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Linkage of E2F1 transcriptional network and cell proliferation with respiratory chain activity in breast cancer cells

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Mitochondria are multifunctional organelles; they have been implicated in various aspects of tumorigenesis. In this study, we investigated a novel role of the basal electron transport chain (ETC) activity in cell proliferation by inhibiting mitochondrial replication and transcription (mtR/T) using pharmacological and genetic interventions, which depleted mitochondrial DNA/RNA, thereby inducing ETC deficiency. Interestingly, mtR/T inhibition did not decrease ATP levels despite deficiency in ETC activity in different cell types, including MDA-MB-231 breast cancer cells, but it severely impeded cell cycle progression, specifically progression during G2 and/or M phases in the cancer cells. Under these conditions, the expression of a group of cell cycle regulators was downregulated without affecting the growth signaling pathway. Further analysis suggested that the transcriptional network organized by E2F1 was significantly affected because of the downregulation of E2F1 in response to ETC deficiency, which eventually resulted in the suppression of cell proliferation. Thus, in this study, the E2F1-mediated ETC-dependent mechanism has emerged as the regulatory mechanism of cell cycle progression. In addition to E2F1, FOXM1 and BMYB were also downregulated, which contributed specifically to the defects in G2 and/or M phase progression. Thus, ETC-deficient cancer cells lost their growing ability, including their tumorigenic potential *in vivo*. ETC deficiency abolished the production of reactive oxygen species (ROS) from the mitochondria and a mitochondria-targeted antioxidant mimicked the deficiency, thereby suggesting that ETC activity signaled through ROS production. In conclusion, this novel coupling between ETC activity and cell cycle progression may be an important mechanism for coordinating cell proliferation and metabolism.

Cell proliferation is a process that simultaneously demands macromolecular synthesis in a greater amount and ATP supply at a higher rate to increase the total biomass and, finally, divide the cells into two daughter cells.[1] To meet these demands, actively dividing cells utilize mitochondria as biosynthetic organelles rather than powerhouses, which are primarily involved in oxidative phosphorylation (OXPHOS).[2] However, this does not necessarily mean that OXPHOS function is not important for actively dividing cells such as cancer cells. Indeed, OXPHOS still makes a significant contribution to ATP supply in cancer cells,[3] thereby supporting cancer cell proliferation and survival.

In some cases, mitochondria appear to play more active roles in tumorigenesis; for example, upon the activation of oncogenes, the cancer cells exploit the electron transport chain (ETC) and elevate the production of reactive oxygen species (ROS).[4–6] In some hereditary cancers, truncated mitochondrial enzymes caused by mutations may have direct oncogenic properties that affect tumor susceptibility.[7]

In addition, it is also possible that basal activities of mitochondria may play some fundamental role in cancer cells, such as sustaining their proliferation potential, given that cell proliferation is degraded greatly when mitochondrial replication and transcription (mtR/T) are inhibited.[8] Because the inhibition of mtR/T primarily leads to deficient OXPHOS function,[4,9–11] this proliferation defect could be explained simply by an insufficient production of ATP. However, we recently discovered that ATP levels remained normal under mtR/T inhibition in several cancer cell types, but their proliferation was still severely impeded. This finding implies that an unknown mechanism regulates cell proliferation depending on the levels of ETC activity. Therefore, in this study, we explored the details of the aforementioned defect in proliferation under mtR/T inhibition, which led us to propose a novel role for ETC activity in cell cycle progression. To the best of our knowledge, this is the first demonstration of a functional linkage between ETC activity and the core regulatory mechanism of cell cycle progression.

Materials and Methods

Full Methods and Materials are available as Supporting Information.

Results

Cell proliferation arrest is induced under electron transport chain-deficient conditions in human breast cancer cells. First, we observed mitochondrial DNA (mtDNA)-less cells, so-called pseudo-ρ0 cells.[9] The mtDNA exclusively encodes RNA and proteins required for the formation of ETC complex; therefore, the defects in pseudo-ρ0 cells are considered to be associated with ETC functions and limited to their relevant activities. This hypothesis has been supported by extensive studies of ρ0 cells,[10,12] thereby providing a valuable model for the evaluation of the significance of ETC functions in cells.

In this experiment, we examined pseudo-ρ0 populations obtained from several human breast cancer cell lines, including the MDA-MB-231 (MDA) cell line, after treating them with ethidium bromide (EtBr), which is an mtR/T inhibitor that is used routinely to selectively deplete mtDNA,[13] as described previously.[11] Figure 1(a–c) shows the effects of EtBr treatment on the condition of mitochondria in MDA cells, which demonstrates that mtDNA and mitochondria-encoded cytochrome b (Cyt.b) mRNA were decreased (Fig. 1a,b). The mitochondrial membrane potential ($\Delta\Psi$m), a direct indicator of ETC functionality, was virtually lost within 5 days (Fig. 1c). However, the amount of ATP remained at a normal level (Fig. 1d), presumably due to the compensation by glycolytic activity. These observations agree with previously described features of ρ0 cells,[10,11] which support the use of ρ0 or ETC-deficient cells for studying the roles of ETC functions uncoupled from ATP synthesis.

Cell proliferation was markedly inhibited under the ETC-deficient conditions described above (Fig. 1e). The G2 and/or M phases were specifically interrupted in the MDA/ρ0 cells with concomitant enlargement of the cells (Fig. 1f–h). The short-term treatment of cells with EtBr had no effect, as indicated in the DNA histogram (Fig. 1g, 0 *vs* 30 min); this excludes the possibility that EtBr treatment interferes with the

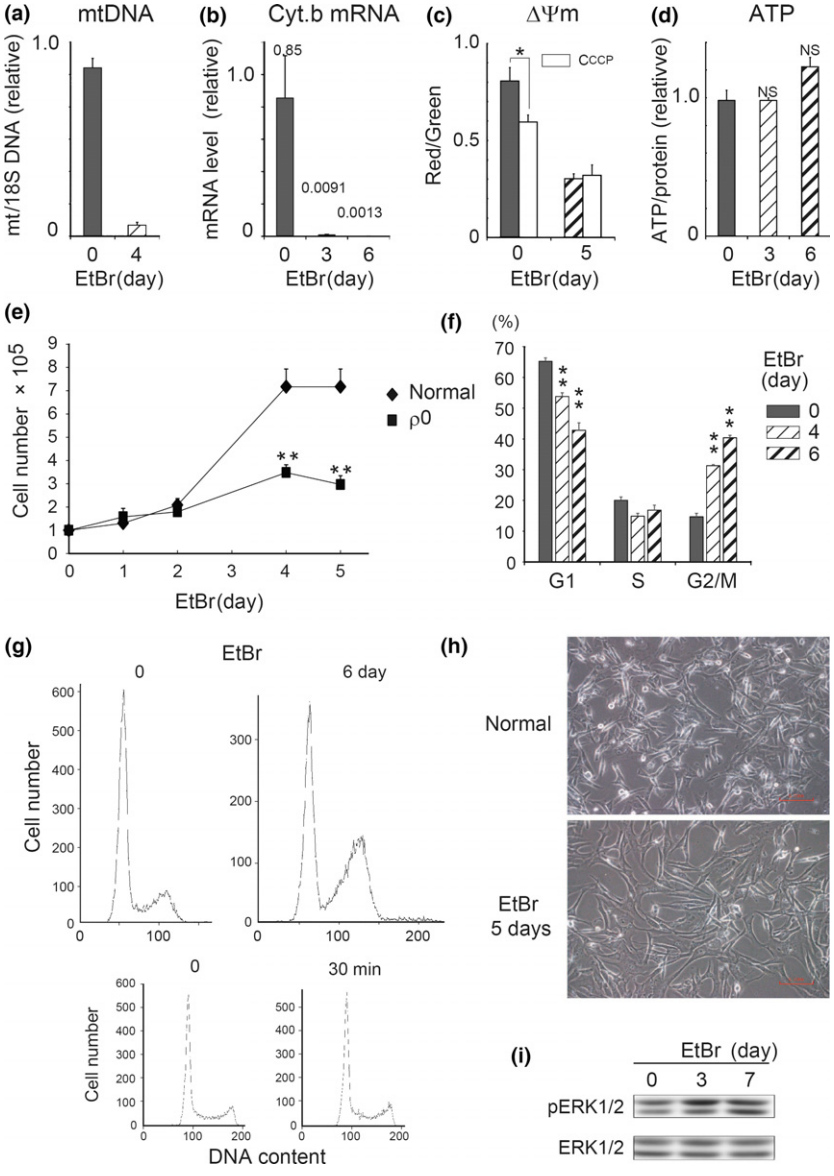

Fig. 1. Cell cycle arrest induced in electron transport chain (ETC)-deficient MDA-MB-231 (MDA) cells. Cells exposed to ethidium bromide (EtBr) (250 ng/mL) on the days indicated (MDA/ρ0) were analyzed. (a) mitochondrial DNA (mtDNA) relative to genomic DNA (18S) (mt/18S DNA) quantified using qPCR. (b) Levels of cytochrome b (Cyt.b) mRNA as analyzed by qRT-PCR. (c) Mitochondrial membrane potential ($\Delta\Psi$m) monitored with Mito-ID. (d) ATP levels were determined using an ATP determination kit and normalized against the protein levels. (e) Cell proliferation in the presence (ρ0) and absence (normal) of EtBr. (f) Cell cycle distribution determined using DNA histograms obtained by flow cytometry. (g) Representative DNA histograms for normal (0 day) and ρ0 (6 days) cells. Bottom: Histograms did not change with the incubation of cells with EtBr (250 ng/mL) for 30 min. (h) Morphology of cells incubated with or without EtBr (250 ng/mL) for 5 days. Magnification: ×100. (i) Activation-dependent phosphorylation of ERK1/2 as detected by western blot. The pair shown is for the phosphorylated (p) form and for the total protein. $^*P < 0.05$ and $^{**}P < 0.01$.

subsequent staining of cells with PI. According to our results of the dye exclusion test, cell viability was above 90% during the observation of cell proliferation (data not shown) and the sub-G1 population comprised <1% in all of the DNA histograms, which implied that apoptosis was negligible. These observations indicate that the ETC-deficient cells almost completely lost their proliferative capacity even when ATP production was at normal levels, thereby suggesting a previously unknown role for the ETC in cell proliferation. Interestingly, cell-signaling molecules, such as ERK1/2, which are central molecules in controlling cell proliferation, were active at levels comparable to those under normal conditions (Fig. 1i). In addition, the suppression of cell proliferation did not appear to be associated with the DNA damage response (see below), although high doses of EtBr affected nuclear DNA as an intercalator. Collectively, mitotic catastrophe, a mechanism that senses mitotic failure and leads to cell death, such as necrosis, or senescence, might occur under the conditions.

Similar suppression of proliferation was also observed in pseudo-ρ0 cells from other cell lines; namely, T-47D (Fig. 2a) and MCF7 (Fig. S1a). Furthermore, in T-47D/ρ0 cells, the cell cycle was interrupted at G2 and/or M phases (Fig. 2b), as found in MDA/ρ0. However, G1/S arrest was dominant in MCF7/ρ0 cells (Fig. S1b), which was probably attributable to the upregulation of p21CIP1 and p27KIP1 cyclin-dependent kinase inhibitors (CKI) at the mRNA and protein levels, respectively (Fig. S1c,d). These inhibitors were not induced in MDA/ρ0 cells. In this context, it should be noted that MCF7 retained wild-type p53, whereas T-47D and MDA did not.[14] In a further study, we explored the defects in cell cycle progression under ETC deficiency, especially the CKI-independent mechanisms that resulted in the defects in G2 and/or M phase progression in MDA and T-47D/ρ0 cells.

Downregulation of a set of cell cycle regulators in electron transport chain-deficient cells. To obtain insight into the mechanisms described above, we studied changes in gene expression

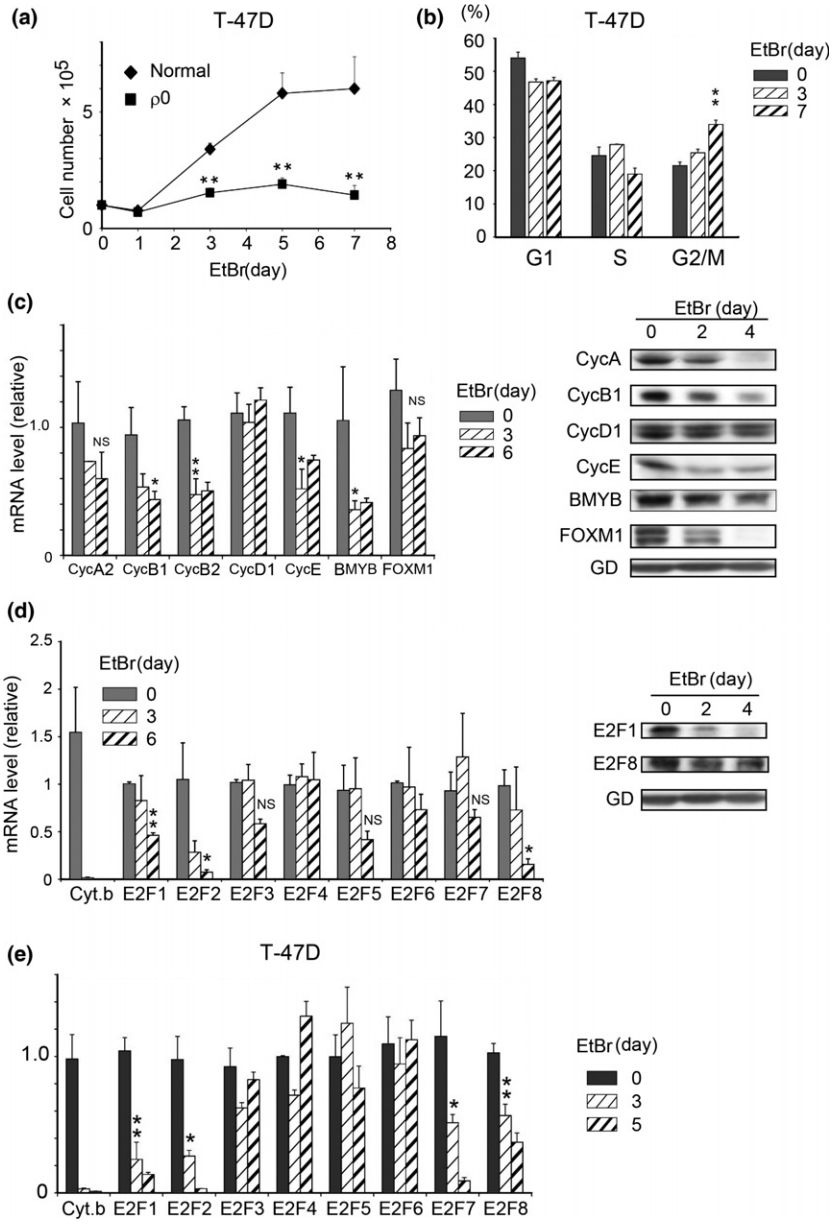

Fig. 2. Downregulation of cell cycle regulators in electron transport chain (ETC)-deficient MDA and T-47D cells. Cell proliferation (a) and cell cycle distribution (b) determined as described in Figure 1(e, f) in ethidium bromide (EtBr)-treated T-47D human breast cancer cells. (c, d) MDA/ρ0 cells, as described in Figure 1, were analyzed on the days indicated. (c) Left: mRNA levels of cyclins (A2, B1, B2, D1 and E), BMYB and FOXM1 quantified using qRT-PCR. Right: protein levels of the corresponding mRNA detected by western blot. GD was the loading control. (d) Left: mRNA levels of Cyt.b and E2F1–8 evaluated by qRT-PCR. Right: Western blot analysis of E2F1 and 8. GD was the loading control. (e) mRNA levels of Cyt.b and E2F1–8 quantified by qRT-PCR in T-47D/ρ0 cells. $*P < 0.05$ and $**P < 0.01$. NS, not significant.

in response to the inhibition of mtR/T. Initially, we analyzed microarray data using murine mammary epithelial cells and found that many proliferation-related genes were downregulated under ETC-deficient conditions. Intriguingly, many of these genes have been previously identified as transcriptional targets of E2F.[15,16] These genes included cyclins (A2, B1 and E1) and other components involved structurally and/or functionally in cell cycle progression (Table S1).

Downregulation of a similar set of E2F-targeted genes, including cyclins A2, B1, B2 and E, was noted in the MDA/ρ0 cells (Fig. 2c). In addition to these cyclins, *Foxm1* and *Bmyb*, having roles in G2 and M phase regulation as components of the transcriptional regulator complex with MuvB,[17] were downregulated; their expression levels were also attenuated in the MDA/ρ0 cells (Fig. 2c). The expression of cyclin D1, which was absent in the list of downregulated genes, was affected slightly only at the protein level; therefore, it was used as a control due to its relative insensitivity to ETC activity.

Downregulation of E2F1 in electron transport chain-deficient cells. Interestingly, in addition to the aforementioned cell cycle regulators, E2F family members (activator E2F1 and 2) were included in the list of downregulated genes (Table S1). In the MDA/ρ0 cells, E2F1, 2 and 8 (a repressor E2F) exhibited significant sensitivity to ETC deficiency in terms of their mRNA levels (Fig. 2d). At the protein level, the expression of E2F1 was particularly sensitive; it decreased dramatically within 2 days of the treatment (Fig. 2d). The expression level of E2F2 was very low; therefore, its expression was undetectable in MDA cells by immunoblotting. Thus, the E2Fs, particularly E2F1, were found to be ETC-sensitive transcriptional regulators. Changes in the expression patterns of these E2Fs were also observed in the pseudo-ρ0 cells from T-47D (Fig. 2e) and MCF7 (Fig. S1e, left), thereby suggesting that the phenomenon is independent of CKI induction or defects in cell cycle progression, at G1/S boundary or during G2 and/or M phases, but it is primarily dependent on ETC deficiency.

E2F1 downregulation as a trigger for the downregulation of cell cycle regulators and cell proliferation. Our results mentioned earlier suggest that E2F1 was first downregulated in response to ETC deficiency, followed by its target cell cycle regulators, which eventually suppressed the cell proliferation. As expected, the knockdown of E2F1 with siRNA decreased the expression of the set of cell cycle regulator genes that responded to ETC deficiency, except for cyclin E and the negative control of cyclin D1 (Fig. 3a–c). Therefore, cell proliferation was significantly influenced by E2F1 silencing, whereas little change was observed using siRNA for E2F2 and 8 (Figs 3d, S2a; E2F8).

Significance of BMYB and FOXM1 downregulation for defects in cell cycle progression during the G2 and/or M phases. The expression of the G2 and M phase regulators, BMYB and FOXM1, was also sensitive to ETC deficiency (Fig. 2c); they appear to be under the transcriptional control of E2F1 similar to cyclins A and B (Fig. 3b). However, unlike the two cyclins, the siRNA for E2F1 was ineffective in downregulating the expression of BMYB and FOXM1 at the protein level (Fig. 3c), thereby implying that their expression was subject to an additional layer of regulation at the protein level as well as the transcriptional regulation by E2F1 (Fig. 3e). Therefore, to assess the roles of BMYB and FOXM1, we performed

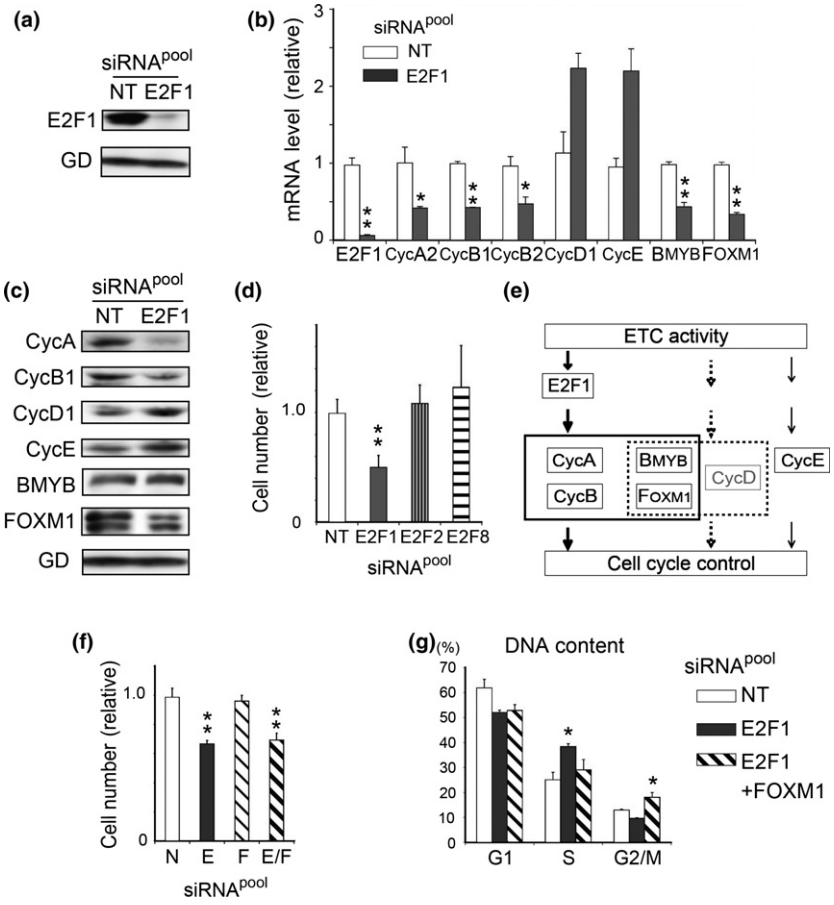

Fig. 3. Downregulation of cell cycle regulators in E2F1-knockdown cells. MDA cells were treated with 50 nM of ON-TARGETplus Human E2F1 siRNA-SMARTpool (E2F1) or non-targeting Pool siRNA (NT). (a) Validation of E2F1 knockdown by western blotting in cells incubated with siRNA for 48 h. (b) mRNA levels of cyclins (A2, B1, B2, D1 and E), BMYB and FOXM1, which were analyzed together with that of E2F1 by qRT-PCR. (c) Western blot analysis of proteins corresponding to the mRNA in (b). GD was the loading control. (d) Effects of E2F1 knockdown (7 days) on cell proliferation compared with those of E2F2 and E2F8. (e) Schematic representation of the electron transport chain (ETC)-dependent cell cycle control mechanisms (see text). (f) Effects of double knockdown of E2F1 and FOXM1 (6 days) with the siRNA on cell proliferation. siRNA: N; NT, E; E2F1, F; FOXM1, E/F; E2F1+ FOXM1. (g) Cell cycle distribution under the knockdown of E2F1 alone or in combination with FOXM1 for 6 days. $*P < 0.05$ and $**P < 0.01$.

silencing of *FOXM1* and *BMYB* with siRNA for themselves instead of E2F1 (Fig. S2a; FOXM1, BMYB).

Knockdown of FOXM1 (F) or BMYB (M) alone had no obvious effects either on the cell number or on the cell cycle distribution (cell number, Figs 3f, S2b; cell cycle distribution, Fig. S2d). When they were knocked down simultaneously with E2F1, we detected proliferation inhibition (Figs 3f, S2b; E/F and E/M). However, the effect was almost the same as that obtained by the single knockdown of E2F1 (E), which suggests that the proliferation potential of the cells was affected principally by E2F1, rather than by FOXM1 or BMYB.

The cell cycle distribution was influenced by the levels of FOXM1 and BMYB. Thus, silencing of E2F1 alone resulted in the accumulation of cells in the S phase, but the simultaneous silencing of FOXM1 or BMYB together with E2F1 elicited a slight but significant shift in the accumulation of cells from the S phase to the G2 and/or M phases. This suggests that these two proteins have important roles in regulating the progression of G2/M phase (Figs 3g, S2c). Triple knockdown of the three genes suggests that the effects of BMYB and FOXM1 knockdown were redundant (Fig. S2e), which is consistent with a previous demonstration that BMYB and FOXM1 function in a common mechanism that operates at G and M phases.[17] In summary, our results suggest a mechanism for cell proliferation suppression in ETC-deficient cells, where the downregulation of E2F1 causes defects in cell cycle progression during the G2 and/or M phases when coupled with the downregulation of BMYB and FOXM1.

Altogether, the ETC activity was first coupled with the control of cell proliferation via the regulation of cell cycle-related gene expression. In an emerging mechanism, the ETC activity modulates multiple pathways, including E2F1-mediated transcription (Fig. 3e, box; solid line) and non-transcriptional mechanisms (Fig. 3e, box; broken line), which regulate the expression of components of the core cell cycle machinery.

Recapitulated electron transport chain-deficient phenotypes in mitochondrial transcription factor A-knocked down cells. We further investigated the novel link between the ETC activity and cell cycle regulation using additional ETC-deficient cells, which we generated by the genetic manipulation of mitochondrial transcription factor A (TFAM), a regulator of mitochondrial transcription. This protein has an additional role as an architectural protein, which is essential for the maintenance of mtDNA; therefore, it also serves as a limiting determinant for the mtDNA copy number.[18,19] Thus, the suppression of TFAM expression (Fig. S3a,b) led to both mtDNA and mtRNA depletion, and a consequent deficiency in ETC function in the same manner as EtBr treatment (Fig. 4a–c), as

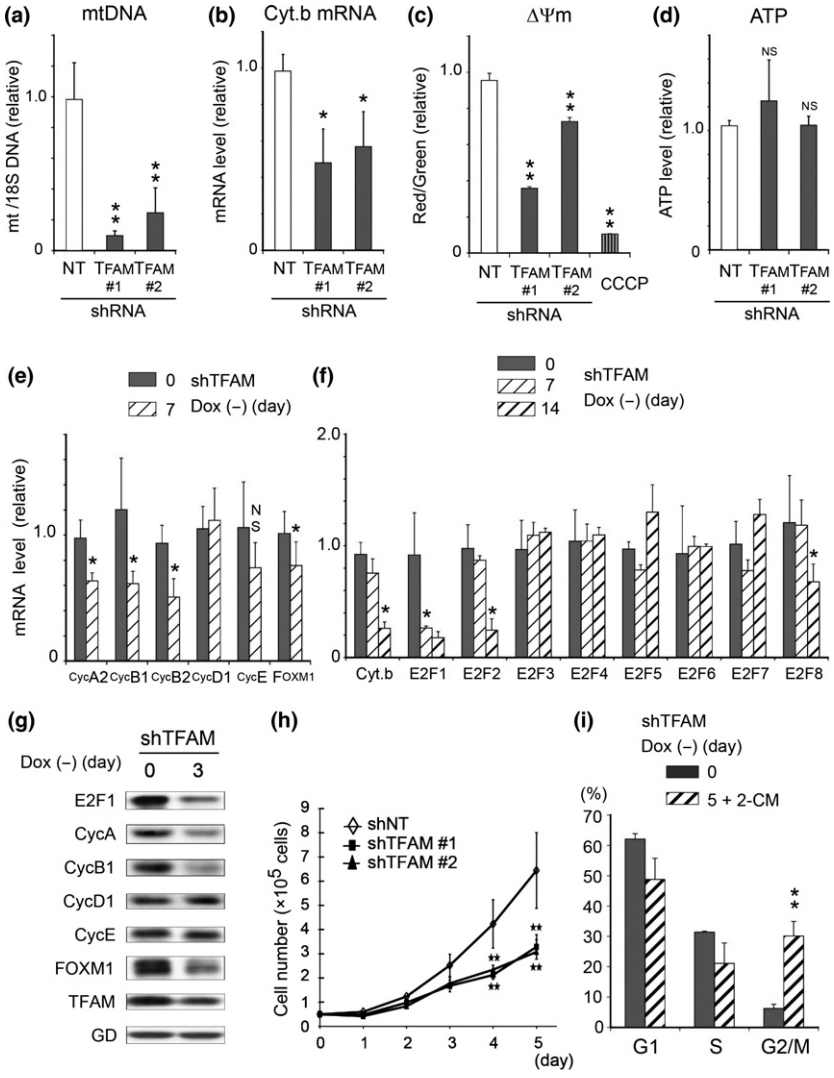

Fig. 4. Downregulation of cell cycle regulators and cell proliferation in mitochondrial transcription factor A (TFAM)-knockdown cells. TFAM-knockdown MDA cells were established as described in Figure S3(a,b). Levels of mtDNA relative to genomic DNA (18S) (mt/18S DNA) (a) and Cyt.b mRNA (b), mitochondrial membrane potential ($\Delta\Psi$m) (c), and ATP levels (d) were determined as described in Figure 1(a–d) after incubating the cells in the absence of Dox for 7 days (a) and 48 h (b–d). (e, f) mRNA levels of cyclins (A2, B1, B2, D1 and E) and FOXM1 (e), as well as those of Cyt.b and E2F1–8 (f) quantified by qRT-PCR in cells where TFAM was knocked down by incubating in the absence of Dox (Dox [−]) for the days indicated. (g) Western blot analysis of representative proteins corresponding to the mRNA in (e) and (f). GD was the loading control. (h) Proliferation of cells with TFAM knockdown by incubating with Dox (−) for the days indicated. (i) Cell cycle distribution of control (0) and TFAM-knockdown cells (Dox [−] 5 days) in the presence of 2-CM (20 μM) (5 + 2-CM), as described in Figure S3(c). $*P < 0.05$ and $**P < 0.01$. NS, not significant.

described previously.[20] In contrast to the pseudo-ρ0 cells (Fig. 1b), the depletion of mtRNA (Cyt.b) was only partial under TFAM knockdown. Further depletion of mtRNA was achieved by using 2′-C-methyladenosine (2-CM), an inhibitor of mitochondrial RNA polymerase-mediated transcription (Fig. S3c). Similar to the treatment with EtBr (Fig. 1d), the ATP levels in the TFAM-knockdown cells were almost the same as those in the control (Fig. 4d).

Importantly, TFAM knockdown decreased the expression of the set of E2F-target cell cycle regulator genes described above (Fig. 4e–g *vs* Fig. 2c,d), and it suppressed cell proliferation (Fig. 4h), thereby strongly supporting the involvement of ETC activity in the control of cell proliferation. Enrichment of the G2/M population was observed under decreased mitochondrial activity and it was statistically significant in the presence of 2-CM (Fig. 4i). Moreover, using the genetically ETC-deprived cells, we demonstrate the importance of ETC activity in the long-term growth of cancer cells *in vitro* and *in vivo*. Figure 5(a) indicates that TFAM knockdown impeded anchorage-independent cell growth *in vitro*. More importantly, TFAM knockdown considerably mitigated tumor growth in our *in vivo* orthotopic implantation experiments (Fig. 5b).

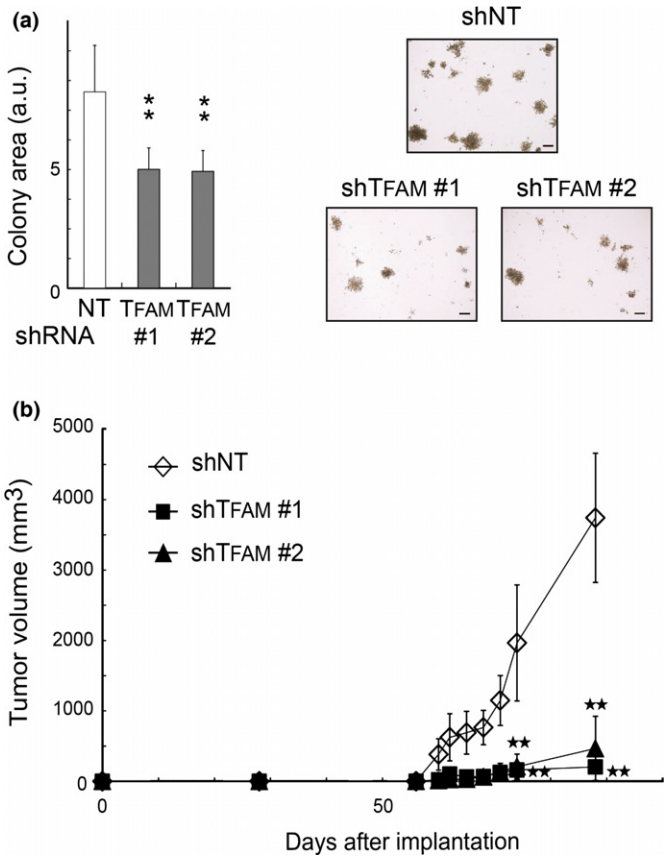

Fig. 5. Suppression of anchorage-independent cell growth and tumor growth *in vivo* by mitochondrial transcription factor A (TFAM) knockdown. (a) Anchorage-independent cell growth of the TFAM short hairpin RNA (shRNA)-expressing MDA cells, as described in Figure S3a,b. Cells grown in methylcellulose under Dox (−) conditions for 4 weeks were photographed (Right; scale bar: 300 μm) and the areas of colonies in images were quantified using ImageJ software (left). Values represent the mean ± SD. $^{**}P < 0.01$. (b) TFAM shRNA-expressing cells established from MDA/GFP cells were implanted into the mammary fat pads of SCID mice and the tumor size was monitored. Each data point represents the mean ± SD based on four or five xenografts. $^{**}P < 0.01$.

Significance of decreased intracellular reactive oxygen species in electron transport chain-deficient cells. Finally, we investigated whether intracellular redox changes derived from mitochondrial sources were involved in the mechanism of ETC-dependent cell proliferation control. The generation of mitochondrial ROS (mtROS) is correlated with levels of $\Delta\Psi$m or ETC activity,[21,22] and ETC-deficient ρ0 cells are incapable of producing mtROS.[4,9–11] Our results agree with these previous findings in that the ROS levels measured with H_2DCFDA were significantly lower in the MDA/ρ0 cells compared with the control cells, where strong fluorescence was observed in the intracellular organelles (Figs 6a, S4a; [−]). The probe detected both cytoplasmic ROS and mtROS,[23] but the majority of the fluorescence appeared to be derived from mtROS under the experimental conditions employed in this study. This is because the fluorescence was largely co-localized with that of a mitochondrial indicator in the cells (Fig. S4a; [−] high magnification). In addition, the fluorescence was sensitive to manipulations of the ETC activity with ETC inhibitors and supplements (Fig. S4b–d). In conclusion, our results suggest that mtROS production was reduced in the MDA/ρ0 cells similar to the other cell types.[6,11–13]

To understand the significance of this decrease in mtROS during the suppression of proliferation in ETC-deficient conditions, we attempted to reduce mtROS levels using an antioxidant. We utilized mitoquinol (Mitq), a reduced form of the mitochondria-targeted coenzyme Q (CoQ) analog, which is a well-characterized mitochondria-targeted antioxidant (mt-antioxidant).[24] As expected, Mitq, but not CoQ, effectively decreased ROS levels in the MDA cells (Figs 6b, S4a). It was remarkable that the treatment of cells with Mitq but not CoQ consistently recapitulated the changes in gene expression for the cell cycle regulators under ETC-deficient conditions (Fig. 6c [MDA] and Fig. S1e; right [MCF] *vs* Figs 2c–e, 4e,f) as well as cell proliferation suppression with concomitant defects in cell cycle progression during the G2 and/or M phases (Fig. 6e,f). Therefore, Mitq had almost the same effects on gene expression and cell cycle arrest as EtBr and TFAM knockdown. Gene expression levels decreased significantly at 24 h prior to inhibition of proliferation, which was apparent after several days, thereby indicating that the changes in gene expression were a response to the lower mtROS levels and not because of the inhibition of proliferation. We confirmed that Mitq treatment had no obvious effects on $\Delta\Psi$m (Fig. S5a), which excluded the possibility that Mitq affected the functionality of the ETC. Thus, mtROS is the most likely mediator of the effects of ETC activity during the regulation of cell cycle progression. Mechanistically, our results suggest that mtROS levels affected the stability of the E2F1 protein (Fig. 6g).

Finally, we investigated whether the DNA damage response (DDR) and spindle assembly checkpoint (SAC) signaling were involved in the transcriptional response of cells under ETC-deficient (EtBr-treated) or mtROS-decreased (Mitq-treated) conditions. Although treatment with cisplatin (CDDP) clearly increased the immunoreactivity to phospho-histone H2A.X (γH2AX), a key indicator of DNA damage, treatment of cells with EtBr and Mitq did not increase the activity (Fig. S5b). In addition, treatments with CDDP or paclitaxel (PTX), which induce DDR and SAC, respectively, did not downregulate the E2F transcriptional network (Fig. S5c). Therefore, it is unlikely that these stress response pathways played a role in the transcriptional response of cells to ETC-deficient or mtROS-decreased conditions.

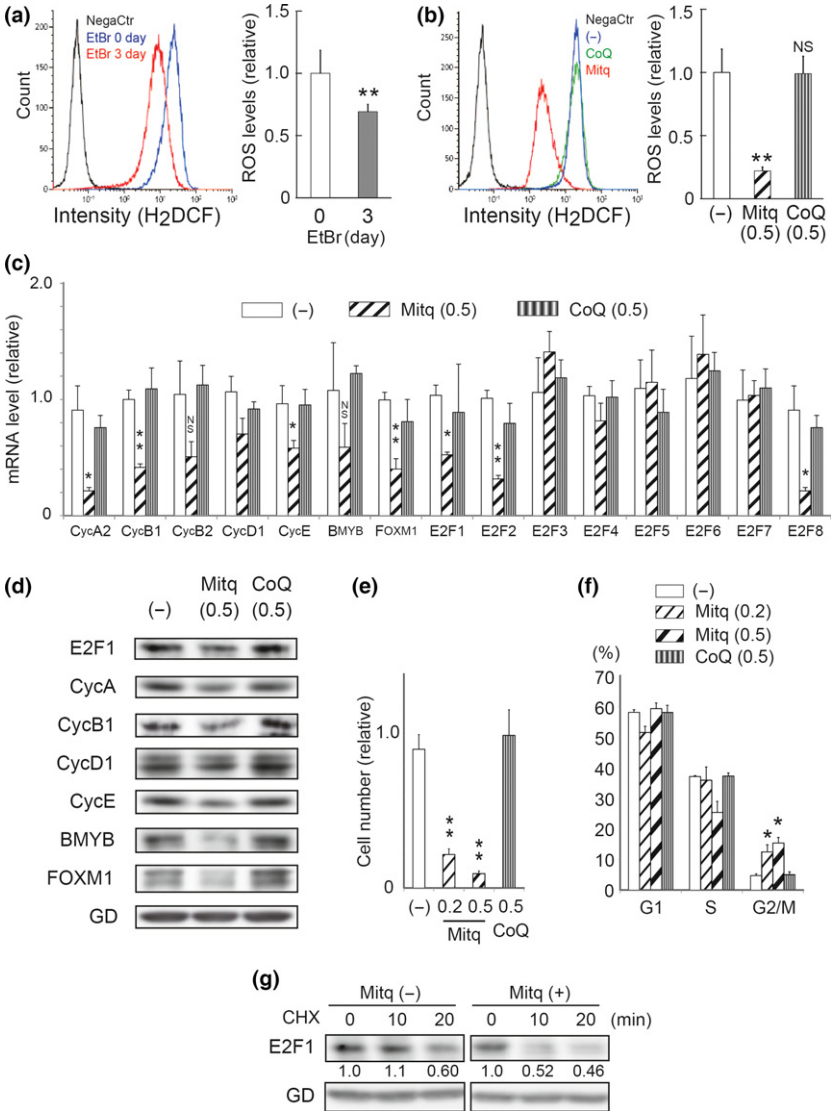

Fig. 6. Decrease in the intracellular reactive oxygen species (ROS) levels in electron transport chain (ETC)-deficient cells and its significance in controlling cell proliferation. (a) ROS levels of normal and pseudo-ρ0 cells determined using H_2DCFDA for flow cytometry. Left: Representative histogram showing the H_2DCFDA florescence intensity obtained from calcein-labeled MDA/ρ0 cells (black [NegaCtr]): ethidium bromide (EtBr) treatment 0 days (unstained), blue: 0 days (stained), red: 3 days (stained). Right: The H_2DCFDA florescence intensity normalized against that of calcein blue in an individual cell was determined in at least 10 000 cells and plotted. (b) MDA cells were treated with 0.5 μM Mitq or CoQ for 24 h, and the ROS levels were determined, as described in (a). Left: Histogram (black [NegaCtr]): unstained control, blue (–): untreated and stained control, red (Mitq): Mitq-treated and stained, green (CoQ): CoQ-treated and stained. (c) mRNA levels of cyclins (A2, B1, B2, D1 and E), BMYB, FOXM1 and E2F1–8 in MDA cells treated with 0.5 μM of Mitq and CoQ for 24 h, which were quantified by qRT-PCR. (d) Western blot analysis of representative cell cycle regulators with MDA cells treated as described in (c) for 48 h. GD was the loading control. (e) Proliferation of MDA cells treated with 0.2 and 0.5 μM Mitq, and 0.5 μM CoQ for 6 days. (f) Cell cycle distribution of MDA cells treated as described in (e) for 48 h. (g) MDA cells treated with 0.5 μM Mitq for 2 h were incubated with 100 μg/mL cycloheximide (CHX) for the times indicated. E2F1 expression levels were examined by conducting western blot analysis, quantified using Image J software, and the relative intensities are shown after normalization against GD. $^*P < 0.05$ and $^{**}P < 0.01$. NS, not significant.

Discussion

In this study, we identified a novel role for ETC activity in the control of proliferation of cancer cells. These findings may also be relevant to proliferation control in normal cells under physiological conditions. The principal mechanism that underlies this effect is the sensitivity of E2F1 expression to ETC activity or mtROS, which couples ETC activity with the cell cycle regulatory mechanism via an E2F1-organized transcriptional network. In this context, it is interesting to note that E2F1 also engages in regulating oxidative metabolism by repressing the key genes that regulate mitochondrial functions, including the respiratory chain.[25] Thus, according to our results and this previous study, a reciprocal regulatory mechanism may be present between E2F1 and the functions of organelles. In this scenario, when ETC activity declines, E2F1 expression levels decrease (Fig. 2c), which leads to the derepression of E2F1-mediated transcriptional repression and the subsequent upregulation of the mitochondrial functions, including ETC activity. This constantly maintains ETC activity above a specific level in cells under physiological conditions. Thus, E2F1 potentially functions as a connector and coordinator between cellular proliferation and metabolic pathways, which appears to be critically important for cellular homeostasis.

A similar study of cell proliferation under mtR/T inhibition was performed previously using HeLa cells.[10] The results agree with ours in that the slowdown of cell proliferation under mtR/T inhibition was unlikely to be attributable to ATP depletion, but it could be attributable to the downregulation of p21CIP1 due to lower ROS levels in the cells. In ρ0 cells derived from hepatoma cells, lower levels of cyclins A and D1 were observed as well as increased levels of CDK inhibitors, such as p16, p27 and p21,[26] which is similar to the case for MCF7/ρ0 cells. Therefore, the specific mechanisms that underlie the suppression of proliferation through mtR/T inhibition may differ among cell types or genetic backgrounds, particularly the p53 status, although no mention was made regarding the E2F transcriptional network in these previous studies. According to our study, downregulation of the E2F transcriptional network was observed in several different cell types in addition to breast cancer cells irrespective of the p53 status under ETC-deficient conditions (unpublished data); therefore, the regulation appears to be a fundamental response that can be modulated by an additional response such as CKI induction.

At present, the mechanism that allows mtROS to regulate the expression of E2F1 remains largely unknown. However, considering the effect of Mitq (Fig. 5),[27] it is probable that a redox change in mitochondria is involved, which is coupled with the stability of the E2F1 protein (Fig. 6g). Given the autoregulatory control of E2F1 expression,[28] decreases in E2F1 protein levels are assumed to reduce the activity of the E2F1 promoter, thereby leading to the downregulation of the entire E2F1-mediated transcriptional network.

In addition to elucidating the detailed mechanisms, understanding the negative impact of mtR/T inhibition on cancer cell proliferation may be of practical value, particularly the effects on MDA cells (Fig. 1). MDA is a representative cell line established from triple-negative breast cancer (TNBC), a subtype of the highly malignant cancers that lack specific cell-surface receptors needed for targeted therapeutic treatments and with a poor prognosis. According to the intrinsic impairment of the ETC, which was identified recently in multiple TNBC cell lines,[29] it is expected that TNBC will be exceptionally sensitive to mtR/T inhibition, which should further reduce the ETC activity of TNBC cells and effectively suppress their proliferation, thereby providing a promising lead in the fight against the highly malignant cancer.

Gene manipulation is a potential approach for inhibiting mtR/T, as suggested by a previous study where TFAM was knocked down in a Kras-driven mouse model of lung adenocarcinoma.[4] Our study extends the possibility of practical applications in human cancers (Figs 4, 5). Methods may be developed to scavenge mtROS as an alternative to gene manipulation that inhibits mtR/T or ETC; in many therapeutic applications, a practical goal of mtR/T inhibition is to decrease mtROS, which act as non-relevant signaling molecules that bring about various adverse conditions. Recently, mt-antioxidants have attracted increasing interest for their potential use in cancer therapy.[30–33] In this study, we demonstrated the effectiveness of Mitq in inhibiting TNBC cell proliferation based on new mechanistic insights (Fig. 6), which further highlights the potential use of mt-antioxidants in cancer therapy. We are hopeful that our results will contribute to the development of improved cancer therapies.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Doc. S1. Materials and Methods, Abbreviations and References**.**

Fig. S1. Defects in cell cycle progression and downregulation of cell cycle regulators in electron transport chain (ETC)-deficient and Mitq-treated MCF7 cells.

Fig. S2. Effects of BMYB and FOXM1 knockdown on cell proliferation.

Fig. S3. mitochondrial transcription factor A (TFAM) knockdown by expression of shRNA.

Fig. S4. Reactive oxygen species (ROS) detection under treatment with EtBr or Mitq, or with an electron carrier, inhibitors and substrates for electron transport chain (ETC).

Fig. S5. Effects of Mitq on $\Delta\Psi$m and effects of signaling activators of DNA damage response and spindle assembly checkpoint on the E2F1 transcriptional network.

Table S1. List of E2F target genes downregulated in pseudo-ρ0 cells.