the pituitary sections had been incubated with anti-DMNT1 (1/100 Cell signalling) overnight, these slices were washed, and further incubated with Alexa 594 anti-rabbit secondary antibodies (1/2000; Invitrogen; Carlsbad, USA) for
1 hour. Images were then obtained using the confocal microscope Axioplan (Carl Zeiss, Jena, Alemania) and collected with a 60 X objective. The analysis of these images was carried out using ImageJ software (1.51o Wayner Rasband National Institute of Health, USA).

2.8 Sequential RNA and DNA co-extraction
Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Sequential DNA co-extraction was performed from the interphase and organic layer obtained after centrifugation. Then, the genomic DNA (gDNA) was precipitated with 100 % ethanol and the pellet was washed with sodium citrate/ethanol buffer (0.1 M sodium citrate in 10% ethanol, pH 8.5) and 70 % ethanol and dissolved in 50 μl of NaOH 8 mM, with the RNA and gDNA integrity and concentration being measured using a NanoDrop spectrophotometer (Thermo Scientific, Uniscience, USA).

2.9 Semiquantitative RT-PCR analysis
Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Thereafter, 1 μg of total RNA was used as a template for reverse transcription using EpiScript™ Reverse Transcriptase (Epicentre, Madison, WI) with random hexamer primers (Thermo Fisher Scientific, Waltham, MA USA). The cDNA obtained was subjected to PCR amplification using GoTaq DNA Polymerase (Promega, Madison, WI), and following this, PCR protocols were performed according to the manufacturer’s instructions. PCR amplification was performed in 10 μl using a specific primers sets (Table 1), and the PCR amplified products were visualized by electrophoresis onto 2% agarose gels by GelSampleRed™ (Biotium) staining, with the band densitometric analysis being carried out using ImageJ software.

2.10 Global DNA methylation analyses whit with Mspl/Hpall endonucleases
Global DNA methylation was evaluated by restriction endonuclease digestion of 1000 μg of genomic DNA digested with 10 μl of reaction mixture of restriction endonucleases Mspl or Hpall (10 U; Fermentas, St. Leon-Rot, Germany) in separate reactions in appropriate buffers and digested at 37 °C for 16 h. Both endonucleases have the same restriction site (CCGG), although Hpall is also sensitive to DNA methylation. The reaction products were resolved by electrophoresis onto 0.8 % agarose gel. Band densitometric analysis was carried out using ImageJ software, and the percentage of methylation was calculated using the formula: global DNA methylation content = (Hpall-Mspl) x 100/ undigested genomic DNA [39].


2.11 TP53 promoter methylation analysis by methylation-sensitive restriction enzymes
To evaluate the TP53 promoter methylation status, firstly gDNA samples were digested with methylation sensitive HpaII restriction endonuclease (New England Biolabs, Ipswich, MA, USA), which can recognize unmethylated CCGG motifs, at 37 °C for 4 h in a 10 µl reaction volume. This was followed by inactivation at 65°C for 20min. Afterwards, the digested gDNA samples were amplified with primers (Table 1) flanking the CCGG motifs in the promoter region of the P53 gene. The PCR amplified products were analysed by electrophoresis onto 2 % agarose gel with undigested gDNA with amplification control, and band densitometric analysis was obtained using ImageJ software. The percentage of promoter methylation was calculated considering that the value of the undigested gDNA sample quantification represented the total amplified DNA template (100 % of the initial template copies were amplified) and, the value of HpaII-digested samples represented the total amplified DNA. The percentage of methylation was calculated using the formula: promoter methylation content = (HpaII) x 100/ undigested gDNA [40].

2.12 Measurement of creatine kinase activity
Creatine kinase (CK) activity was determined in pituitary homogenates by measuring creatine formation by the colorimetric method [41] using a commercial kit of CK (Doles, Goiania, Brazil) according to the manufacturer's instructions. The results from pituitary homogenates of 3 animals per experimental group were expressed as µmol of creatine/min/mg protein.

2.13 Protein carbonyl content
The protein oxidative damage was measured by determining the of protein carbonyl group content (PCC), based on the reaction with dinitrophenylhydrazine (DNPH) [42]. Normal and tumoral pituitary gland supernatants were treated with 4 mmol DNPH dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 20 % TCA and centrifuged for 5 min at 10000 xg. Next, pellets were washed with 1 ml ethanol: ethyl acetate (1:1 v/v) and re-dissolved in 6 M guanidine prepared in 2.5 N HCl, and samples were incubated at 37 °C for 5 min to assure complete dissolution of the pellet, with the resulting sample being examined at 360 nm. Differences between DNPH-treated and HCl-treated samples were used to estimate the PCC. Results were calculated as pmol of carbonyls groups/mg protein, using the extinction coefficient of 0.022 µM-1cm⁻¹ for aliphatic hydrazones.
2.14 Mitochondrial membrane potentials and ROS production

JC-1 (MitoProbe™ JC-1 Assay Kit for Flow Cytometry-M34152) is a lipophilic cation, which selectively accumulates, either within the mitochondrial matrix as a green (527 nm) fluorescent monomer at depolarized membrane potentials, or as J-aggregates with orange-red (590 nm) fluorescence at hyperpolarized membrane potentials [43]. DCFH-DA (Molecular Probes™ Reactive Oxygen Species Detection Reagents-mp36103) is a cell-permeant dye that once inside the cell is cleaved by intracellular esterase into its non-fluorescent form DCFH. This probe, which is no longer membrane permeant, may be further oxidized by ROS to its fluorescent form DCF [44]. Here, fluorescence emission from DCF (green) was detected at a wavelength of 530 nm.

In order to determine the mitochondrial membrane potential and ROS production, dispersed pituitary cells from normal and estrogen-treated animals were incubated with 500 µl of DMEM supplemented with 10 % foetal bovine serum (150000 cells / treatment). Then, 0.5 µl of JC-1 (Stock solution 2 µM) or 2.5 µl DCFH-DA (Stock solution 10µM) were added to each sample and incubated at 37 °C for 30 min under 5 % CO₂, after which, pituitary cells were washed twice at 37°C with filtrate phosphate buffer saline (PBS), resuspended in a 100 µl final volume and analysed by flow cytometry (BD FACS Canto II). The accumulation of JC-1 in pituitary cells, measured by an increase in fluorescence, together with the intensity ratio of orange J-aggregates to green monomers were used to monitor the mitochondrial membrane potential change induced during estrogen-induced pituitary tumoral development. CCCP (0.5 µl/ Stock solution 50 µM) and H₂O₂ (2.5 µl/ Stock solution 100 µM) were used as positive controls for membrane polarity and for stimulating ROS production, respectively.

2.15 Measurement of the respiratory chain enzyme activities

Complex I activity was measured by the rate of NADH-dependent ferricyanide reduction at 420 nm (1 mM⁻¹cm⁻¹) [45] with the cytochrome c oxidase activity (complex IV) being also determined spectrophotometrically measuring cytochrome c oxidation at 550 nm [46, 47]. The above-mentioned methods were slightly modified, as detailed in a previous report [48]. The activities of the respiratory chain complexes were calculated as nmol/min⁻¹/mg protein, and measured using a temperature-controlled Varian Cary 50 spectrophotometer (Varian, Inc., Palo Alto, CA, USA).

2.16 Reduced Glutathione Determination

Reduced Glutathione levels were measured according to Browne and Armstrong [49] in tissue homogenates after protein precipitation with 20 % trichloroacetic acid. Pituitary samples were
diluted in 100 mM PBS pH 8, containing 5 mM EDTA, and incubated with Ortho-flthalaldehyde (1 mg/ml methanol) at room temperature for 15 min. Fluorescence was measured using 350 nm excitation and 420 nm emission in pituitary homogenates from 5 animals for each experimental group. A standard curve of reduced glutathione was used for concentration determination, and the results were expressed as nmoles GSH/mg of protein.

2.17 Citrate synthase activity
Citrate synthase (CS) was measured at 410 nm at room temperature as described previously [50]. Pituitary homogenates from normal and estrogen-treated animals were incubated in the reaction buffer containing 100 mM Tris-HCl pH 8, 0.1 mM acetyl-CoA, 0.2 mM DTNB, and 0.1 (v/v) Triton X-100. The assay was initiated by the addition of 10 ml of 20 mM oxalacetate and, 10 min later, the absorbance was read on a microplate reader (Bio-Rad, Hercules, CA, U.S.A.).

2.18 Lactate determination
Normal and estrogen-induced tumoral pituitaries were suspended in 4X volumes of lactate assay buffer using a homogenizer sitting on ice, and centrifuged at 13000 g at 4 °C to remove any insoluble material. Then, supernatants were collected and endogenous LDH, which could degrade lactate, was removed by sample deproteinization using trichloroacetic acid and centrifuged at 13000 xg at 4 °C. Finally, the pH solution (pH 7.4) was adjusted with 1 M of cold Tris solution (pH 8.8), and lactate levels were immediately determined using the colorimetric assay LACT2 (Roche Diagnostic GmBH, Mannheim, German). To obtain detectable levels of lactate was 0.2 mmol/l and also for statistical purposes, the pituitary glands from 4 animals were pooled for each experimental condition. In addition, muscle tissue (10 mg) from the same animals was processed in parallel and used as an internal control of lactate determination.

2.19 Statistical analysis
Statistical analysis was carried out using an analysis of variance with Fisher’s post-test (InfoStat version 2015; Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina). The results were given as the means ± SEM, and the data were obtained from at least in three replicates measured from three independent experiments. The significance levels were chosen at P< 0.05. Graphical design was performed using the Prism 5 software (GraphPad, La Jolla, CA, USA).
3. Results

3.1. Modifications in mitochondrial mass, dynamics and morphology emerged during the pituitary tumour development.

Cellular proliferation is an energy-consuming process, making very interesting to understand how the mitochondrial function is altered in tumoral growth. Therefore, our interest was focused on the evaluation possible mitochondrial adaptations that occur during the estrogen-induced pituitary tumour development.

An essential process that modifies the mitochondrial mass is biogenesis, which can adjust energy production by synthesis of new organelles and their components. This cellular program is stimulated by the enhancement of NRF-1, NRF-2 (nuclear respiratory factors 1 and 2) and TFAM (transcription factor A, mitochondrial) transcriptional activity, which are the main factors responsible for mitochondrial gene expression [51]. The transcript levels of all of these mitochondrial biogenesis promoters were markedly induced and sustained during tumour development (Figure 1A-D). In addition, as the mitochondrial number, structure and function are continuously modified by functional requirements to adapt to different cell demands, we also analysed the ultrastructural mitochondrial features. As shown in Figure 1E, an enlargement in the mitochondrial area was detected from the earliest stages of pituitary tumour development, which was also accompanied by a sustained and significant rise in CS activity, as a mitochondrial mass marker, with tumour development from 20 to 60 days (Figure 1F). However, the morphometric analysis of mitochondria in normal and tumoral pituitaries revealed a slight increase in the number of mitochondria per area only for the E10 condition, when examined by TEM (p<0.05 Figure 1G-H).

Mitochondrial morphology is tightly regulated by the coordination of fusion and fission events, so that variations in their balance can cause excessive mitochondrial fragmentation or elongation. These processes involve dynamin-related protein 1 (DRP1) and mitochondrial fission factors MFF and FIS1, which constitute the core of mitochondrial fission machinery, and the dynamin-like GTPases, mitofusin 1 and 2 (MFN 1 and MFN2), and optic atrophy type 1 (OPA1) proteins, which mainly regulate mitochondrial fusion processes [52]. Thus, to try to understand the mitochondrial dynamics implications, we evaluated the expression of the fusion proteins MFN-2 and OPA-1 by Western blot (WB). The expression of both proteins was progressively intensified during pituitary tumour development (Figure 1I; Suppl Figure 1), in association with a prevalence of a circular shape at the expense of a decreased mitochondrial elongation, as detected by ultrastructural analysis (p<0.05; Figure 1J-L). We found an increased protein level of DRP-1
phosphorilated serine 637 (Figure 1I), indicating the inhibition of mitochondrial fission [53]. These results, supported also by our previous findings regarding MFN-1 and DRP-1 proteins suggest an imbalance of the protein involved in mitochondrial dynamics, thereby favouring fusion processes [17].

3.2. Adaptation of the mitochondrial functionality occurred during experimental pituitary tumour development

It has been frequently supposed that tumoral cells may show defective OXPHOS [54]. Thus, we next evaluated the enzymatic activity of complexes I (NADH: ubiquinone oxidoreductase) and IV (cytochrome c oxidase), which comprise two complexes of the three energy-conserving core of the electron transport chain (ETC). As shown in Figure 2A, the Complex I activity was mainly unaltered, showing a rise only at the beginning of tumour progression (E10), in agreement with the increased mitochondrial number. In contrast, a marked increase in Complex IV activity was detected, starting from E20 and continuing over time (p<0.05; Figure 2B). These results may suggest that OXPHOS activity is not compromised during tumour development.

The enzyme Creatine Kinase (CK) also plays a main role in cellular energy buffering and transport, thereby balancing its availability between the cell compartments [55]. The determination of CK activity showed a significant increase of its enzyme activity, practically for all the pituitary tumour growth periods analysed (Figure 2C). These data may reveal that, despite presenting functional ETC complexes as mentioned above, the pituitary tumoral cells may need energy buffering between compartments.

A common feature of tumoral cells is the enhanced rate of glycolysis [56, 57]. As shown in Figure 2D, a sustained increment in lactate production was detected during pituitary tumour development (p<0.05). The data strongly suggest a shift to a glycolytic metabolism. Furthermore, this finding might indicate that glycolysis and OXPHOS are both essential for pituitary tumour progression, or also may suggest that glycolysis must provide further advantages, probably resulting in rapidly ATP supply or providing carbons for biomass generation [58].

The assessment of the mitochondrial membrane potential (MMP) is also frequently used to indicate mitochondrial health, as a sign of proton circuit efficiency in OXPHOS [9]. Consequently, we analysed the time-course changes in the red/green fluorescence ratio of the JC-1 mitoprobe during pituitary tumour development. Significant differences in this ratio were detected, showing an increase in the MMP from E40 (Figure 2E), which is consistent with several
3.3. ROS production and oxidative damage in pituitary tumour development

Through different approaches we analysed the oxidative stress status during the pituitary tumour development. As shown in Figure 3A, the cellular ROS levels progressively increased from E20, reaching a 2 fold increase at the E60 endpoint. ROS can harm almost all classes of subcellular components, so we investigated whether the higher ROS production detected under our experimental conditions was accompanied by oxidative damage of relevant biomolecules. With regard to proteins, carbonylation is considered to be the main modification occurring after severe oxidative damage [64] and, as shown in Figure 3B, a notable increase in the amount of carbonyl groups was detected from the early stages of tumoral progression (E10-E20), with values attaining a 3 fold increase compared to basal pituitaries. Interestingly, these protein damage signals were not sustained until the last stages evaluated, as a recovery in the basal values was detected (Figure 3B).

Another relevant biomolecule susceptible to oxidative damage is DNA. Hence, we initially evaluated p-γH2AX protein expression as a hallmark of DNA damage, which demonstrated gradual increases from the start of tumoral development, but recovering to normal levels at E60 (Figure 3C; Suppl Fig 2). Concerning ROS damage to DNA, 8-OHG is one of the most common markers used for the evaluation of oxidative DNA lesions [65]. Therefore, we detected a striking increase 8-OHG protein expression levels by WB from the early stages of pituitary tumour development (Figure 3C; Suppl Fig 2). This result was also supported by immunohistochemistry, as a noticeable rise was recorded in the amount of pituitary cells displaying an augmented nuclear 8-OHG protein level (Figure 3D). Moreover, this assay also revealed relevant cytoplasmic 8-OHG protein localization, probably indicating oxidized mitochondrial DNA.

In the context genetic integrity, the enzyme 8-Oxoguanine DNA glycosylase/AP lyase 1 (OGG-1) plays an important role in protection against cellular damage by ROS [66]. Consequently, a significant rise in mRNA OGG1 levels was detected from E10 (Figure 3E), indicating the activation of oxidative repair responses during the development of pituitary tumours.

The maintenance of a cell proliferation rhythm and cell survival depends on the redox homeostasis between ROS production and elimination [67]. In fact, cells have a variety of antioxidant defense systems, with one of the most common being glutathione, which acts as central sensor of oxidative stress and is considered to be the major non-enzymatic antioxidant
The determination of the quantity of glutathione revealed a significant decrease in its availability from E10 to E40, although basal values gradually recovered by E60 (Figure 3F). These data may show an increased glutathione detoxification activity, yet also with a decreased capacity in order to replenish the glutathione pool that usually maintains the reducing cycle.

3.4. Oxidative stress response is activated in pituitary tumour cells

Several of the above-mentioned oxidative damage kinetics returned to basal values, suggesting the re-establishment of healthy biomolecules levels and an antioxidant system at E60, indicating the activation of a cellular detoxification signalling pathway as a response to oxidative injuries. In this regard, the transcription factor Nrf-2, is a master regulator of the cellular response under oxidative agents and has a coordinator role in the regulation of redox balance [16]. Subsequent to our observations related to the oxidative stress in the pituitary tumour, we evaluated the total and phosphorylated Nrf-2 (p-Nrf-2) protein expression levels in nuclear fractions from normal and tumoral pituitary glands. It can be observed in Figure 3G that a gradual increase in both protein levels was detected during the pituitary tumour growth (p<0.05; Suppl Fig 2), with this response also being accompanied by a significant rise in mRNA Nrf2 levels starting from E10 up to E40 (p<0.05, Figure 3H). Additionally, we evaluated the expression of an Nrf-2 downstream effector, the oxidative stress-associated HO-1, which revealed increases at E10 and E60 (Figure 3I; Suppl Fig 2). These findings showed a clear activation of the oxidative stress response during pituitary tumour development, probably leading to the extensive surviving capabilities of pituitary cells.

3.5. Hypermethylation of the p53 promoter accompanied energetic and metabolic shifts in pituitary tumours

Epigenetic control of nuclear gene expression is highly sensitive to the mitochondrial function [69, 70]. Therefore, an intriguing question was whether metabolism alteration in the pituitary gland could be accompanied by changes in the epigenetic status. Thus, to assess whether cellular adaptation during pituitary tumour development also involved epigenetic modifications, we initially compared global DNA methylation profiles during pituitary tumour development. By restriction endonuclease digestion of DNA pituitary extracts, a decrease was observed in the overall DNA methylation during tumour progression (Figure 4A-B). Then, a decrease in the protein expression levels of DNMT1 (DNA methyltransferase 1) was detected from E20 (Figure 4C-D), together with a reduction in its levels of mRNA in E60 (Figure 4E-F)
Hypomethylation is a common feature of the tumorogenesis epigenome. However, it also frequently harbours hypermethylation of specific tumour growth regulator promoters [71, 72]. Several genes linked to the most important tumour suppressors Rb and p53 were previously found to have been inactivated in pituitary tumours by epigenetic mechanisms [73-75]. In this regard, p53 is a relevant transcription factor that also regulates a metabolic rewiring in order to suppress proliferation pathways of stressed or damaged cells, suggesting that p53 may contribute to global metabolic functions distinct from its well-characterized responses to specific cellular stresses [76, 77]. Through the evaluation of the TP53 promoter methylation status, we detected significant signs of hypermethylation (Figure 4H) accompanied by a gradual restoration of mRNA p53 basal levels during tumoral progression (Figure 4I). These results may indicate that a progressive expression loss can be sustained by epigenetic modification in order to help maintain a metabolic adaptation status through gene expression modulation in pituitary tumour progression regulation.
Discussion

A deep understanding of how mitochondrial alterations contribute to cell growth and tumorigenesis is an underexplored research area beyond Warburg effect, regarding its significance in tumoral development behaviour. We have previously described that experimental pituitary tumours are characterized by a benign growth which involves, in addition to proliferation, an increased cellular senescence as evidence of a cell arrest mechanism together with no apoptosis signs [17]. In this context, it is important to mention that despite the large number of metabolic aberrations thus far identified for tumorigenesis, apparently none of these is common to all tumour types. Hence, to have a clear understanding of the physiological basis of pituitary adenoma pathogenesis, we studied several aspects of mitochondrial morphology and energy metabolism through different approaches during the in vivo development of experimental pituitary tumours. It was found that pituitary cells showed clear evidence of oxidative stress in association with oxidative damage, accompanied by bigger and round mitochondria probably related to an increased fusion process and augmented biogenesis. These events took place in unison with an important Nrf2 stress response pathway activation, which may have led to the attenuated oxidative damage found during tumour development, providing survival advantages to pituitary cells. These neoplasms also presented a progressive increase in lactate production, suggesting a metabolic shift towards glycolysis. Furthermore, data obtained from epigenetic analysis might suggest that pituitary neoplasm harbours features of the cancer epigenome, due to a global hypomethylation and focal hypermethylation of p53. We envisage that these data may reflect pituitary cell metabolism regulation by adapting to different energy requirements and signalling events in a pathophysiological situation in order to obtain protection from damage and to enhance survival chances. We propose that the mitochondria function and oxidative stress or damage might play critical a role in pituitary tumour progression, thus targeting mitochondrial alterations and signalling could be a promising strategy for the development of selective antitumoral therapy in the case of endocrine tumours.

Distinct subpopulations of cancer cells coexist in tumours and contribute to functional heterogeneity. Each subpopulation seems to rely on different metabolic programs to maintain their energy. In experimental pituitary tumours, the coexistence of proliferative events together with the activation of growth control mechanisms may surely result in a particular combination of cell signalling pathways. As a greater amount of energy must be needed to carry out the initial burst observed in pituitary tumour proliferation [17], this might explain the increase in
mitochondrial mass, biogenesis and ETC complex functionality [78]. In addition, the rapid production of ATP by glycolysis may be an optimal energy source in the growing context [57]. However, our present results also revealed an important oxidative stress effect with a concomitant oxidative damage induction during pituitary tumour development. Whereas it is known that excessive damage accumulation can lead to cell death [79], during pituitary tumour development a relevant increase in the Nrf2 detoxification pathway was seen in the current study. Hence, it is possible that attenuation of damage could contribute to the survival of pituitary cells [80, 81], thereby requiring an alternative energetic metabolism that may even support the senescent cellular arrest we have previously described [17].

The extent to which the control of dynamics, mass and subcellular spatial mitochondrial organization is deregulated has been less explored than other mitochondrial aspects in tumorigenesis [10]. Also, the mitochondrial structure alterations are heterogeneous and not specific to any neoplasm or microenvironment conditions, even more research about mitochondrial mass in primary tumours in vivo is scarce. Here, a rise in energetic demand due to proliferative stimulus, and also the possibility of mitochondrial damage may explain the increase in biogenesis [82, 83] in association with the augmented mitochondrial mass revealed by the CS analysis during pituitary tumour progression. Despite the increased mitochondrial biogenesis and mass, there was only a slight increase in the mitochondria number. Concomitantly, a gradual promotion of the mitochondrial fusion process was detected, suggesting that new mass may constantly be incorporated by fusion during tumour development, implying modifications reflected in bigger and rounded mitochondria as the pituitary tumour developed, as was also reported by others authors [28, 29, 31].

Whereas mitochondrial fission permits renewal and redistribution inside the cells [84, 85]; fusion allows cell interaction and communication. In addition, fusion has also been associated with higher a mitochondrial efficiency [86, 87] since it may promote more a rapid diffusion of the matrix metabolites required to drive more efficient OXPHOS and also stimulate complementation of mtDNA mutations [88]. Additionally, the so-called “stress-induced mitochondrial hyperfusion” [89, 90] may also prevent the mitochondrial fragmentation and dismantling of the mitochondrial network, which sensitizes cells to death via apoptosis [89, 91] as a response to damage. Since an enduring overall shift toward fusion events may lead to the establishment of arrested cellular senescent cells [92], therefore, from a functional standpoint, we believe that -in pituitary tumours-
a profuse and long-term mitochondrial fusion might promote survival of this master gland by avoiding dysfunctions in injured mitochondria while preserving a suitable bioenergetic capacity.

The tumour cell metabolism also differs from that of normal cells, but a comprehensive examination of the metabolic status of pituitary tumours has not yet been performed. Here, we demonstrated that pituitary tumours had functional ETC activity, yet displayed a progressive increased in the glycolysis rate compared with normal cells. In particular, glycolysis seems to be the main energy pathway involved in slow-growing solid tumours [93, 94]. The Warburg effect is considered to be a mandatory metabolic change that allows cells to divide and proliferate [12], which sustains growth rates through rapidly increasing ATP levels and supports biomass generation [58, 95]. Thus, the progressive rise in lactate production may provide carbons to generate biomass [57], since we have already reported that pituitary tumour cells become hypertrophic and covered with organelles engaged in protein synthesis [96]. In this regard, it has been reported that the majority of untreated pituitary tumours have an increased HIF-1α expression [97], which could be related with the expression of glycolytic genes [98], and also linked to decreased p53 [76].

Defects in mitochondrial respiration are not necessarily the cause of tumour glycolysis, as it has been reported that OXPHOS is not impaired in most tumoral cells [99-101]. Thus, a more extensive analysis of either energy production or consumption and substrate preferences needs to be performed to determine whether mitochondrial respiration is defective in pituitary cells. However, in our study, these cells seemed to be quite active as both I and IV complex activities rose during pituitary tumor growth, which we interpreted as a functional MRC state. The increase in complex I activity, particularly at E10, might be mainly associated with a first line of response to a bigger energy demand, together with an elevation in the mitochondrial number.

Considering MRC efficiency, complex IV has been suggested to be a major regulation site for oxidative phosphorylation [102]. Hence, the higher complex IV activity demonstrated here may support our suggestion of an active respiration rate in pituitary tumors. However, it has been reported that at high intramitochondrial ATP/ADP ratios can inhibit cytochrome c oxidase [103]. Thus, even when MRC substrates continue to be oxidized, this does not imply a concomitant ATP production. Several studies have suggested that enhancing respiratory rates without ATP synthesis is advantageous under some conditions [104-107].
The inhibition of proton flux through ATP synthase may lead to an increase in MMP. Those increased complex IV activity might suggest the existence of functional MRC pumping protons into the interspace membrane, thereby contributing to maintain elevated MMP hyperpolarized organelles, rather than causing depolarization and possibly leading to apoptosis. In the present work, we noted a gradual increase in MMP polarization in pituitary cells during tumoral development, in agreement with other reports for different tumours [59-63, 108-110], and this alteration may result in a sub-optimal value for ATP production [111]. In this context, we propose that sustained growth rates and ATP levels during pituitary tumor growth could be preferentially attributable to a concurrent increase in glycolysis [57] and CK buffer activity [55, 112], while pituitary tumors may accumulate damaged or defective mitochondria. Consequently, we believe that the mitochondrial integrity in pituitary tumors might be in part a consequence of the balance toward mitochondrial fusion processes, which are probably will be coupled with the hyperpolarized MMP when chronic damage could be attained at E40 and E60.

It is probable that tumoral cells do not use aerobic respiration and MMP in the same way as normal cells. In fact, MMP maintenance is particularly significant for mitochondrial health, because depolarized potentials could lead to apoptosis and avoiding cell death is a central tumour ability acquisition [113, 114]. As we have previously reported that pituitary tumours exhibited almost null apoptotic figures [17], it is possible to argue that maintaining hyperpolarised MMP [113, 115, 116]; which is also related to the increased mitochondrial size detected [117] or elevated fusion [118, 119], might help prevent cell death Consequently, the increased MMP polarization in pituitary tumours above mentioned could be the outcome response of a conjunction of some of the respiration mechanisms that might confer selective survival advantage in addition to rapid ATP generation. Conversely, this choice may also lead to an increase in ROS production, but the activation of the Nrf-2 antioxidant pathway detected in our study, would make the pituitary tumour cells more tolerant to oxidative stress [120]. Summing up, we postulate that sustained changes in tumour cellular bioenergetics together with the damage accumulation detected during tumoral progression, lead to a metabolic reprogramming in the pituitary gland, with stronger adaptive responses that seemingly work as a survival program. Nevertheless, further analysis must be carried out to elucidate if the MRC is working properly and whether the rate of ATP is accurate.

ROS can contribute to mitochondrial signalling in cell growth, cellular senescence and differentiation [121]. However, ROS may also have a causative role in the progression of
tumourigenesis [15, 122, 123], as they increase under tumoral contexts [124, 125]. In agreement, in this investigation, a progressive rise in ROS production was detected during pituitary tumour development, which may have been primarily derived from mitochondria [14]; although ineffectiveness in ETC, greater metabolic demand, incomplete ROS scavenging, replicative stress and affected mitochondrial dynamics cannot be discarded [121, 126, 127].

In the present work, we detected clear signs of oxidative damage in different biomolecules. Among these, we showed an increased protein oxidative damage, DNA damage signals and an augmented oxidized DNA during pituitary tumour development, which could imply important mutagenic lesions and can cause genomic instability [65, 128]. Also, the 8OH-dG cytoplasmic signs of DNA damage detected suggest that mtDNA, whose vulnerability is prone to proto-oncogenic mutations [129], may be compromised in pituitary tumours. In particular, oxidative damage was detected earlier in proteins and DNA than the increase in ROS levels. A plausible explanation for this could be that additional sources of reactive species, not specifically those detected by DCF, inflict oxidative damage to biomolecules from the start of tumour induction [130].

We speculate that the oxidative damage detected throughout pituitary tumoral development sets the platform for early cell transformation in these adenomas, and at the same time may contribute to triggering the growth arrest through cellular senescence [131, 132], thereby setting a status of oxidative stress that may have an impact on the pathogenesis of pituitary tumorigenesis.

Redox homeostasis is a central process for cell survival and antioxidant enzymes have been shown to have altered levels in tumours, with most of these found to be deficient [133, 134]. Under the present experimental conditions, the decrease in GSH levels during the initial days of tumour progression suggests that the GSH pool might not be replenished concomitantly, which may also be an additional signal of oxidative stress [68]. However, the GSH levels were in fact gradually restored by the end of tumoral progression in our model.

GSH production is supported by Nrf2 expression through the control of GSH synthesis [135]. Remarkably, we observed a solid trigger of Nrf2 expression and activation with an up-regulated expression of stress response genes, such as HO-1 [136]. This Nrf2 pathway stimulation might be a response to oxidative insults and may support cellular detoxification through metabolic rewiring to sustain the antioxidant systems in pituitary tumorigenesis. An oxidative damage
restoration kinetic during pituitary tumour development has been seen in protein damage, also connected with Nrf2 activity, as it enhanced the recognition, repair, and removal of damaged proteins [137]. In parallel, we detected an increased expression of the repair enzyme OGG1 involved in the removal of 8-OHG from DNA [138] from the beginning of tumour development. As OGG1 expression is also susceptible to Nrf2 activation [139], Nrf2 may influence the pituitary cells fate by guiding them into antioxidant response pathways for the replacement and repair of damaged components.

It is intriguing how cells can balance the requirements of growth and homeostasis, with the redox balance strongly influencing cell proliferation rhythm [67]. Many Nrf2-regulated metabolic pathways confer growth advantages to tumour cells [137, 140] and constitutive activation of the Nrf2 pathway has been detected in human tumours [141, 142]. This activation is associated with a poor prognosis [143] and has also been considered to be a resistance mechanism to chemo- or radiation therapy because it eliminates therapy-induced ROS, thereby promoting cell survival [144]. In the present study, we observed that the main signs of oxidative damage appeared during the first 10 days of tumoral induction. Then, the restoration kinetics observed suggest that after 20 days the cells might undergo a detoxification response, probably through a readjustment of their metabolism. The Nrf2-mediated antioxidant response activation during the progression of pituitary tumour development can result in damage alleviation which might help to confer pituitary cells a non-lethal condition that may, at least in part, explain the decrease in proliferation and upsurge of cellular senescence [141].

Despite activation of the Nrf2 stress response pathway, the ROS levels in pituitary tumors still increased even at E60. However, the fact that ROS tends to accumulate during pituitary tumour development, does not necessarily imply any further oxidative damage, as defences systems may not have been overcome [120]. As ROS are needed for signalling [120] for example they can mediate Nrf2 stabilization [145], an emerging model is proposed that tumoral cells raise the production of ROS to trigger localized pro-tumorigenic signalling, but stabilize the increased ROS with a higher antioxidant activity to preserve the redox balance [120]. In this regard, ROS level detection and oxidative damage during pituitary tumour growth may not have a linear correlation. Instead, the relative contribution of the antioxidant equipment and the rate of removal of ROS may also need to be taken into account [146-148]. Hence, the antioxidant response resulting from Nrf2 activation in pituitary tumours could be considered to be a relevant event that may contribute to the slow and benign characteristics of pituitary neoplastic growth.
Tumor suppressor p53 is able to regulate certain metabolic pathways, promoting respiration while constraining glycolysis in order to achieve tumour growth prevention [76]. Thus, the glycolysis rise detected in pituitary tumours might be promoted by a loss of p53 function due to the gradual reversion to mRNA p53 basal levels observed after E10 in the present work, in agreement with data we have previously reported for p53 protein expression [17]. Related to this, we also have previously suggested that an increased activity of p53 at the beginning of experimental pituitary tumour development may be associated with the cellular senescence arrest trigger, which reduces afterwards to be replaced by p21 accumulation, as previously proposed by others [149]. Concomitantly, a detoxification process may appear as new requirement for survival.

Interestingly, when p53 is strongly up-regulated, the antioxidant response genes are down-regulated [150]. Hence, the weak p53 induction observed in the present work might result in Nrf2 stabilization by p21, thus arresting the cell [151] and allowing the induction of DNA damage-repair and an attempt to lower intracellular ROS, leading to a general cytoprotective response [152, 153]. Previously, we have described an accumulation of p21 expression occurring together with growth deceleration during pituitary tumour development, which - in this context - could also be related to the Nrf2 antioxidant response. It is possible that critical cross-talk interactions between Nrf2 and the inhibition of proliferation induced by oxidative-stresmay occur during pituitary tumour development [150]. These results suggest that these adaptation mechanisms during tumorigenesis may not be limited to alterations in enzyme activity, but also involve gene expression regulation [154]. This discovery contributes significantly to an emerging network of signalling between p53 and Nfr2, and supports the idea that pituitary tumour cells may have a low p53 activity that promotes redox homeostasis, repair and cell survival [155].

Mitochondrial function and metabolism affect gene expression by epigenetic regulation [69, 70]. Studies have demonstrated that epigenetic alterations are another force affecting the expression of genes implicated in neoplastic development [156, 157], thereby comprising pituitary tumorigenesis [74, 75, 158]. Here, we found a global hypomethylation of DNA probably due to a deregulation of DMNT 1, suggesting a methylation loss by a passive mechanism during pituitary tumour progression [159]. In addition, as previously suggested by Pease, Ling, Mack, Wang and Zada [73], we detected an increased TP53 promoter methylation in pituitary tumours, which might be associated with p53 mRNA regulation as described. These results may imply that pituitary neoplasm harbours features of the cancer epigenome which might present global hypomethylation and focal hypermethylation [160, 161]. However, this regulation mechanism is
not a simple on-off switch, but rather responds in a continuous manner to the changing metabolic needs of the cell. Hopefully, our pituitary tumour development model might permit us to reach conclusions about time-lapse regulation and switches. Although the functional consequences of this epigenetic modification are unclear, we propose that via epigenetic adjustment, pituitary cells could maintain a new metabolic state and thus favour cell survival even in the presence of a hostile oxidative microenvironment.
Conclusions

In summary, our data suggest that the mitochondrial metabolism plasticity that takes place in pituitary tumours leads to an improved fitness of the resulting cells in order to cope with the damage in the context of tumoral development. The metabolic shift might be an important mechanism to support the remarkable “growth advantage” that a tumour cell needs to overgrow its contemporaries which favours cells that have increased resistance to the disadvantageous oxidative microenvironment.

From the collective data presented, we propose that cooperative actions of p53 and Nrf2 might lead to cell repair and evasion of apoptosis during pituitary tumour development. Certain gene features may alter a set of different signalling pathways alone or in conjunction with each other, leading to pituitary tumoral cells exhibiting redox homeostasis restoration in an attempt to avoid further damage and support cell survival, thereby consolidating a strategy of cell death evasion.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements
The authors wish to thank Dr Carolina Leimgruber, EE Nestor Boetto, Ms Lucía Artino and Mr Marcos Almirón for their excellent technical assistance. They would also like to thank native speaker Dr Paul Hobson for revising the manuscript and Dr Gastón Soria for providing reagents.

This work was supported by the Fondo Nacional de Ciencia y Tecnología (AGENCIA-FONCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT–UNC) and Fundación Florencio Fiorini.
References

[43] A. Barbu, N. Welsh, J. Saldeen, Cytokine-induced apoptosis and necrosis are preceded by disruption of the mitochondrial membrane potential (Deltapsi(m)) in pancreatic RINm5F cells: prevention by Bcl-2, Mol Cell Endocrinol 190(1-2) (2002) 75-82.
32


[65] H. Kasai, Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis, Mutat Res 387(3) (1997) 147-63.


34


TABLES

Table 1 Primer sequences for gene expression and promoter methylation analysis

LEGENDS OF FIGURES

Figure 1. Alterations in mitochondrial features in experimental pituitary tumours

(A-D) Representative image of NRF1, NRF2 and TFAM RNA relative expression during tumoral pituitary development determined by RT-PCR. (E) Mitochondrial size by TEM. (F) Citrate synthase content determined by absorbance from control and estrogen-treated animals. (G) Mitochondrial morphometry by TEM. (H) Representative electron micrographs of normal and tumoral pituitary glands (E10): symbols highlight the mitochondria present in the area. (I) Fission (p-DRP1) and fusion (MFN2 and OPA-1) protein expression from cytosolic extracts of control and tumoral glands. (J) Mitochondrial circularity by TEM. (K) Representative electron micrographs from normal and tumoral pituitaries depicting mitochondrial morphological alterations after 60 days of estrogen stimulation. (L) Mitochondrial elongation from control and estrogen-treated animals. The β-actin mRNA and α-tubulin protein expression confirmed that equal loading occurred in all lanes. Data are from a representative experiment from a total of three independent experiments with similar results. Values are expressed as mean ± SEM. p<0.05, *vs Basal. ANOVA followed Fischer post hoc test. Scale bar: 2 µm.

Figure 2. Modification in mitochondrial functionality during pituitary tumours.

(A-B) Respiratory chain complexes I and IV enzymatic activity during pituitary tumoral development. (C) CK levels from control and estrogen-induced pituitary tumors assessed by a colorimetric assay. (D) Lactate concentration from control and tumoral pituitaries analysed by a colorimetric assay; skeletal muscle was used as positive control. (E) Mitochondrial membrane potential determined by JC-1 assay in pituitary cells during estrogen-induced tumoral development. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was used as positive control. Values are expressed as mean ± SEM. p<0.05, *vs Basal, ANOVA followed Fischer post hoc test.

Figure 3. Signs of oxidative stress and DNA oxidation in pituitary tumours.

(A) Reactive species production in normal and tumoral pituitary cells; H2O2 was used as positive control. Data are expressed as percentage of control. (B) The oxidative damage to proteins was measured by quantifying carbonyl groups during tumoral development. (C) 8OHdG and p-γH2AX protein expression levels in nuclear extracts from normal and tumoral pituitaries. (D) Paraffin
embedded adenohypophysis sections depicting nuclear 8OHdG immunocytochemical detection in control and tumoral pituitaries (E10 and E60). (E) OGG1 mRNA expression levels and (F) Reduced glutathione expression from control and tumoral pituitary cells determined by a colorimetric assay. (G) Nrf2 and p-Nrf2 protein expression from nuclear fractions determined by WB. (H) Nrf2 RNAm expression levels during estrogen-induced pituitary tumour development. (I) HO-1 protein expression from cytosolic extracts evaluated by WB. The β-actin mRNA and α-tubulin and Lamin B protein expression confirmed equal loading occurring in all lanes. Images correspond to a representative experiment from a total of three with similar results. Values are expressed as mean ± SEM. p<0.05, * vs Basal, ANOVA followed Fischer post hoc test. Scale bar: 20 µm.

Figure 4: Analysis of global DNA and p53 gene promoter methylation during experimental pituitary tumour development.

(A-B) Representative electrophoresis and densitometric analysis of non-undigested DNA (−), MspI- and HpaII-digested DNA samples are shown. (C-D) Representative image and analysis of DNMT1 protein expression during pituitary tumoral development are shown. (E) Representative electrophoresis of DNMTs (1, 3A and 3B) and TET (1, 2, 3) mRNA expression levels evaluated by RT-PCR is presented. (F) Densitometric analysis of DNMT1/β-actin mRNA ratio. (G) DNMT1 subcellular localization in normal and tumoral cells. DNMT1 immunolabeling depicts a punctuate staining at nuclear level (arrowheads) with a diffuse cytosolic staining in basal and at E10. A lack of DNMT1 nuclear localization was observed at E60. (H) Representative electrophoresis and densitometric analysis of TP53 gene promoter methylation is shown. The (−) symbol indicates undigested DNA samples and (+) HpaII-digested DNA samples. (I) Representative image and densitometric analysis of p53/β-actin mRNA ratio determined by RT-PCR. Data are expressed as mean ± SEM. * p<0.05 vs Basal, ANOVA followed by Fischer post hoc test. Bar: 10µm.

Supplemental Figure 1: Mitochondrial dynamic protein expression during experimental pituitary tumour development

(A-B-C) Representative images and densitometric analysis of OPA-1, MFN-2 and p-DRP1/α tubulin ratio, determined by WB. Data are expressed as mean ± SEM. * p<0.05 vs Basal, ANOVA followed by Fischer post hoc test.

Supplemental Figure 2: Oxidative stress and DNA damage markers in pituitary tumours. (A-B-C-D-E) Representative images and densitometric analysis of 8OHdG, p-γH2AX, Nrf2, p-Nrf2 and HO-
1/Lam B ratio, determined by WB. Data are expressed as mean ± SEM. * p<0.05 vs Basal, ANOVA followed by Fischer post hoc test.

Table 1: Primer sequences for gene expression and promoter methylation analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Note</th>
<th>Sequence (5' to 3')</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRF1</td>
<td>NM_001100708.1</td>
<td>Gene expression</td>
<td>(F) ACG ATG GGC GGG AGG ACC TT</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) TCC AAC GGC TGC TGC GGT TT</td>
<td></td>
</tr>
<tr>
<td>NRF2</td>
<td>NC_005110.4</td>
<td>Gene expression</td>
<td>(F) TAA GCC AGG CCA TAG ACA TC</td>
<td>292</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) CGA GGA ACC CCC TGA AA</td>
<td></td>
</tr>
<tr>
<td>TFAM</td>
<td>NM_031326.1</td>
<td>Gene expression</td>
<td>(F) AAA TGG CTG AAG TTG TTC TAAT TG</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) AGC TTC TTG GTG CCC AAT CCC AATG</td>
<td></td>
</tr>
<tr>
<td>OGG1</td>
<td>NC_005103.4</td>
<td>Gene expression</td>
<td>(F) CTA AGA AGA CAG AAG GCT AGG TAG</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) TGA CTT TGA TTT GGG ATG TTT GC</td>
<td></td>
</tr>
<tr>
<td>Nrf2</td>
<td>NM_031789.2</td>
<td>Gene expression</td>
<td>(F) CAG TCT TCA CCA CCC CTG AT</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) CCA AAC TTG CTC CAT GTC CT</td>
<td></td>
</tr>
<tr>
<td>DNMT1</td>
<td>NM_053354.3</td>
<td>Gene expression</td>
<td>(F) AGG ACC CAG ACA GAG AAG CA</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) GTA CGG GAA TGC TGA GTG GT</td>
<td></td>
</tr>
<tr>
<td>DNMT3A</td>
<td>NM_001003958.1</td>
<td>Gene expression</td>
<td>(F) AGG AAC CCC ATC CGG GTG CTA</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) AGC GGT CCA CTT GGA TGC CC</td>
<td></td>
</tr>
<tr>
<td>DNMT3B</td>
<td>NM_001003959.1</td>
<td>Gene expression</td>
<td>(F) GTG AAG CGG ATG ATG GAG AT</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) CCT CCG AGA CTT GGT AGC TG</td>
<td></td>
</tr>
<tr>
<td>TET 1</td>
<td>XM_006223880.2</td>
<td>Gene expression</td>
<td>(F) TGT CAC CTG TTG CAT GGA TTT</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) TTG GAT CTT GGC TTT CAT CC</td>
<td></td>
</tr>
<tr>
<td>TET 2</td>
<td>XM_006224264.2</td>
<td>Gene expression</td>
<td>(F) AAT CGC CTT CGG ATT CAG ACA CTC</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) CTT GAC CTC CGA TAC ACC CAT TTA GC</td>
<td></td>
</tr>
<tr>
<td>TET 3</td>
<td>XM_006236793.2</td>
<td>Gene expression</td>
<td>(F) TGG CTG CAG ACT TGA TCT TCC</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) ACC CGG CTC TAT GAA ACC TT</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>NM_030989.3</td>
<td>Gene expression</td>
<td>(F) CTT CGA GAT GTT CCC AGA GC</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) CTT CGG GTA GCT GGA GTG AG</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_031144</td>
<td>Gene expression</td>
<td>(F) ACC CAC ACT GTG CCC ATC TA</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R) CGG AAC CGC TCA TTT GC</td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>NC_005109.4</td>
<td>Promoter methylation</td>
<td>(F) CGT AGC TCC TCC GAC GTC TT</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>analysis</td>
<td>(R) GCG AGC CTA TCG GAA GGT G</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Mitochondrial mass, dynamics and morphology

A

B

C

D

E

F

G

H

I

J

L

K
Figure 2. Mitochondrial metabolism plasticity in pituitary tumour development

A

B

C

D

E

Mitochondrial potential membrane (relative to Basal)

0.5 1.0 1.5 2.0

Basal E10 E20 E40 E60 CCCP

Lactate (mmol/l)

0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0

Basal E10 E20 E40 E60 Muscle

Complete N-activity mg/mg protein

0 5 10 15 20

Basal E10 E20 E40 E60

Catalase activity nmol/min/mg protein

100 200 300 400 500

Basal E10 E20 E40 E60
Figure 3. Oxidative stress response in experimental pituitary tumours

A

Fluorescence intensity (italic, % of Basal)

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>E10</th>
<th>E20</th>
<th>E40</th>
<th>E60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>250</td>
<td>300</td>
</tr>
</tbody>
</table>

B

Carboxylated proteins

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>E10</th>
<th>E20</th>
<th>E40</th>
<th>E60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>75</td>
<td>100</td>
<td>125</td>
<td>150</td>
</tr>
</tbody>
</table>

C

Western blot analysis

283 kDa
15 kDa
66 kDa

Basal E10 E20 E40 E60

8OHdG
p-H2AX
Lam B

D

Immunohistochemistry

E

Oxidative stress markers

Basal E10 E20 E40 E60

Glutathione levels

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>E10</th>
<th>E20</th>
<th>E40</th>
<th>E60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>12</td>
<td>14</td>
<td>16</td>
<td>18</td>
</tr>
</tbody>
</table>

F

Glutathione levels in % of tissue

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>E10</th>
<th>E20</th>
<th>E40</th>
<th>E60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>7</td>
<td>9</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>
Figure 4. The global DNA and p53 methylation status in pituitary tumour progression

B. Global DNA methylation

C. DNMT1 protein expression

D. DNMT1/α-actin ratio

E. DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3

F. DNMT1/β-actin mRNA ratio

G. Immunofluorescence microscopy

H. p53 expression

I. p53/α-actin mRNA ratio