Oncomrogenic Properties of a Spermatogenic Meiotic Variant of Fer Kinase Expressed in Somatic Cells

Etai Yaffe, Elad Hikri, Yoav Elkis, Ortal Cohen, Ariela Segal, Adar Makovski, Alexander Varvak, Sally Shpungin, and Uri Nir

Abstract

The kinase Fer and its spermatogenic meiotic variant, FerT, are coexpressed in normal testes and cancerous tumors, but whether they exert related roles in spermatogenic or malignant cells has not been known. Here, we show that Fer and FerT reside in the mitochondria of spermatogenic cells and are harnessed to the reprogrammed mitochondria of colon carcinoma cells. Both kinases bound complex I of the mitochondrial electron transport chain (ETC) in spermatogenic and in colon carcinoma cells, and silencing of either Fer or FerT was sufficient to impair the activity of this complex. Directed mitochondrial accumulation of FerT in nonmalignant NIH3T3 cells increased their ETC complex I activity, ATP production, and survival, contingent upon stress conditions caused by nutrient and oxygen deprivation. Strikingly, directed mitochondrial accumulation of FerT endowed nonmalignant cells with tumor-forming ability. Thus, recruitment of a meiotic mitochondrial component to cancer cell mitochondria highlights a pivotal role for reprogrammed mitochondria in tumorigenesis. Cancer Res; 74(22): 6474–85. ©2014 AACR.

Introduction

The study of mitochondrial bioenergetics has been dedicated to understanding the central role of the mitochondrion in cellular ATP generation. Generation of ATP occurs via the respiratory electron transport chain (ETC), which is coupled to the mitochondrial oxidative phosphorylation (Oxphosph.) processes (1). However, in addition to its essential role in cell energy and metabolism, the mitochondrion is now recognized as a multifunctional organelle, which participates in a variety of cellular processes including proliferation, death, and senescence (2, 3). Deregulation of mitochondrial functions, in particular at the level of the ETC, is associated with several pathologies, including cancer (4). Cancer cells adopt mitochondrial alterations and metabolic reprogramming to sustain their unique metabolic needs and produce the molecules and energy required to promote tumor growth (5). Specifically, while normal mammalian cells primarily use mitochondrial Oxphosph. for ATP production, cancer cells remodel their glycolytic and mitochondrial machineries so that glycolysis is upregulated but only marginally replenishes the mitochondrial tricarboxylic acid (TCA) cycle and the Oxphosph. processes, even under aerobic conditions (6). This phenomenon of prevailing aerobic glycolysis is termed the "Warburg effect" (7–10). It should be noted that in parallel to the documented relevance of aerobic glycolysis to cancer cell metabolism, recent studies have also established the importance of mitochondrial processes in malignant transformation (11).

Fer is an intracellular tyrosine kinase, which resides in both the cytoplasm and nucleus of mammalian cells (12). Together with c-Fes, Fer constitutes the F-BAR domain–containing subfamily of intracellular tyrosine kinases that share a unique structure with an extended N-terminal tail (previously reviewed in refs. 13, 14). Fer is involved in the regulation of several cellular processes. These include among others "outside-in" signaling that regulates cytoskeleton organization and cell adhesion (13, 15–17). Fer has also been implicated in the response of cells to stress cues. It was shown to rescue cells from ionic radiation (18) and to mediate the migration of fibroblasts in response to reactive oxygen species (ROS; ref. 19). Several lines of evidence also suggest a supportive role of Fer in the progression and growth of malignant tumors. The kinase was detected in all human malignant cell lines analyzed (20, 21), and its levels in malignant prostate tumors are significantly higher than those detected in benign growths (22). Furthermore, downregulation of Fer impairs the proliferation of prostate, breast, and colon carcinoma cells (23), induces death in colon carcinoma and non–small cell lung cancer (NSCLC) cells (24, 25), abolishes the ability of prostate carcinoma PC3 and V-Sis–transformed cells to form colonies in soft agar (22), and prevents the metastatic spread of breast and lung adenocarcinoma tumors (26, 27). At the clinical level, high Fer expression levels have been linked to poor prognosis of hepatocellular carcinoma (HCC; ref. 28), clear cell renal cell
cancerous cells suggest that directed accumulation of FerT in mitochondria of non-reprogrammed mitochondria of colon carcinoma cells and revealed. Protein in cancer cell proliferation and survival have not been molecular mechanisms underlying the role of this meiotic

In the current study, we show that FerT and Fer populate the reprogrammed mitochondria of colon carcinoma cells and that directed accumulation of FerT in mitochondria of non-cancerous cells suffices to endow them with the ability to form tumors in vivo.

Materials and Methods

Antibodies and reagents

The following antibodies were used for either immunoblotting or immunocytochemistry: Affinity-purified anti-N-terminal Fer (anti-N-Fer) antibodies (24), affinity-purified anti-SH2 Fer/FerT antibodies (anti-Fer/FerT; ref. 35), anti-complex I NDUFA9 subunit monoclonal antibody (MitoSciences), anti-VDAC, anti-mitochondria monoclonal antibody (Meridian), anti-porin 31HL (Ab-2) monoclonal antibody (Calbiochem), anti-ATP5A mitochondrial ATP synthase alpha subunit monoclonal antibody (Santa Cruz Biotechnology), anti-mitochondria monoclonal antibody (Meridian), VDAC, anti-mitochondria monoclonal antibody (Meridian), and anti-lamin B monoclonal antibody (Santa Cruz Biotechnology).

Sperm cell isolation, capacitation, and protein lysate preparation

Ejaculated bull semen was obtained from the Israeli Veterinary Institute and washed three times by centrifugation (780 × g, 10 minutes) in NKM buffer (110 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L N-morpholino propanesulfonic acid, pH 7.4). In vitro capacitation and protein lysate preparation of bull sperm were carried out as described before (36).

Mouse mature caudal spermatozoa were obtained by dissecting the cauda epididymis of male mice (ICR strain) followed by its puncture for 15 minutes in a dish containing 3-mL PBS to enable sperm release out of the epididymal tissue. The sperm suspension in PBS was then filtered through a nylon mesh with 50-μm pores and centrifuged at 1,000 × g for 5 minutes; the pellet was resuspended either in a lysis buffer for protein analysis or in fresh PBS solution.

Immunocytochemistry

Cells were spread, fixed, and stained with the appropriate primary antibody, as described (37). The immunostained slides were inspected under an AxioimagerZ1 (Zeiss) fluorescence microscope or a confocal fluorescence microscope (Olympus FV1000).

Cell culture, normoxia, nutrient starvation, and hypoxia growth conditioning

Cell lines (HCT116, NIH3T3, SW620) were obtained from the ATCC. Cell authentication was carried out using a morphology check by microscope and by characterization of the DNA profiles using short tandem repeat analysis. HCT116 and SW620 cells were grown in minimum essential medium (MEM) containing 10% heat-inactivated FBS and 1% nonessential amino acids (Biological Industries). NIH3T3 cells were grown in DMEM. All cells were grown at 37°C with 5% CO₂.

For nutrient starvation, DMEM was aspirated 24 hours after cell seeding and replaced by Hank's Balanced Salt Solution (Biological Industries) for 24 hours.

For growth under hypoxia, plates with NIH3T3 cells grown in DMEM were transferred for 24 hours to anaerobic culture jar containing CO₂-generating envelope (GasPak EZ, BD) that reduces the oxygen level in the jar to 1% within 30 minute.

Isolation of mitochondria and preparation of cytoplasmic and mitochondrial extracts

Intact mitochondria were isolated from hearts, livers, and testes of ICR mice and from HCT116 colon carcinoma cells. Tissues were excised from 10-week-old wild-type mice and collected into 10 volumes of ice-cold isolation buffer (IB), and mitochondria were isolated as described (38).

Immunoprecipitation

Purified mitochondria were lysed, and complexes I and V were immune-captured from 1 mg protein using specific beads according to the manufacturer’s (MitoSciences) instructions. Total mitochondrial lysates or immunoprecipitates were resolved by SDS-PAGE and immunoblotted with specific antibodies.

siRNA-mediated silencing of the Fer and FerT genes

siRNA-mediated silencing in cells was carried out as described (39). siRNA sequences are presented in Supplementary Table S1.

ROS measurements

ROS levels were measured as described (39).

Measurement of the mitochondrial OXPHOS complex I activity

Activity of the mitochondrial OXPHOS complex I (NADH dehydrogenase) was determined using the Microplate Assay for Human Complex I Activity (MS141-MitoSciences), according to the manufacturer’s instructions.
Plasmid construction and DNA transfection of cells
A Myc-tag encoding sequence was ligated to the 5’ of the human FerT cDNA (24) that was already cloned in the p-EFIRE-P (pIREs) expression vector (40), constructing the p-EFIRE-MyfFerT (MycFerT) plasmid. The mitochondrial targeting sequence (MTS) derived from the integral mitochondrial protein cytochrome c oxidase subunit VIII (11) was then ligated to the 5’ of the Myc-human FerT cDNA in the above described vector to form the p-EFIRE-MTS-MycFerT (MTS-MycFerT) plasmid. A kinase-dead FerT (MTS-MycFerT<sub>201R</sub>) (41) was constructed by introducing a point mutation to the p-EFIRE-MTS-MycFerT plasmid, using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) with the following primers: 5’-cttccacaaatctcttgccag-3’, 5’-gcatatgggagattgctg-3’ according to the manufacturer’s instructions. The MycFerT, MTS-MycFerT, and MTS-MycFerT<sub>201R</sub> encoding plasmids were used to stably transfect NIH3T3 cells, using the transfection reagent Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions.

Quantification of cellular ATP and NAD levels
Cells were suspended in 400 μL cold 0.4 mol/L perchloric acid solution in 1.5-mL tubes. The mixture was incubated on ice for 15 minutes and was then centrifuged at 12,000 × g to remove the precipitate. The supernatant was neutralized with 4 mol/L K<sub>2</sub>CO<sub>3</sub> solution to pH 7.5, followed by incubation for 15 minutes on ice. Following centrifugation (10,000 × g), the supernatants were immediately used for chromatographic analysis. The chromatography apparatus consisted of Hitachi Elite LaChrom system equipped with diode array detector, column oven, autosampler, and a quaternary pump. All chromatographic analyses were performed at 30°C using SUPELCO SIL LC-18-S HPLC column (5 μm particle size, L × I.D. 25 cm × 2.1 mm), flow rate 0.2 mL/min under isocratic elution conditions with the following buffer composition [50 mmol/L potassium phosphate, 100 mmol/L triethylamine, pH 6.5 (adjusted with phosphoric acid):acetonitrile (98.5:1.5)]. Each analysis cycle was set to 30 minutes. The chromatographic flow was monitored at 260 nm and integrated using EZChrom Elite Software.

Cell survival assays
Death levels in transfected NIH3T3 cells were determined by using the MultiTox-Fluor Multiplex Cytotoxicity Assay (G9201, Promega) according to the manufacturer’s protocol or by Annexin V and propidium iodide (PI) fluorescent staining of cells, as described (24, 29).

Tumor formation assay in mice
All animal experiments were performed according to the guidelines of the Bar-Ilan Institutional Animal Care and Use Committee. Mice were housed five per cage with unlimited access to food and water and exposure to 12-hour light/dark cycles. Ten 8-week-old female immunocompromised nude mice (Harlan) were subcutaneously injected with 4 × 10<sup>6</sup> NIH3T3-transfected cells and tumor volume was determined as described before (37).

Immunohistochemical analysis of frozen tumor sections
Tumors from nude mice were dissected and fixed as previously described. Sections were stained with the appropriate antibody by a standard immunohistochemical (IHC) staining protocol (37) using anti-c-Myc (9E10, sc-40, Santa Cruz Biotechnology) followed by incubation with a secondary Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes, Invitrogen). Nuclei were visualized by staining with 0.1 μg/mL Hoechst.

The immunostained sections were viewed with an Axiosimager z1 microscope.

Statistical analysis
Statistical analysis was performed using the paired and unpaired Student t tests with a P < 0.05 considered significant.

Results
Fer and FerT reside in the mitochondria of spermatogenic and malignant cells
We previously showed that downregulation of Fer increases ROS level in colon carcinoma cells (39). To examine whether FerTsimilarly inhibits the generation of ROS in malignant cells, the protein was knocked down in colon carcinoma cells that endogenously express both Fer and FerT, using different, specific siRNA sequences (Fig. 1A and Supplementary Table S1). This led to a significant increase in the cellular level of ROS,

Figure 1. Downregulation of Fer or FerT induces ROS levels in colon carcinoma cells. A, protein level of Fer and FerT in HCT116 colon carcinoma cells 48 hours after transfection with control or specific siRNA. Fer and FerT were detected using anti-Fer/FerT antibodies by Western blot analysis. B, ROS levels were measured 48 hours after transfection of cells with the different siRNAs. Histograms represent means ± SE (n = 4).
to an extent that was similar to the one measured in Fer-depleted cells (Fig. 1B and Supplementary Fig. S1A and S1B). The stimulatory effects of Fer and FerT depletion on the elevation of ROS production suggested the regulatory involvement of these proteins in cellular ROS generating processes. These can take place either outside or within the mitochondria (42). Additional support for the presence of Fer or FerT in the mitochondria is provided by the association of Fer with Stat3 (31) and the recent detection of Stat3 in the mitochondria of mammalian cells (11). We therefore examined the accumulation of Fer and FerT in the mitochondria of normal cells.

Because both Fer and FerT accumulate in spermatogenic cells (32, 43), we first determined whether the two proteins are coexpressed in mature spermatozoa, as they are in malignant cells. We initiated our study with mature spermatozoa, which can be reliably isolated and identified. Both enzymes were detected in whole-cell lysates prepared from mouse caudal epididymal spermatozoa and from bovine ejaculated sperm (Fig. 2A).

To determine the subcellular localization of Fer in sperm cells, ejaculated bovine sperm cells were immunocytochemically stained with affinity-purified anti-N-Fer antibodies. The observed immunostaining was confined to the sperm midpiece section, which primarily represents the mitochondrial sheath of these cells (44). Furthermore, the staining of Fer overlapped the immunostaining of the mitochondrial ATP synthase 5a subunit (ATP5A) when examined by fluorescence microscopy (Fig. 2B). To further substantiate the overlapping signals of the Fer and ATP5A subunit immunostaining, confocal microscopy analysis was applied. This approach showed clear colocalization of Fer and the mitochondria (Fig. 2C), thereby indicating the presence of Fer in the mitochondria of sperm cells.

To further corroborate this finding and to examine whether also FerT resides in the mitochondria of spermatogenic cells, we performed a subcellular fractionation analysis. Proteins were extracted from cytoplasmic and mitochondrial fractions prepared from mouse testicular cells. Fer and FerT were found to reside in the mitochondrial fraction, whose purity was confirmed using specific mitochondrial markers: voltage-dependent anion channel (VDAC) protein (associated with the outer mitochondrial membrane) and NDUFA9 [NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9–associated with complex 1 of the ETC] proteins. α-Tubulin, which normally resides in the cytoplasm and the regulator of chromosome condensation 1 (RCC1), whose accumulation is confined to the cell nucleus, served as extra-mitochondrial markers (Fig. 3A). Similarly, Fer was present in mitochondrial fractions prepared from mouse liver and heart tissues, which prominently express this tyrosine kinase (Fig. 3B and C).

Fer and FerT are both expressed in colon carcinoma cells (24). We therefore examined whether these 2 proteins also reside in the mitochondria of malignant cells. Similarly to testicular cells, both Fer and FerT were detected in mitochondria purified from the HCT116 colon carcinoma cells (Fig. 3D).

To more directly verify the presence of Fer in the mitochondria of malignant cells, affinity-purified anti-N-Fer antibodies were applied in an immunocytochemical analysis. Staining of HCT116 colon carcinoma cells with anti-N-Fer antibodies and with a specific anti-mitochondrial monoclonal antibody

Figure 2. Expression of Fer and FerT in spermatozoa. A, sperm cell lysates were prepared from ejaculated bovine sperm incubated in capacitation medium or left uncapsulated and from mouse caudal epididymal spermatozoa. Proteins were resolved by 10% SDS-PAGE, and Fer and FerT were detected using anti-Fer/FerT antibodies in a Western blot analysis. AKAP3 was used as a sperm-specific marker. B, immunocytochemical staining of bovine-ejaculated sperm for Fer (anti-N-Fer antibodies; red) and ATP synthase 5c subunit (anti-ATP5A; green). The diffraction interference contrast (DIC) and the merged images are shown in the bottom of the figure. Bars, 10 μm. These images represent one of three independent experiments, which gave similar results. C, confocal microscopic images of immunoocytochemically stained bovine-ejaculated sperm for Fer (red) and ATP5A (green). Bars, 10 μm. Merged images are shown at the bottom. Images boxed in red and white are enlarged in the right top and bottom images, respectively. These images were obtained with the Zeiss LSM 510 confocal microscope with consecutive sections of 0.7 μm, and they represent one of four independent experiments, which gave similar results.
revealed overlapping staining of the two antibodies, demonstrating the presence of Fer in the mitochondria of malignant cells (Fig. 3E). Collectively, these observations substantiate the accumulation of Fer and FerT in the mitochondria of colon carcinoma cells.

**Fer and FerT associate with complex I of the ETC in testicular and colon carcinoma cells and support complex I activity**

The mitochondrial presence of Fer or FerT, and the increased ROS production upon downregulation of either of
Figure 4. Fer and FerT associate with the mitochondrial ETC complex I and support its activity. A, protein extracts were prepared from cytoplasmic (cyto) and mitochondrial (mito) fractions of mouse testicular cells. ETC complex I was immunoprecipitated from the mitochondrial extract (IP:mitoCxI). Proteins from all samples were resolved by SDS-PAGE. Fer and FerT were detected with anti-Fer/FerT antibodies by Western blot analysis. NDUFA5 (complex I) and ATP5A (complex V) served as markers for immunoprecipitation selectivity. B, protein lysates were prepared from cytoplasmic and mitochondrial fractions of mouse liver and hearts. Complex I was immunoprecipitated from the mitochondrial fractions, and all marked proteins were detected using specific antibodies in a Western blot analysis. C, protein lysates were prepared from cytoplasmic and mitochondrial fractions of SW620 CC cells. Complex I (top) and complex V (bottom) were immunoprecipitated from the mitochondrial fractions. Precipitation with IgG-conjugated beads (IP: IgG beads, top) served as a control for the specific immunoprecipitation of complex I (top). IP-Supp. in the bottom denotes the supernatant of the immunoprecipitation with anti-complex V antibody. All presented proteins were detected using specific antibodies in a Western blot analysis. D, complex I was immunoprecipitated from mitochondrial fraction of HCT116 CC cells. All proteins were resolved by SDS-PAGE, and the presented proteins were detected using specific antibodies by Western blot analysis. All presented images represent one of three independent experiments, which gave similar results. E, knockdown of Fer or FerT impairs the ETC complex I activity. HCT116 cells were treated with either control, scrambled (siRNA-scr), siRNA directed toward Fer mRNA (siRNA-Fer), or siRNA directed toward FerT mRNA (siRNA-FerT). Complex I was isolated from each sample, and its activity was measured spectrophotometrically, as described in Materials and Methods. The activities in Fer- and FerT-depleted cells were normalized to the control (siRNA-scr) activity, which was given a value of 100. Histograms represent means ± SE (n = 3).
Figure 5. Mitochondrial presence of FerT salvages complex I activity and ATP accumulation under stress conditions. A, NIH3T3 cells were transfected with either the empty pIRES vector (NIH3T3-pIRES; a–d) or with a plasmid encoding MTS-MycFerT (NIH3T3-MTS-MycFerT; e–h). Cells were fixed and stained with Hoechst 33342 to visualize nuclei (blue), with anti-complex IV for mitochondria (red), and with anti-Myc for FerT (green). (Continued on the following page.)
these proteins, suggested their association with the respiratory ETC components in malignant cells. Because generation of mitochondrial ROS is linked to ETC complex I activity (45), we examined the association of Fer and FerT with this complex and with complex V, which functions downstream in the ETC. Complexes I and V were immunoprecipitated from normal and from malignant cells, using a monoclonal antibody that captures components of these complexes. To ensure specificity of the immunoprecipitation, precipitated proteins were challenged with antibodies directed toward mitochondrial proteins that are part of either complex I or complex V or that associate with the mitochondrial membrane. These included the complex I component, NDUFA9, ATP5A, which is a part of the ETC complex V, and VDAC. As expected, NDUFA9 co-immunoprecipitated with the anti-complex I antibody from mitochondrial extracts of testicular, heart, and liver cells. ATP5A, which served as negative controls, was not precipitated by this antibody (Fig. 4A), indicating the specificity of the assay. Interestingly, while Fer and FerT coprecipitated with complex I from the mitochondria of testicular cells (Fig. 4A), Fer did not associate with complex I precipitated from mitochondria of normal heart or liver (Fig. 4B).

To examine the association of Fer and FerT with mitochondrial complex I of malignant cells, the complex was immunoprecipitated from SW620 and HCT116 colon carcinoma cells. Both Fer and FerT, as well as Stat3, coprecipitated with complex I prepared from mitochondria of the two malignant cell types, whereas VDAC, which served as a negative control in these experiments, was not precipitated (Fig. 4C and D). However, the three proteins did not coprecipitate with complex V prepared from SW620 cells (Fig. 4C). Thus, similarly to testicular cells, Fer and FerT associate with complex I in the mitochondria of malignant colon carcinoma cells.

On the basis of the above results, we hypothesized that Fer or FerT take part in the mitochondrial functioning in colon carcinoma cells. To test this notion, we examined the effect of Fer or FerT depletion on the functioning of the ETC complex I. Fer or FerT were knocked down using specific siRNAs, and the activity of the complex was measured in solubilized mitochondria. Downregulation of either Fer or FerT in colon carcinoma cells decreased complex I activity by approximately 40% (Fig. 4E).

**Directed mitochondrial accumulation of FerT salvages complex I activity, ATP production, and cell survival under nutrient- and oxygen-deprived conditions**

To further explore the outcome of mitochondrial accumulation of Fer and FerT in somatic cells, we directed ectopic accumulation of the two enzymes in mitochondria of NIH3T3 cells. Myc-tagged Fer and FerT were each fused in the N-terminus of the protein to a mitochondrial targeting sequence (MTS) derived from the cytochrome c oxidase subunit VIII, an integral mitochondrial protein. NIH3T3 cells were stably transfected with the Fer- and FerT-expressing vectors or with the empty vector alone (NIH3T3-pIRES cells). The mitochondrial accumulation of the two ectopic proteins was examined using immunofluorescence. While Fer could not be stably and ectopically overexpressed in the mitochondria of NIH3T3 cells (not shown), the expression of MTS-MycFerT was stable and was confined to the mitochondria of the transfected cells (NIH3T3-MTS-MycFerT cells; Fig. 5A).

The directed mitochondrial accumulation of FerT might endow the transfected cells with an increased efficiency of complex I activity and ATP production. We therefore measured the mitochondrial complex I activity and the steady-state levels of ATP in NIH-pIRES and NIH3T3-MTS-MycFerT cells grown under normal and stress conditions. The most significant difference between complex I activity in mitochondria of NIH3T3-MTS-MycFerT cells in comparison to NIH-pIRES cells was observed when starvation conditions were applied. Notably, this significant effect of FerT on complex I was observed for kinase active FerT but not for kinase-inactive FerT mutant (MTS-MycFerT<sup>521/521</sup>; Fig. 5B, ref. 41). The enhanced activity of complex I was further corroborated by an observed increase in the level of the complex I primary metabolite NAD (45) in starved NIH3T3-MTS-MycFerT cells (Fig. 5C). Notably, the difference in complex I activity under starvation coincided with the relative levels of ATP which were measured in the two cell types grown under this stress condition. A profound increase of 37% in the cellular ATP level was seen in NIH3T3-MTS-MycFerT cells compared with the NIH3T3-pIRES cells, when the 2 cell types were grown under nutrient and growth factor deprivation conditions (Fig. 5D).

To examine whether NIH3T3-pIRES and NIH3T3-MTS-MycFerT cells undergo apoptotic death under stress conditions, the presence of apoptotic markers was analyzed in lysates prepared from the 2 cell types. The apoptotic marker cleaved caspase-3 was detected only in hypoxic NIH3T3-pIRES cells but not in NIH3T3-MTS-MycFerT, nor was this marker detected in NIH3T3-pIRES or NIH3T3-MTS-MycFerT cells grown under nutrient starvation condition (Fig. 6A). The increased apoptotic death in hypoxic NIH3T3-pIRES cells was further corroborated by an increased staining of these cells by Annexin V and PI under hypoxia (Fig. 6B). A higher percentage of dead NIH3T3-pIRES cells in comparison to NIH3T3-MTS-MycFerT cells grown under nutrient starvation was seen when the multiplex cytotoxicity assay had been applied (Fig. 6C). Thus, directed expression of FerT increases the survival of NIH3T3 cells under stress growth conditions.

(Continued)
Directed mitochondrial accumulation of FerT is required and sufficient to drive tumor formation in vivo

To directly examine the role of mitochondrial FerT in tumor initiation and/or progression, we examined the ability of NIH3T3 cells expressing FerT either bearing (NIH3T3-MTS-MycFerT, cells) or lacking MTS (NIH3T3-MycFerT cells) to drive the formation of tumors in vivo. NIH3T3 are immortal cells in culture, and we therefore assumed that the ultimate criterion for deciphering an effect of FerT on their malignant potency is to examine their ability to form tumors in mice. Control NIH3T3-pIRES cells, NIH3T3- MTS-MycFerT, or NIH3T3-MycFerT cells were subcutaneously injected into immunocompromised (nude) mice. Strikingly, in all 10 injected animals, 28 days postinjection, MTS-MycFerT–expressing cells started to develop highly vascularized tumors, which after 42 days, reached an average size of 1 cm³. Notably, the NIH3T3- MycFerT cells–expressing FerT, which lacks MTS (Supplementary Fig. S2A), developed small tumors in only 3 of 10 injected mice by day 42. No tumor developed during this period of time by NIH3T3-pIRES cells harboring the empty expression vector, pIRES (Fig. 7A and B, Supplementary Fig. S2B and Supplementary Table S2). Immunoblotting of tumor protein lysates and immunostaining of tumor sections confirmed the ectopic presence of MTS-MycFerT in the elicited tumors (Fig. 7C and D). Thus, guided mitochondrial accumulation of FerT is required and sufficient to endow nonmalignant cells with tumor-forming capacity.

Discussion

Reprogrammed metabolism and altered energy generation pathways are hallmarks of the abnormal survival and proliferation of malignant cells (7–10). Although functionally modified, cancer cell mitochondria are not globally dysfunctional but are rather reprogrammed to support the unique metabolic needs of malignant cells (46, 47). Moreover, mitochondrial ATP production replenishes aerobic glycolysis in cancer cells by its coupling to the functioning of the key glycolytic enzyme, Hexokinase II, which is physically associated with the mitochondrial outer membrane anchored protein, VDAC1 (48). Hence, cancer cell mitochondria may adopt unique tools to direct and support their modified functioning. In the current study, we show that Fer and its spermatogenic, meiosis-specific variant, FerT, can provide such a function. We found that Fer and FerT associate with complex I of the mitochondrial ETC in spermatogenic and in colon carcinoma cells. Accordingly, downregulation of either one of these proteins impaired the functionality of this complex and increased cellular ROS levels. Thus, Fer and FerT are part of the metabolic reprogramming system that restrains the accumulation of ROS and their potential damaging effects (49) in colon carcinoma cells. In spermatogenic cells, the association of Fer and FerT with complex I and the maintenance of low ROS levels should reduce the risk of DNA damage caused to chromosomal DNA undergoing extensive recombination in primary spermatocytes that harbor relatively high levels of Fer and FerT (50). In sperm, Fer and FerT may enhance the production of ATP, which propels the motility of this cell, and aid the sperm cells, avoiding energy depletion and death due to low availability of nutrients and oxygen.

Computational analysis failed to identify MTS in either Fer or FerT. Thus, the translocation of these enzymes to the mitochondria of spermatogenic or colon carcinoma cells may
depend on their cotransport with a protein that is present in these cells and does bear MTS. A similar possibility was raised for the Stat3 protein, which lacks MTS and translocates to the mitochondria (11).

Our findings portray Fer and FerT as mitochondrial proteins that can support energy generation and restrict ROS production in sperm and in malignant cells. The harnessing of Fer and FerT to the reprogrammed mitochondria of malignant cells enables these cells to improve their energy production under restricted availability of essential nutrients like glucose. This enables cancer cells to cope with stress conditions that are prevalent in solid tumors when malignant cells outgrow the carrying capacity of the local vasculature. Several reported findings envisage a proliferation-supportive role of Fer, in
malignant cells grown under stress conditions. For example, the requirement of Fer for the deregulated proliferation of cancer cells in vitro is most profoundly manifested when the cells are grown in agarose under nonanchoring conditions accompanied by restricted nutrient and oxygen availability (51). Furthermore, shRNA knockdown studies carried out with mouse xenografts models showed that Fer plays an important role during the initiation of primary tumors (51) and is essential for the development of secondary metastases (26, 27). This might reflect, at least in part, a required contribution of Fer to the initiation and early stages of tumor formation, during which the nutrient and oxygen supplying vasculature has not yet been established. However, the molecular mechanisms through which Fer exerts this supportive activity in tumor formation remained elusive. On the basis of our current study, we propose that Fer and FerT support tumor initiation and formation through their mitochondrial functions. Similar role was reported for the Fer-interacting mitochondrial protein Stat3 (31), which is required for the proliferation of RAS-transformed cells grown in agarose, under nonadherent and nutrient-restrictive conditions (11). In accordance with this notion, we found in the current study that mitochondrial FerT augments the activity of the ETC complex I in transfected cells and that this is accompanied by a significant increase in the cellular ATP pool, thereby enabling cellular survival, under nutrient and oxygen deprivation conditions. Thus, FerT enables colon carcinoma cells to survive nutrient and oxygen deprivation conditions, which are experienced by these cancerous cells during tumor formation. This is most probably carried out by an FerT-directed upregulation of the initiation rate of the mitochondrial oxidative phosphorylation process as is reflected by an increase in NAD and ATP levels, under these stress conditions. Notably, while the increased survival of FerT-expressing cells under nutrient deprivation could be linked to an increased activity of complex I, the increased survival of NIH3T3-MTS-MycFerT cells under hypoxia seems to be complex I independent and may reflect an antiapoptotic activity of FerT in the mitochondria. The effects of FerT on the mitochondrial complex I activity could be either kinase-dependent or -independent. Using a kinase-inactive FerT, we found that the effect of FerT on complex I is, at least in part, kinase activity–dependent.

The significance of the mitochondrial role of FerT in tumor formation is most profoundly demonstrated by the ability of MTS-MycFerT to endow NIH3T3 cells with the capacity to efficiently form fast-growing tumors in all mice injected with these FerT-expressing cells. Unlike this observation, MTS lacking FerT drove the formation of slowly growing tumors in only 30% of the injected animals. It should also be noted that FerT was previously shown to exert only minor tyrosine kinase activity in the cytoplasm of transfected somatic cells and that this activity is significantly lower than that exerted by Fer (21, 31). We therefore assume that the major effect of FerT on tumor formation by the transfected cells results predominantly from its mitochondrial activity, rather than from a putative deregulated tyrosine kinase activity in the cytoplasm.

Collectively, our data suggest that modified mitochondrial functioning can endow immortal cells with the ability to form tumors. Thus, reprogrammed mitochondria can drive the initiation and progression of tumors. Identification of key components in the mitochondrial reprogramming process should therefore offer new targets for cancer intervention.

Disclosure of Potential Conflicts of Interest

Y. Elkis is a consultant at UriFer and is also an advisory board member of UriFer. U. Nir is the CTO at UriFer. Y. Elkis and U. Nir have ownership interests (including patents) in UriFer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: E. Yaffe, E. Hikri, Y. Elkis, A. Segal, S. Shpungin, U. Nir


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Elkis, O. Cohen, A. Segal, A. Makovski, S. Shpungin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Yaffe, Y. Elkis, O. Cohen, A. Segal, A. Varvaki, U. Nir

Writing, review, and/or revision of the manuscript: E. Yaffe, Y. Elkis, O. Cohen, A. Segal, S. Shpungin, U. Nir

Study supervision: U. Nir

Grant Support

This research was supported by grants awarded by the Florence Calb, Milstein family, Meir Yosef Nügi, Sheizman Louis, and Seladi Family Foundations. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 14, 2014; revised August 11, 2014; accepted August 27, 2014; published OnlineFirst September 18, 2014.

References


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Etai Yaffe, Elad Hikri, Yoav Elkis, et al.


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