Stimulation of Mitochondrial Activity by p43 Overexpression Induces Human Dermal Fibroblast Transformation

Stéphanie Grandemange,1 Pascal Seyer,1 Angel Carazo,1 Philippe Bécue,3 Laurence Pessemesse,1 Muriel Busson,1 Cécile Marsac, Pascal Roger,2 François Casas,1 Gérard Cabello,1 and Chantal Wrutniak-Cabello1

1UMR 866, Différenciation Cellulaire et Croissance (INRA-UMII-ENSAM), Unité d’Endocrinologie Cellulaire, Institut National de la Recherche Agronomique; 2Laboratoire d’Anatomie et Cytologie Pathologiques, Hôpital Laënnec, Montpellier, France; 3Laboratoire de Biologie Cellulaire du Développement, EA 3446, Proliferateurs de Peroxyomes, Université Henri Poincaré-Nancy I, Faculté des Sciences, Vandœuvre-lès-Nancy, France; and 4CERTO, Faculté Necker-Enfants Malades, Paris, France

Abstract
Mitochondrial dysfunctions are frequently reported in cancer cells, but their direct involvement in tumorigenesis remains unclear. To understand this relation, we stimulated mitochondrial activity by overexpression of the mitochondrial triiodothyronine receptor (p43) in human dermal fibroblasts. In all clones, this stimulation induced morphologic changes and cell fusion in myotube-like structures associated with the expression of several muscle-specific genes (Myf5, desmin, connectin, myosin, AchRcs). In addition, these clones displayed all the in vivo and in vitro features of cell transformation. This phenotype was related to an increase in c-Jun and c-Fos expression and extinction of tumor suppressor gene expression (p53, p21WAF1, Rb1). Lastly, reactive oxygen species (ROS) production was increased in positive correlation to the stimulation of mitochondrial activity. The direct involvement of mitochondrial activity in this cell behavior was studied by adding chloramphenicol, an inhibitor of mitochondrial protein synthesis, to the culture medium. This inhibition resulted in partial restoration of the normal phenotype, with the loss of the ability to fuse, a strong decrease in muscle-specific gene expression, and potent inhibition of the transformed phenotype. However, expression of tumor suppressor genes was not restored. Similar results were obtained by using N-acetylcysteine, an inhibitor of ROS production. These data indicate that stimulation of mitochondrial activity in human dermal fibroblasts induces cell transformation through events involving ROS production. (Cancer Res 2005; 65(10): 4282-91)

Introduction
Besides their involvement in fuel metabolism, the importance of mitochondria in cell physiology has been extended to the regulation of cell proliferation, differentiation, and apoptosis, all these being processes strongly impaired during the development of oncogenesis. Indeed, inhibition of mitochondrial gene expression induces a potent decrease in cell proliferation (1, 2). In addition, studies done in erythroleukemia, neurons, or myoblasts established that impairment of organelle activity also inhibits cell differentiation (3–5). Our own studies provided evidence that the latter influence resulted from actual regulation of differentiation by mitochondrial activity through the control of myogenin expression (5). Lastly, it is now well established that release of cell death–inducing factors by the organelle is a key event in the induction of cell apoptosis (6) and that disruption or resistance of the apoptotic pathway is an evident “hallmark of cancer” (7).

Many reports have also pointed out the occurrence of mitochondrial dysfunctions in several pathologies, including diabetes (8), degenerative diseases such as myopathies (9), and Parkinson's or Alzheimer's diseases (10, 11). Similarly, alterations of organelle activity have been consistently reported in tumoral tissues (12) and mitochondrial genome mutations have been observed during oncogenic processes (13). Although the significance of these associations between mitochondria and cancer are not really understood, several data clearly suggest that organelle dysfunction could be involved in the induction or development and maintenance of events leading to oncogenic processes. In particular, cybrids obtained by fusion between the cytoplasm of transformed cells and the nucleus of normal cells display the same oncogenic properties as initial cancer cells, thus suggesting that cytoplasmic factors are sufficient to induce tumorigenic events (14, 15).

Among the cytoplasmic factors, the possible involvement of mitochondria is well supported by various studies indicating that experimental inhibition of organelle protein synthesis or depletion of mitochondrial DNA strongly decreases in vitro or in vivo oncogenic phenotype (16, 17). In addition, mitochondria are the major site of free radical production in the cell and this production is frequently altered in tumor tissues (18, 19). Although elevated concentrations of reactive oxygen species (ROS) can induce apoptosis (20), these observations have led to the use of antioxidant molecules in cancer cell therapy (21).

The major influence of triiodothyronine (T3) on mitochondrial activity has been partly explained by the discovery of p43, a mitochondrial T3 receptor (22). This receptor is synthesized through the use of an internal AUG occurring in the c-erba1 transcript, also encoding a T3 nuclear receptor (23). This truncated (43 kDa) form of the thyroid hormone receptor c-ErbA1 (TRα) is located in the mitochondrial matrix (22) and acts as a T3-dependent transcription factor of the mitochondrial genome (24). Besides the importance of this pathway for cell physiology, these findings lead to the possibility that organelle activity can be directly stimulated by p43 overexpression.

In this study, we used this opportunity to study the influence of stimulated mitochondrial activity induced by p43 overexpression in human dermal fibroblasts. We report that such stimulation leads to cell transformation, owing to down-regulation of the expression of tumor-suppressor genes and up-regulation of c-Jun and c-Fos expression, and acquisition of a defective myogenic phenotype...
linked to the induction of Myf5 expression, a myogenic factor involved in muscle cell specification. In addition, these events are associated with ROS overproduction and are partially overcame by chloramphenicol, an inhibitor of mitochondrial protein synthesis.

Materials and Methods

Cell culture and treatment conditions. Primary human dermal fibroblasts from two young female normal controls were used in these experiments. They were independently grown in DMEM supplemented with gentamicin (100 IU/ml) and FCS (10%) at 37°C and 5% CO2.

The pattern of cell fusion was assessed before and after lowering the medium serum concentration at cell confluence (0.5%), a classic procedure used to induce differentiation in myoblasts.

For proliferation assays, cells were plated in 60 mm dishes in triplicate at a density of 5 x 10^5 cells per dish. They were grown in medium containing FCS at 10% and counted daily over a 5-day period.

To inhibit mitochondrial protein synthesis when needed, chloramphenicol (100 µg/ml) was added to the culture medium and cells were cultured during three passages in the presence of the drug. To assess the involvement of ROS production in this observed phenotype, transformed cells were treated with the antioxidant N-acetylcyctisteine (NAC, 0.25, 0.5, and 1 mM/L; Sigma, St. Louis, MO).

Stable transfection. Human fibroblasts constitutively expressing c-erbAα1 were obtained by stable transfection of the plBV FE6 expression vector (22). Control fibroblasts were obtained by stable transfection of the plBV ‘empty’ vector. Ten micrograms of each plasmid carried G418 resistance were transfected using the calcium phosphate phosphate procedure 24 hours after plating. The medium was changed 24 hours later after PBS washes, and amplification was done after 10 days in the presence of G418.

Growth in soft agar and tumorigenicity in nude mice. A double-layer culture technique was used for the study of growth in soft agar. Cells (5 x 10^5 cells per 60 mm dish) were seeded in 3 ml medium containing 0.3% agar (Diagnostics Pasteur, Marnes-la-Coquette, France) and placed over a bottom agar consisting of 4 ml medium containing 0.6% agar. Cells were fed weekly with 1 ml medium. Two weeks after seeding, colonies were scored.

For analysis of tumorigenic potentials in vivo, cells were dispersed with trypsin, washed thrice with PBS to remove serum, and resuspended at 1 x 10^6 cells/ml in a medium without serum. About 2 x 10^5 cells were injected s.c. into 7-week-old athymic mice. Tumor occurrence and size was monitored weekly for up to 3 months.

Cytoimmunofluorescence and immunohistochemical studies. P43 mitochondrial localization and expression of muscle-specific markers [myosin heavy chain (MHC) and desmin] were assessed by cytoimmunofluorescence. After methanol fixation and appropriate washings, cells were stained with a rabbit polyclonal antibody raised against c-ErbA (RHTII; ref. 22), a mouse anti-human mitochondria monoclonal antibody (MAB1273, Chemicon International, Temecula, CA), or a mouse anti-MHC monoclonal antibody (1F11, given by Dr. F. Pons [Institut National de la Sante et de la Recherche Medicale, Montpellier, France]), or a mouse anti-desmin antibody [1F11, given by Dr. F. Pons (Institut National de la Sante et de la Recherche Medicale, Montpellier, France)], or a mouse anti-MHC monoclonal antibody (MAB1273, Chemicon International, Temecula, CA), or a mouse anti-desmin antibody (1F11, given by Dr. F. Pons [Institut National de la Sante et de la Recherche Medicale, Montpellier, France]) or a mouse anti-MHC monoclonal antibody (1F11, given by Dr. F. Pons [Institut National de la Sante et de la Recherche Medicale, Montpellier, France]). Nuclei were stained with Hoechst 33258 (1 µg/ml).

Cell culture and treatment conditions. Primary human dermal fibroblasts from two young female normal controls were used in these experiments. They were independently grown in DMEM supplemented with gentamicin (100 IU/ml) and FCS (10%) at 37°C and 5% CO2.

Measurement of cytochrome oxidase activity. Cells plated in coated dishes were harvested in 1 ml PBS and then centrifuged for 5 minutes at 12,000 x g. For enzymatic activity determination, the pellet was resuspended in 50 to 150 µl lysis buffer (10 mMol/L Tris pH 7.5) and lysed by three cycles of freezing/defreezing. Total proteins were measured on an aliquot using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Results

P43 overexpression in human dermal fibroblasts stimulates mitochondrial activity and induces morphologic changes. After stable independent transfections of the c-erbAα1 expression vector and selection, 11 clones belonging from the two donors were obtained. As they displayed a similar phenotype, two of them were used for the complete study (MT1 and MT2). They were characterized by an increase in the level of p43, a truncated form of the T3 nuclear receptor acting as a T3 mitochondrial receptor, associated to a clear decrease in the level of the nuclear receptor (Fig. 1A). Cytoimmunofluorescence studies confirmed that overexpressed p43 was specifically addressed to mitochondria (Fig. 1B).

As previously shown in other cell types (5, 22), cytochrome oxidase activity was stimulated in the two selected clones (MT1: ×5; MT2: ×15, relative to control cells, P < 0.05 and P < 0.01, respectively; Fig. 1C). Moreover, in the two clones, we found a significant increase in ROS production (Fig. 1D and E).
In addition to these mitochondrial alterations, we observed important morphologic changes in p43-overexpressing cells. Whereas normal cells and cells transfected with the empty vector displayed a typical dermal fibroblast morphology, cells expressing p43 acquired a compact morphology, with a reduction in the cytoplasmic area (Fig. 2A). In addition, they spontaneously fused in plurinucleated structures looking like myotubes for the MT1 clone or myoballs for the MT2 clone. Fusion processes were strongly enhanced after decreasing serum concentration in the culture medium, a procedure classically used to induce myoblast differentiation (Fig. 2B). However, the striated structure observed in myotubes was not apparent.

As we observed fusion events in MT1 and MT2 cells, we checked for the possible expression of genes encoding muscle-specific proteins. Cytoimmunofluorescence studies indicated that, as expected, control cells did not express myosin or desmin. However, MT1 and MT2 cells expressed these two muscle-specific proteins (Fig. 2C-F), but not T-troponin (another muscle-specific protein; data not shown). Western blotting experiments confirmed that MHC and desmin expression was restricted to p43-overexpressing cells (Fig. 2G). In addition, the frequency of cells expressing these proteins increased in relation to the increase in the value of the fusion index (Fig. 2D and F). In contrast to other myogenic factors, Myf 5 transcripts were easily detected in MT1 and MT2 clones (Fig. 2H) but not in control cells [data not shown from reverse transcription-PCR (RT-PCR); Fig. 2I]. In addition, only p43-overexpressing cells expressed AChRα (subunit α of acetylcholine receptor) mRNA, a specific marker of muscle cells (Fig. 2H). Taken together, these data clearly indicate that p43 overexpression in human dermal fibroblasts induces a myogenic phenotype characterized by cell fusion and expression of genes encoding muscle-specific proteins. However, our data point out the acquisition of a defective phenotype with absence of MyoD, myogenin, and T-troponin expression and the lack of a striated structure.

P43 overexpression in human dermal fibroblasts induces cell transformation. In addition to these morphologic changes, we first observed that, in contrast to control cells, MT1 and MT2 clones formed multilayers of cells (Fig. 3A), thus suggesting a loss of contact inhibition. In addition, MT1 and MT2 cells displayed a large increase in their proliferation rate relative to control cells (Fig. 3B) as their number was five times greater than in control cells after 4 days of culture. To test the possible induction of cell transformation by p43 overexpression, we next studied anchorage-independent growth potential, another well-established property of transformed cells. When plated in semisolid medium, the control cells remained in suspension as single cells and never formed colonies, whereas the MT1 (P < 0.05) and MT2 (P < 0.01) clones grew in large colonies (Fig. 3C). The number and size of these colonies were greater in MT2 cells characterized by higher mitochondrial activity than in MT1 cells. Furthermore, MT1 and MT2 cells displayed a lower requirement for serum growth factors. Their proliferation rate was maintained in media with 0.5% FCS in contrast to control cells that became quiescent as indicated by an exclusive accumulation in the G0-G1 phase (Fig. 3D).

Lastly, as a final examination of p43 transforming potential, we tested the ability of MT2 cells to promote tumor formation in athymic nude mice. After cell injection in the right flank, three of five mice injected with MT2 cells and two of five with MT1 cells displayed large tumors (Fig. 4A). By contrast, none of the mice injected with control cells developed detectable tumors. Analysis of these tumors revealed significant myosin and desmin expression and the occurrence of few plurinucleated structures (Fig. 4B). However, when cultured, tumor cells displayed exactly all the features of MT2 or MT1 clones including extensive cell fusion (data not shown).

These results indicate that stimulation of mitochondrial activity by increasing amounts of p43 is able to promote all in vivo and in vitro properties of cell transformation. These findings clearly
highlight the oncogenic potency of p43, a mitochondrial transcription factor synthesized from the proto-oncogene c-erbAα1 transcript.

P43 overexpression induces changes in gene expression leading to the transformed phenotype. As cell transformation is associated to decreased expression or to a loss of tumor suppressor functionality and/or to proto-oncogene activation, we studied p53, p21WAF1, Rb (tumor suppressor genes), c-Jun, and c-Fos expression. P53, p21WAF1, and Rb transcripts were easily detected by Northern blot and by RT-PCR (data not shown) in control cells, but not in MT1 and MT2 clones (Fig. 5A). Western blot analyses confirmed the absence of p53, Rb, and p21WAF1 proteins in these cells (Fig. 5B). In addition, we found that c-Jun and c-Fos protein levels were up-regulated by p43 overexpression (Fig. 5C). Altogether, these data provide evidence of the extinction of three tumor suppressor genes and the induction of expression of two proto-oncogenes in p43-overexpressing cells, thus explaining cell transformation.

Inhibition of mitochondrial protein synthesis or reactive oxygen species production partially restores a normal cell phenotype. To assess the role of the increase in mitochondrial activity induced by p43 overexpression in the initiation of the myogenic and transformed phenotypes of dermal fibroblasts, we used chloramphenicol, an inhibitor of mitochondrial protein synthesis, to inhibit the organelle activity of MT1 and MT2 clones. As expected, chloramphenicol strongly inhibited mitochondrial activity assessed by measurement of cytochrome oxidase activity (Fig. 6A). As shown in Fig. 6B and C, this treatment effectively abolished the ability of MT1 and MT2 cells to fuse in plurinucleated structures, but did not restore the normal fibroblastic morphology. This influence was associated with a strong decrease in the
expression of genes encoding muscle-specific proteins, Myf5 and AchRα, assessed by Northern blot (Fig. 6D) and MHC by Western blot (Fig. 6E).

Similarly, chloramphenicol strongly reduced the proliferation rate of MT1 and MT2 cells (day 5 of culture: 20-fold reduction in the number of cells; Fig. 7A), moderately reduced the proliferation rate of control cells (day 5: 2-fold reduction) as well as their ability to grow in semisolid agar (Fig. 7B) or in low serum medium (Fig. 7C), and restored contact inhibition, as reflected by the absence of multilayer growth in culture (data not shown). However, this treatment did not restore the expression of the three tumor suppressor genes and did not inhibit the expression of the two oncogenes (data not shown).

As chloramphenicol significantly reduced ROS production (Fig. 8A), to assess the involvement of these molecules in MT1 and MT2 phenotypes we studied the influence of NAC, an antioxidant molecule. We found that NAC treatment mimicked the chloramphenicol effect by strongly reducing MT1 and MT2 proliferation rate (Fig. 8B) and ability to grow in soft agar (Fig. 8C).

Finally, these results suggest that the stimulation of mitochondrial activity by p43 overexpression is directly involved, through an increase in ROS production, in the acquisition of the defective myogenic phenotype, and induced cell transformation. However, as inhibition of mitochondrial activity did not completely reverse transformation-associated events (extinction of tumor suppressor genes and oncogene overexpression) some features seem to be irreversible, as also shown by the persistence of morphologic changes.

Discussion

In this study, we provide data establishing the induction of cell transformation by stimulation of mitochondrial activity through p43 overexpression: (a) stimulation of mitochondrial activity is associated to human dermal fibroblast transformation in association with the acquisition of a defective myogenic phenotype; (b) inhibition of organelle activity partially reverses the transformed defective myogenic phenotype.

P43 overexpression stimulates mitochondrial activity. C-erbAα1 simultaneously encodes a nuclear and a mitochondrial T3 receptor, synthesized through the use of an internal AUG occurring in the TRα transcript (29). Consequently, TRα transfection might result theoretically in a simultaneous increase in nuclear and mitochondrial T3 receptor levels. In fact, we found a decrease in the nuclear receptor protein level. This point is under investigation, but one possibility is that a stimulation of mitochondrial activity could reduce stability of the nuclear receptor; alternatively, it could affect the expression, translation, or degradation of a putative RNA-binding protein involved in the selection of the first or second AUG leading to nuclear or mitochondrial receptor synthesis. In addition, we found that c-ErbAα1 overexpression results in a sharp increase in p43 levels in human dermal fibroblasts, a result not observed with the same intensity in myoblasts or preadipocytes (data not shown).

However, we have already reported that despite the occurrence of nearly similar levels of TRα transcripts in the two tissues, p43 protein levels are very important in brown adipose tissue, whereas they are not detected in adult brain (22, 30). Similarly, whereas TRα expression is lower in liver (31), p43 levels are particularly abundant.

**Figure 3.** Overexpression of p43 promotes tumorigenic events in vitro. A, phase contrast microscopy (>100) shows the potentiality of p43-expressing cells to form foci in culture. Cells were grown in medium containing 10% (v/v) FCS for 5 days after confluence. B, effects of p43 overexpression on cell proliferation in medium containing 10% (v/v) FCS. Cell numbers were determined daily. Points, mean of four independent experiments; bars, SE. C, phase contrast microscopy (>100) of control or p43-expressing cells grown in soft agar medium (left). Quantification of colonies growing in each dish of soft agar medium from 1 × 10⁵ control, MT1, and MT2 cells (right). Columns, mean of three independent experiments; bars, SE; ND, none detected. D, cell cycle profile of control, MT1, and MT2 cells growing in medium containing 0.5% serum. Cells were fixed and stained with propidium iodide (PI) and then analyzed by flow cytometric analysis. The percentage of cells in each stage of the cell cycle (G₀, S, and G₂-M) is indicated. Statistically significant differences relative to control cells: *P < 0.05 and **P < 0.01 (Student’s t test).
(22, 30). All these data point to the occurrence of a cell-specific regulation in the use of the internal AUG giving rise to p43.

After having established that the overexpressed p43 protein was correctly addressed into mitochondria, we observed the induction of potently stimulated mitochondrial activity reflected by a 5-fold (MT1) or 15-fold (MT2) increase in cytochrome oxidase activity associated with more moderate stimulation of other parameters reflecting organelle activity (oxygen consumption), in agreement with previous studies (5, 22). In parallel, p43 also increased ROS production, in good correlation with the increase in cytochrome oxidase activity found in MT1 and MT2 clones.

**P43 overexpression induces a defective myogenic phenotype in human dermal fibroblasts.** Unexpectedly, we found that p43 overexpression induced morphologic changes and especially the ability of cells to fuse in syncytial structures, a phenomenon potentiated by decreasing serum concentration in the culture medium, as currently used to induce myoblast differentiation. These morphologic changes were associated with the expression of muscle-specific genes, such as desmin, connectin (data not shown), AchRα, and MHC. All these changes could be related to the induction of Myf5 expression, a myogenic factor involved primarily in the induction of the myogenic phenotype (32).

**Figure 4.** Overexpression of p43 promotes tumorigenic events in vivo. A, tumor formation in athymic nude mice following s.c. injection with control or p43-overexpressing cells (left) and growth curves of tumors in individual animals (right). B, immunohistochemical studies of myosin and desmin expression in tumor section (×200, left and ×400, right).

**Figure 5.** P43 overexpression abrogates tumor suppressor gene expressions. Northern blot (A) and Western blot (B) analyses of three tumor suppressor gene expressions (Rb, p21WAF1, and p53) in control (pIRV) or MT1 and MT2 (p43-overexpressing) cells at the indicated times (P, C, and LS). 18S is used as a loading control. Data are representative of three independent experiments. C, Western blot analysis of two cellular oncogenes, c-Fos and c-Jun, in control, MT1, and MT2 cells at confluence. Data are representative of three independent experiments.
However, neither MyoD nor myogenin transcripts were detected in these cells, explaining the acquisition of a defective myogenic phenotype characterized by the absence of several muscle-specific proteins, such as T-troponin, and other structural proteins that can explain the lack of the striated structure typical of skeletal muscle.

In agreement with these data indicating that Myf5 expression alone is sufficient to induce a myogenic phenotype, Rudnicki et al. (33) have previously reported that MyoD is not essential for skeletal muscle development in mice, revealing some degree of functional redundancy in the control of the skeletal myogenic developmental program. Indeed, inactivation of MyoD in mice led to an increase in Myf5 mRNA level and to normal or subnormal muscle development in these mice. Furthermore, constitutive expression of one of the four myogenic factors is sufficient to induce a myogenic program in nonmuscle cells (34). In addition, c-Jun has been shown to be a major component of the transcription factor AP-1, is known to fulfill important functions in cell proliferation, differentiation (37, 38), and transformation (39). Several studies have established the transformation potentialities of AP-1 complexes via c-Jun expression in rat embryo cells (40) and the c-Fos requirement for malignant progression in skin tumors (41). Furthermore, inhibition of AP-1 activity reversed cell transformation in JB6 mouse epidermal cells (42). In addition, c-Jun has been shown to be involved in the induction of Myf5 by several regulators (dexamethasone and anisomycin; ref. 43). Consequently, the increase in c-Jun expression in MT1 and MT2 clones could partly mediate the induction of Myf5 and the acquisition of a defective myogenic phenotype.

**P43 overexpression induces human dermal fibroblast transformation.** Besides the induction of a myogenic phenotype, the strong increase in mitochondrial activity resulting from p43 overexpression induces the well-established hallmarks of cellular transformation (7). Overexpressing p43 cells lost contact-mediated growth inhibition, exhibited reduced serum growth requirements, and were fully protected from apoptosis following growth factor deprivation. Furthermore, we found that, unlike control cells, p43-expressing cells formed colonies in semisolid medium and tumors when injected into athymic nude mice. In combination with the simultaneous observation of a defective myogenic phenotype, these data clearly suggest that p43 overexpression induces a rhabdomyosarcoma-like phenotype. Indeed, rhabdomyosarcoma, the most common soft-tissue sarcoma of childhood, is characterized by expression of skeletal muscle markers and alterations of oncosuppressor genes. However, the origin of rhabdomyosarcoma remains unclear (36).

Study of changes in the expression of some genes involved in the induction or prevention of cell transformation revealed some interesting data. First, we found that p43 overexpression stimulates expression of c-Jun and c-Fos, two important members of activator protein 1 (AP-1) complexes. The proto-oncogene c-jun, encoding a major component of the transcription factor AP-1, is known to fulfill important functions in cell proliferation, differentiation (37, 38), and transformation (39). Several studies have established the transformation potentialities of AP-1 complexes via c-Jun expression in rat embryo cells (40) and the c-Fos requirement for malignant progression in skin tumors (41). Furthermore, inhibition of AP-1 activity reversed cell transformation in JB6 mouse epidermal cells (42). In addition, c-Jun has been shown to be involved in the induction of Myf5 by several regulators (dexamethasone and anisomycin; ref. 43). Consequently, the increase in c-Jun expression in MT1 and MT2 clones could partly mediate the induction of Myf5 and the acquisition of a defective myogenic phenotype.
Besides the stimulated expression of these cellular oncogenes, we also found that p43 overexpression fully abrogated the expression of three tumor suppressor genes, p53, Rb, and p21\(^{WAF1}\). As the activation of cellular oncogenes and/or the loss of function of tumor suppressors are involved in oncogenic processes, all these data satisfactorily explain the induction of transformation processes by p43 overexpression. Interestingly, these three tumor suppressor genes seem to be involved in rhabdomyosarcoma. For instance, a simultaneous inactivation of both p53 and Rb in mice induces rhabdomyosarcoma development (44). In addition, according to Aurade et al. (43), p21\(^{WAF1}\) is methylated and hypoexpressed in rhabdomyosarcoma (45). All these data well support the possibility that, by inducing a dramatic decrease in p53, Rb, and p21\(^{WAF1}\) expression, a potent stimulation of mitochondrial activity through p43 expression in dermal fibroblasts could induce a rhabdomyosarcoma-like phenotype.

Furthermore, the repression of c-Fos expression by Rb (46) suggests the existence of a functional link between the extinction of tumor suppressor gene expression and the stimulation of c-Fos expression. These results thus established the oncogenic potentiality of the mitochondrial transcription factor, p43. Since its characterization, the c-erbA\(_{a2}\) gene has been considered as a proto-oncogene due to the occurrence of a mutated version in the avian erythroblastosis virus (47, 48); however, there has been no direct experimental proof of its oncogenic properties. In this study, we showed for the first time that c-erbA\(_{a1}\) overexpression alone is sufficient to induce dermal fibroblast transformation and that, unexpectedly, this influence is mediated by the product of this gene addressed into mitochondria.

Stimulation of mitochondrial activity is directly involved in the acquisition of the defective myogenic phenotype and in the induction of cell transformation. As previously shown, p43 overexpression induced a potent stimulation of mitochondrial activity characterized by stimulation of cytochrome oxidase activity. To find out if this event was directly involved in the cell phenotype changes observed in this study, we used chloramphenicol, an inhibitor of mitochondrial protein synthesis. As expected, chloramphenicol strongly inhibited mitochondrial activity. This influence was associated with strong inhibition of the myogenic phenotype characterized by almost complete abrogation of cell fusion, and potent inhibition of the expression of genes specifically expressed in muscle tissue, probably due to the decrease in Myf5 expression.

In addition, we also found that inhibition of mitochondrial activity by chloramphenicol was associated with a partial reversion of the transformed phenotype. Not only did cell proliferation decrease, but cells did not grow in foci and lost their ability to proliferate in low serum medium. Moreover, their ability to grow in semisolid agar decreased, but expression of tumor suppressor genes or oncogenes was not affected (data not shown).

Altogether, these results show that stimulation of mitochondrial activity is directly involved in the changes occurring in the dermal fibroblast phenotype, but that some genetic programs are probably not reversible after their induction.

ROS production has frequently been implicated in the initiation and promotion phases of tumorigenesis (49). The electron transport chain of mitochondria is considered as a major source of ROS. In this study, stimulation of mitochondrial activity is associated with an increase in ROS cellular levels. In turn, several reports have shown...
that ROS production affects nuclear gene expression by altering the activity of some transcriptional factors (50, 51). In particular, nuclear factor \( \text{n} \) \( \text{B} \) and AP-1 have been identified as regulated by the intracellular redox state. In agreement with our data, c-Fos and c-Jun are induced by relatively small amounts of hydrogen peroxide, superoxide, nitric oxide, and other inducers of oxidative stress (52). In addition, the involvement of ROS production is also well supported by the observation that phenotypic reversion induced by chloramphenicol is associated with a significant reduction in ROS levels (data not shown).

In this study, we report that \( \text{c-erb\alpha} \) \( \text{a} \) overexpression, through increased amounts of the mitochondrial T3 receptor \( \text{p}43 \), induces phenotypic changes in human dermal fibroblasts characterized by the acquisition of defective myogenic features and cell transformation, with a resemblance to rhabdomyosarcoma cells. This work constitutes the first experimental induction of a rhabdomyosarcoma-like phenotype. In addition, we have provided evidence suggesting that \( \text{p}43 \)-induced stimulation of mitochondrial activity is directly involved in these events, probably through ROS production. Overall, we have brought new insights into the possible involvement of mitochondria in oncogenic processes, and we have established that the \( \text{c-erb\alpha} \) gene must indeed be considered as a proto-oncogene.

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