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Mutations in BALB mitochondrial DNA induce CCL20 up-regulation promoting tumorigenic phenotypes

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ABSTRACT

mtDNA mutations are common in human cancers and are thought to contribute to the process of neoplasia. We examined the role of mtDNA mutations in skin cancer by generating fibroblast cybrids harboring a mutation in the gene encoding the mitochondrial tRNA for arginine. This somatic mutation (9821insA) was previously reported in UV-induced hyperkeratotic skin tumors in hairless mice and confers specific tumorigenic phenotypes to mutant cybrids. Microarray analysis revealed and RT-PCR along with Western blot analysis confirmed the up-regulation of CCL20 and its receptor CCR6 in mtBALB haplotype containing the mt-Tr 9821insA allele compared to wild type mtB6 haplotype. Based on reported role of CCL20 in cancer progression we examined whether the hyper-proliferation and enhanced motility of mtBALB haplotype would be associated with CCL20 levels. Treatment of both genotypes with recombinant CCL20 (rmCCL20) resulted in enhanced growth and motility of mtB6 cybrids. Furthermore, the acquired somatic alteration increased the *in vivo* tumor growth of mtBALB cybrids through the up-regulation of CCL20 since neutralizing antibody significantly decreased *in vivo* tumor growth of these cells; and tumors from anti-CCL20 treated mice injected with mtBALB cybrids showed significantly decreased CCL20 levels. When rmCCL20 or mtBALB cybrids were used as chemotactic stimuli, mtB6 cybrids showed increased motility while anti-CCL20 antibody decreased the migration and *in vivo* tumor growth of mtBALB cybrids. Moreover, the inhibitors of MAPK signaling and NF-κB activation inhibited CCL20 expression in mtBALB cybrids and decreased their migratory capabilities. Thus, acquired mtDNA mutations may promote tumorigenic phenotypes through up-regulation of chemokine CCL20.

1. Introduction

Tumor development and progression are multifactorial processes with complex regulation. The members of chemokine superfamily are considered to be important elements that can regulate neoplastic processes in cancer cells. Chemokines and their receptors are expressed by tumor and/or by host cells, in primary tumors, and in specific metastatic loci. Some of them support tumor development and progression mostly by their ability to induce cellular motility while the others can potentially suppress cellular functions that are involved in malignant transformation [1]. Generally, chemokines can play an important role in formation of primary tumors and metastases [2].

An important member of chemokine superfamily is chemokine CCL20. CCL20 was identified in 1997 by three independent groups in screens of human cDNA libraries from liver, monocytes and pancreatic cells and was designated liver and activation-regulated chemokine (LARC) [3], macrophage inflammatory protein-3α (MIP-3α) [4], and Exodus-1 [5], respectively. Thus, in the systematic chemokine nomenclature, LARC/MIP-3α/Exodus-1 is designated as CCL20 (CC chemokine ligand 20) [6]. CCL20 can function as both an inflammatory and a homeostatic chemokine depending on the specific situation and its natural receptor is the CCR6. Their interaction regulates multiple physiological functions, particularly tissue

Abbreviations: mtDNA, mitochondrial DNA; UV, ultraviolet; CCL20, CC chemokine ligand 20; rmCCL20, recombinant protein CCL20; LARC, liver and activation-regulated chemokine; MIP-3α, macrophage inflammatory protein-3α; CCR6, CC chemokine receptor type 6; NF-κB, nuclear factor-κB; IκB, inhibitor of kappa B; ROS, reactive oxidative species; NMSC, non-melanoma skin cancer; MMP, metalloproteinases; Col1A1, collagen, type I, alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; NAC, *N*-acetyl cysteine; tBHP, tert-butyl hydroperoxide; RT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction.

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architecture and compartment-specific migration of white blood cells [7]. Cancer cells can also exploit the CCL20/CCR6 receptor system for mediation of their specific migration and metastasis [8]. It was observed that CCL20 as well as CCR6 play important role in colorectal cancer leading to enhanced proliferation and migration. Compared to normal colon mucosa, CCR6 and CCL20 both were found to be up-regulated in colorectal cancer and colorectal liver metastasis [9]. CCL20 participation in cancer progression was also shown in pancreatic adenocarcinoma where CCL20 expression was significantly higher compared to normal tissue [10–12]. Huang and Geng [13] made similar observation in hepatocellular carcinoma samples where significantly enhanced expression of both CCL20 and CCR6 was seen compared to healthy tissue. CCL20 was also shown to be up-regulated in biopsies of breast cancer patients [14,15], renal cell carcinoma [16], melanoma [17] and squamous cell carcinoma including keratinocytes [18]. Baumforth et al. [19] observed up-regulation of CCL20 caused enhanced migration of regulatory T cells in Hodgkin's lymphoma patients.

Expression of chemokine ligand CCL20 is controlled by nuclear factor-κB (NF-κB) transcription factor [20]. NF-κB plays an important role during cellular responses to inflammatory stimuli and general responses to pathogens in a number of different cell types and is inhibited by the IκB molecule. IκB phosphorylation and its subsequent degradation releases NF-κB triggering transcription of many nuclear genes involved in pro-carcinoma processes, including chemokine CCL20 and targeting NF-κB by its specific inhibitors results in suppression of CCL20 expression in cells [21]. Besides of the NF-κB-dependent CCL20 expression, it is known that the promoter region of CCL20 contains binding sites for the Ets transcription factor which is activated by ERK1/2 suggesting a role of the Ras-MAPK-pathway in CCL20 expression [22].

In our study, we employed a cybrid model for analysis of mtDNA changes found in UV-induced non-melanoma skin cancer (NMSC). We previously discovered a mutation hot spot (9821insA) in mouse *mt-Tr* locus ($tRNA^{Arg}$) in approximately one third of premalignant and malignant skin tumors (squamous papillomas and squamous cell carcinomas). To determine the functional relevance of this particular mutation *in vitro*, cybrid cells with the same nuclear background but containing different *mt-Tr* ($tRNA^{Arg}$) alleles were generated [23]. We demonstrated that a 9821insA can alter the biochemical characteristics of murine cybrids and subsequently can contribute to significant changes in their behavioral capabilities. Moreover, we showed that there are mtDNA-driven differences in ROS production between wild type (mtB6) and 9821insA (mtBALB) cybrids [23,24]. Microarray analysis was conducted in order to elucidate the expression differences between the two cybrid lines differing only in their mtDNA. 44 K mouse whole genome chip revealed roughly 400 differentially expressed genes between the two cybrid lines including genes involved in tumorigenic processes such as matrix matalloproteinases (MMPs) and inflammatory chemokines and their receptors [25].

Chemokine CCL20 and its receptor CCR6 were found up-regulated in mutant cybrids. Based on previously reported role of CCL20/CCR6 in tumor progression we examined whether CCL20/CCR6 is also responsible for the mtBALB haplotype oncogenic features like enhanced proliferation, migration and invasion. Targeting expression of CCL20 by its natural upstream transcription factor NF-κB and Ets; or disruption of CCL20/CCR6 interaction could be a useful strategy in a treatment of cancer. When combined with other therapeutic approaches, the research on chemokines and namely CCL20 in various cancers may provide novel means for improved outcomes.

2. Materials and methods

2.1. Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The IACUC protocol was approved by the University of Arizona Institutional Animal Care and Use Committee (Permit number 07-029). All procedures were performed in every effort to minimize suffering.

2.2. Cybrid cell lines

LMEB3(mtBALB) and LMEB3(mtB6) cells were generated by harvesting the mitochondria from brain synaptosomes of B6 and BALB mice and electrofusing them to a mouse fibroblast LMEB3ρ^0 cell line that lacked its own mtDNA. Two cybrid lines differing only in their mtDNA but having identical nuclear background from murine LA9 cells were produced. A mouse LMEB3ρ^0 cell line was produced by exposure of LM(TK-) cells to ethidium bromide as described previously by [26]. Both lines were maintained in DMEM medium supplemented with 10% FBS and Pen/Strep (Life Technologies, Grand Island, NY) in 5% CO_2 atmosphere at 37 °C.

2.3. DNA sequencing of mitochondrially encoded $tRNA^{Arg}$ (mt-Tr locus) in cybrid lines

Forward (5′-TTCTAGTCACAATTCTATCTCTAGGC-3′) and reverse (5′-GCATTGTAGTAGGTTGAGATTTTGG-3′) primers were designed for PCR to amplify the *mt-Tr* gene from total genomic DNA of both cybrid lines. PCR samples were prepared in a total volume of 50 μl. Each tube contained 1xTaq master Mix (New England BioLabs) containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 25 units/ml Taq DNA polymerase, 5 mM of both primers, 0.2 mM dNTPs each, 5% glycerol, 0.08% NP-40, and 0.05% Tween-20 and 250 ng of DNA template of either mtB6 or mtBALB cybrid cells. The PCR parameters were as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, and elongation at 68 °C for 1 min, with a final elongation at 68 °C for 5 min. PCR products were then purified using the QIAquick PCR purification kit (Qiagen) and sequenced on an Applied Biosystems 3730XL DNA analyzer (Life Technologies, Carlsbad, CA).

2.4. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

mRNA expression analysis was performed as described previously [25] with further modifications. Mouse CCL20 (Mm00444228_m1), CCR6 (Mm99999114_s1) and GAPDH (Mm99999915_m1) primer/probes purchased from ABI (Applied Biosystems, Branchburg, NJ) were used. PCR amplification of GAPDH was used to control the quality of cDNA. CCL20 and CCR6 levels were normalized to GAPDH control. Amplification plots were generated and the Ct values (cycle number at which fluorescence reaches threshold) recorded.

In experiments with rmCCL20, the cybrids were incubated 24 h with 150 ng/ml rmCCL20 (R&D Systems, Minneapolis, MN) and then allowed growing for an additional 24 h in culture. Thereafter, cells were harvested and RNA isolated.

In experiments with inhibitors PD98059 or BAY11-7082, cybrids were pre-treated with 50 μM PD98059 (Sigma-Aldrich, St. Louis, MO) and/or 5 μM BAY11-7082 (EMD Chemicals, Gibbstown, NJ) dissolved in DMSO, or vehicle alone for 60 min in serum free medium. After the treatment, medium was replaced with the normal growing medium (supplemented with 10% FBS) and the same

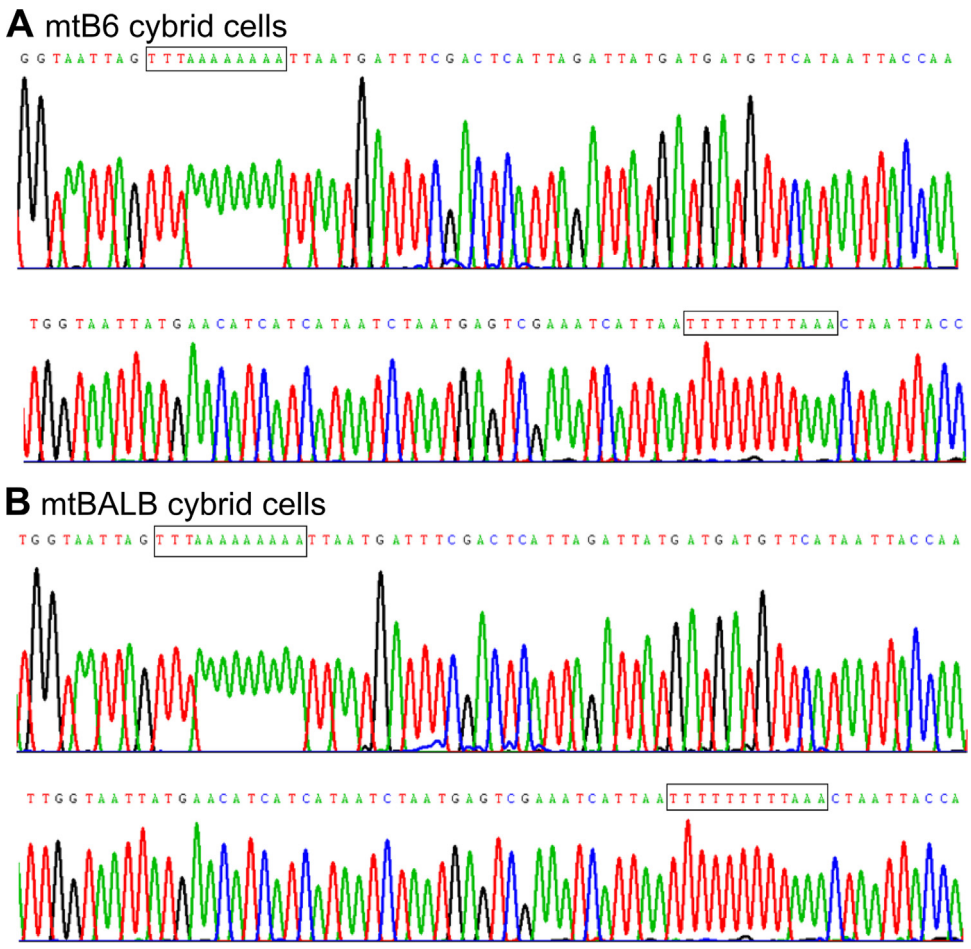

Fig. 1. DNA sequencing of the *mt-Tr* locus in wild type mtB6 and mutant mtBALB DNA samples. (A) Partial DNA sequence of wild-type allele in forward and reverse orientation and (B) 9821insA mutant mtBALB allele in forward and reverse orientation. Five individual wild type mtB6 and mutant mtBALB cybrid clones were analyzed.

treatment was repeated every day, for four days. Thereafter, cells were harvested and RNA isolated.

In experiments with antioxidants vitamin E and NAC, mutant mtBALB cybrid cells were growing for 4 days in media containing 200 μM vitamin E (Sigma Aldrich, St. Louis, MO) and 10 mM NAC (Sigma Aldrich, St. Louis, MO), respectively. Then cells were harvested and RNA isolated.

In experiments to induce ROS levels, wild type cybrid cells were pre-treated either with 20 μM tBHP for 30 min or irradiated with low dose of UVA (2 J/cm^2). After the treatment, medium was replaced with the normal growing medium (supplemented with 10% FBS) and the same treatment was repeated every day, for four days. Thereafter, cells were harvested and RNA isolated.

2.5. Western blot analysis

Cell lysates were prepared from both cybrids using RIPA buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton N-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate and protease inhibitor mixture (Leupeptin, Aprotinin, PMSF). 30 μg of total protein was separated by Any kDTM SDS-polyacrylamide gel electrophoresis followed by transfer to PVDF membrane (Bio-Rad, Hercules, CA). Mouse monoclonal anti-actin clone AC-40 antibody obtained from Sigma Aldrich (St. Louis, MO), mouse monoclonal anti-CCL20 antibody (MAB760) and anti-rat HRP secondary antibody purchased from R&D Systems (Minneapolis, MN) were used for detection. Blots were incubated with ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) and exposed to BioMax XAR film (Kodak, Rochester, NY). The same blot was used for both, CCL20 and β-actin detection.

2.6. Cell proliferation assay

1×10^5 cybrid cells were seeded in 6-well plates. Cells were either incubated in normal growing medium only/supplemented with IgG1 (controls) or in medium supplemented either with 150 ng/ml rmCCL20 or 50 μM anti-CCL20 functional blocking antibody (R&D Systems, Minneapolis, MN) for 24 h, 48 h and 72 h, respectively. IgG1/rmCCL20/anti-CCL20 were added to the cultured medium only once and media were not changed during 72 h. Viable cells were counted daily with a hemocytometer by the trypan blue exclusion method.

2.7. Co-culture and transwell migration assay

Transwell migration assays were performed as described previously [24] with further modifications. Three membrane filters and three individual cybrid clones were used for each condition within one experiment.

In co-culture experiments, 15×10^4 cells were seeded in the lower chamber and allowed to attach in the presence of 10% FBS, then attached cells were rinsed twice with DPBS, and sustained throughout the experiment in 600 μl of DMEM supplemented with 0.5% FBS and 0.1% BSA (Sigma-Aldrich, St. Louis, MO). 25×10^3 tested cybrid cells resuspended in 150 μl of migration buffer were

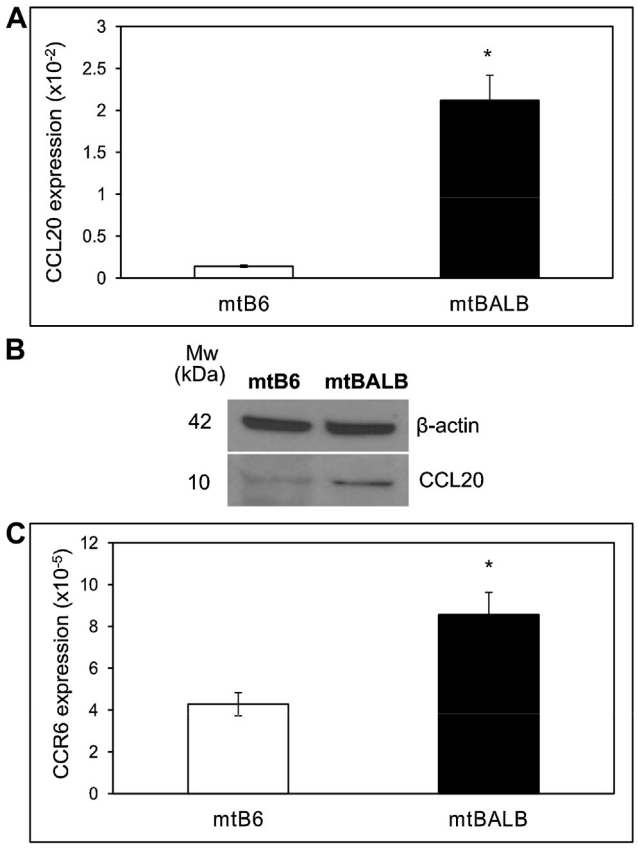

Fig. 2. mRNA expression and protein levels of chemokine CCL20 and its receptor CCR6 in mtB6 and mtBALB cybrid cells. (A) Quantitative RT-PCR histogram showing the mRNA expression levels of chemokine CCL20 in fibroblast cybrid cells. (B) Western blot analysis showing the protein levels of CCL20 in both cybrid cells. (C) RT-PCR histogram showing the mRNA expression levels of CCR6 receptor in cybrid cells. Three individual cybrid clones were assayed for each experiment. At least three independent experiments were executed. The data are graphed as a mean ± SD with * $P \leq 0.0005$.

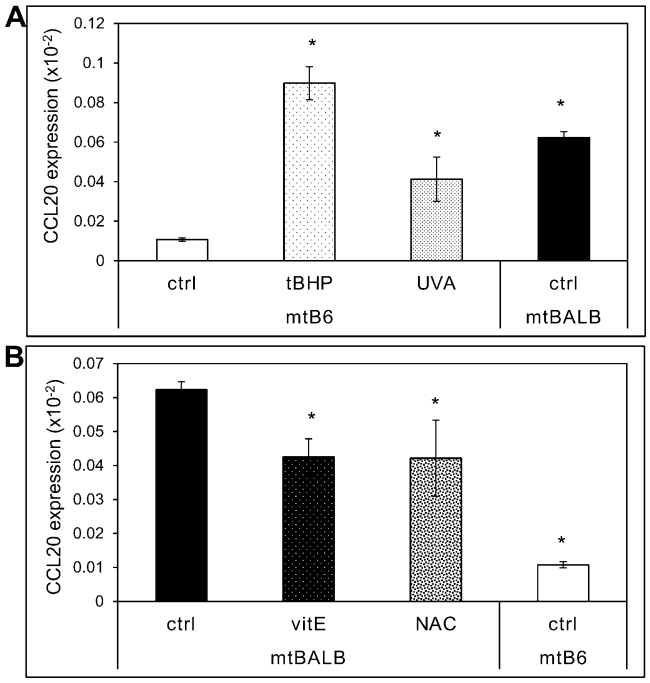

Fig. 3. mRNA expression levels of chemokine CCL20 in cybrid cells after the treatment with either ROS inducers or antioxidants. Quantitative RT-PCR histogram showing the mRNA expression levels of chemokine CCL20 in fibroblast cybrids after treatment with (A) 20 μM tBHP and 2 J/cm^2 UVA radiation and (B) 200 μM vitamin E and 10 mM NAC. The mtB6 cybrids were pre-treated with tBHP for 30 min and irradiated with 2 J/cm^2 UVA every day for a total of 4 days. The mtBALB cybrids were cultured in normal growing media supplemented with antioxidants for 4 days. Three individual cybrid clones were assayed for each experiment. The data are graphed as a mean ± SD.

seeded in the upper chamber which was then assembled into the dish suspended above the lower wells.

In experiments with rmCCL20 protein, 150 ng/ml of rmCCL20 was either added to the tested cybrids or added to the bottom well as a chemo-attractant.

In experiments with inhibitors PD98059 or BAY11-7082, 25×10^3 cybrid cells pre-treated with 50 μM PD98059 or 5 μM BAY11-7082, or vehicle (60 min for a total of 4 days) were resuspended in migration buffer and seeded on the top of insert and assayed in transwell system.

In experiments with functional blocking monoclonal mouse anti-CCL20 antibody (R&D Systems, Minneapolis, MN), 25×10^3 cybrids resuspended in migration buffer supplemented with 50 μM anti-CCL20 or control IgG1 antibody (R&D Systems, Minneapolis, MN) were placed on the upper well of a transwell system and assayed.

2.8. Establishment of tumor xenografts

10×10^6 mtB6 and mtBALB cybrids were resuspended in 0.1 ml of sterile PBS and injected subcutaneously in the right flank of male SCID mice (16 mice/group). The mice were obtained from the University of Arizona Cancer Center SCID house colony at the age of 9 weeks with weight average of 25 g. 24 h after the cell injection, the half of the mice from each group started to get subcutaneous injections of neutralizing anti-CCL20 (tested groups) and the other half IgG1 isotype control (control groups) antibodies (20 μg/injection) every other day during two weeks. Both treatments were injected subcutaneously in the left flank of tested mice. Tumor diameters measured twice weekly at right angles (d_{short} and d_{long}) using electronic callipers were converted to volume by the formula, volume = $(d_{short})^2 \times (d_{long})/2$. Individual mice were sacrificed when the tumor volume reached 2000 mm^3 and the tumors were harvested (1/2 of tumor snap frozen and other 1/2 fixed in NBF).

3. Results

We have previously discovered a mutation hot spot (9821insA) in the mitochondrially encoded tRNA^{Arg} (*mt-Tr* locus) in approximately one third of premalignant and malignant UV-induced skin tumors [23]. To determine the functional relevance of this particular mutation *in vitro*, cybrid cell lines containing different *mt-Tr* (tRNA^{Arg}) alleles were generated [23] and 9821insA mutation in the *mt-Tr* locus of mutant mtBALB cybrids was confirmed by DNA sequencing (Fig. 1). We have further described that mutations in mitochondrial *mt-Tr* locus can alter the biochemical characteristics of murine cybrids which subsequently cause significant changes in their behavioral abilities such as proliferation, resistance to UV-induced apoptosis, enhanced migration and invasion, the phenotypes seen in malignant cells [23,24]. Moreover, we showed that these alterations in mtDNA can produce the differences in expression levels of some nuclear-encoded genes between the wild type mtB6 and mutant mtBALB cells [25], including matrix matalloproteinase MMP-9; chemokines CCL5, CCL8 and structural genes such as collagen 1A1 (Col1A1) and Col1A6.

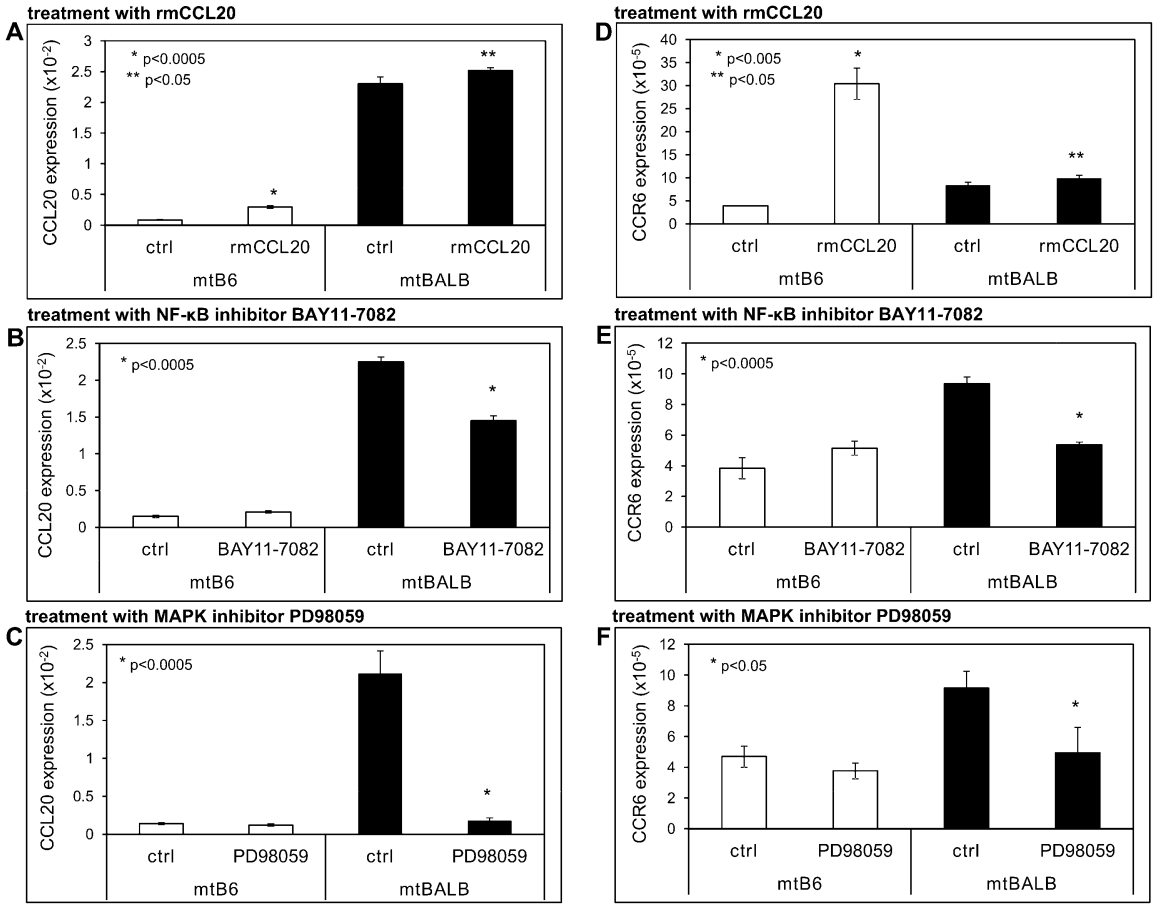

Fig. 4. mRNA expression levels of chemokine CCL20 and its receptor CCR6 in mtB6 and mtBALB cybrid cells after the treatment with rmCCL20 and pathway specific inhibitors BAY11-7082 and PD98059. Quantitative RT-PCR histogram showing the mRNA expression levels of chemokine CCL20 in fibroblast cybrids after treatment with (A) 150 ng/ml rmCCL20, (B) 5 μM NF-κB inhibitor BAY11-7082 and (C) 50 μM MAPK inhibitor PD98059. Quantitative RT-PCR histogram showing the mRNA expression levels of CCR6 receptor in fibroblast cybrids after treatment with (D) 150 ng/ml rmCCL20, (E) 5 μM NF-κB inhibitor BAY11-7082 and (F) 50 μM MAPK inhibitor PD98059. Three individual cybrid clones were assayed for each experiment. At least three independent experiments were executed. The data are graphed as a mean ± SD.

3.1. Chemokine CCL20 and its receptor CCR6 are up-regulated in mtBALB haplotype compare to mtB6 haplotype

Microarray analysis revealed that nuclear encoded CCL20 gene was up-regulated in mtBALB haplotype with a 3.48 fold change compared to mtB6 haplotype. Significantly higher mRNA expression levels and increased protein levels of this chemokine in mtBALB cybrids were confirmed by RT-PCR (Fig. 2A) and Western blot analysis (Fig. 2B). Moreover, mtBALB cells also showed increased levels of CCR6, a known receptor of CCL20 ligand (Fig. 2C).

3.2. Antioxidants vitamin E and NAC were significantly able to suppress the CCL20 mRNA expression in mutant mtBALB cybrid cells while inducers of ROS production such as tBHP and UVA irradiation were able to increase the expression in wild type mtB6 cybrid cells

Treatment of wild type mtB6 cybrids with ROS inducer tBHP was able to induce CCL20 expression to the level of CCL20 expression seen in mutant mtBALB cybrids. Moreover, short exposure of mtB6 cybrids to 2 J/cm^2 of UVA radiation was also able to significantly increase the mRNA expression levels of this chemokine (Fig. 3A). In contrary, treatment of mutant mtBALB cybrids with antioxidants vitamin E and NAC was able to significantly decrease the expression of chemokine CCL20 in these cells (Fig. 3B).

3.3. Recombinant CCL20 protein was able to enhance the expression of chemokine CCL20 in mtB6 haplotype while pathway specific inhibitors decreased its expression in mtBALB haplotype

Pre-treatment of both cybrids with rmCCL20 was able to significantly increase its own CCL20 expression (Fig. 4A), expression of its own CCR6 receptor (Fig. 4D) and expression of MMP-9 about 50% (data not shown). It is known that expression of CCL20 chemokine is regulated by the Ets and NF-κB transcription factors. Thus, specific inhibitors of NF-κB activation (BAY11-7082) and MAPK signaling (PD98059) were used to inhibit the expression of CCL20/CCR6 in cybrid cells. As documented by Fig. 4B, C, E and F, both inhibitors were able to partially inhibit the expression of both CCL20 and its receptor CCR6 in mtBALB cybrids with a more profound effect seen in cells pre-treated with PD98059.

3.4. Recombinant CCL20 protein significantly increased cellular growth of mtB6 cybrids while anti-CCL20 functional blocking antibody decreased growth of mtBALB cybrids

Previously, we have observed that mtBALB cybrids expressing higher levels of CCL20/CCR6 have higher proliferative capabilities than mtB6 cybrids expressing low levels of this chemokine and its receptor [24]. Thus, we sought to explore whether the treatment of mtB6 cybrids with rmCCL20 and mtBALB cybrids with

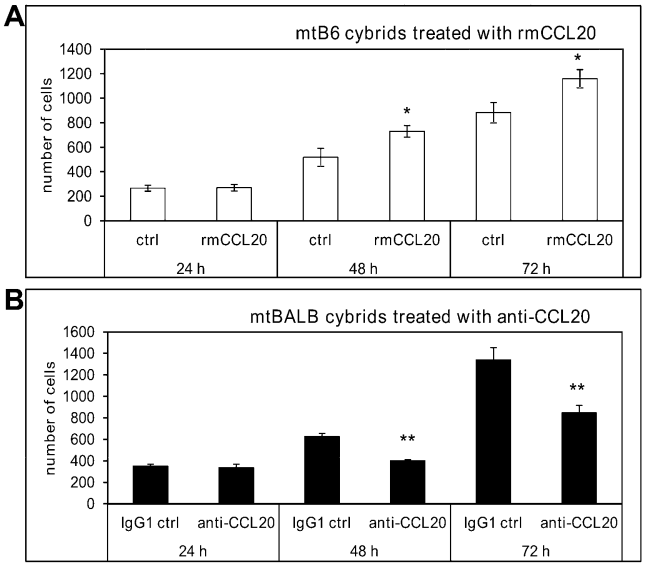

Fig. 5. Chemokine CCL20 affects the proliferative abilities of fibroblast cybrid cells. Cybrid cells were either incubated in normal growing media (crtl) or in media supplemented with either (A) 150 ng/ml of rmCCL20 protein (rmCCL20) or (B) 50 μg/ml anti-CCL20 functional blocking antibody or control IgG for the times indicated. Viable cells are shown. The data are graphed as a mean ± SD of three individual cybrid clones with * $P \leq 0.05$, ** $P \leq 0.005$). Growing experiments were executed at least three times.

functional blocking anti-CCL20 antibody would have an impact on their *in vitro* growing abilities. We have observed that mtB6 cybrids showed enhanced proliferation rate after 48 and 72 h of incubation with rmCCL20 (Fig. 5A) and mtBALB cybrids significantly decreased growth after 48 and 72 h of treatment with neutralizing anti-CCL20 antibody (Fig. 5B).

3.5. Co-culturing of mtB6 cybrid cells with mtBALB haplotype significantly enhanced in vitro migratory capabilities of these cells

Moreover, we sought to elucidate whether the *in vitro* co-culturing of cybrids expressing different levels of CCL20 might significantly affect their migratory capabilities. We used the transwell co-culture system where one cybrid line was used as a chemo-attractant (added to the bottom well) while the other was tested for migration (added on the top of transwell insert). As shown by Fig. 6A, only the migration of mtB6 cybrids (expressing low levels of CCL20/CCR6) was significantly increased when they were co-cultured with mtBALB cybrids (expressing high levels of CCL20/CCR6) used as a chemo-attractant while all other combinations of co-culture experiments did not have any effect on migration of either cybrids.

3.6. In vitro cellular motility of mtB6 haplotype was significantly increased by treating these cybrid cells with the recombinant CCL20 protein

We have previously demonstrated that mtBALB cybrids have significantly enhanced cellular migration through uncoated transwell inserts and invasiveness through matrigel coated inserts compared to mtB6 cybrids [24]. Transwell assays were conducted in order to study the influence of CCL20 on cellular motility. Recombinant CCL20 (rmCCL20) used as a chemo-attractant (rmCCL20 was added to the bottom well) increased the migration of mtB6 cybrids. However, when these cybrids were incubated with rmCCL20 (rmCCL20 was added to the cells placed on the top of insert), the effect on motility of these cybrids was even more profound and the migration was increased almost to the level of mtBALB cybrids

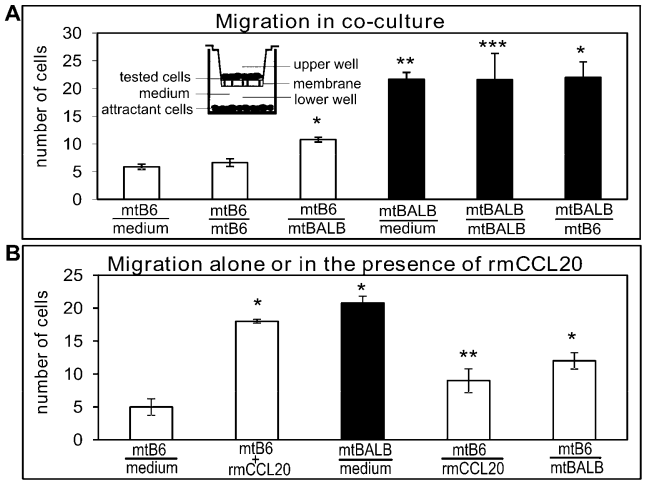

Fig. 6. Migratory capabilities of fibroblast cybrid cells in co-culture system and in the presence of chemokine CCL20. Migration transwell assays were performed using uncoated inserts. The bar graphs represent the average numbers of migrated cells in (A) various co-culture systems: mtB6/medium, mtB6/mtB6, mtB6/mtBALB, mtBALB/medium, mtBALB/mtBALB, and mtBALB/mtB6 (format example: mtB6 (top membrane)/medium (bottom well)); (B) the presence of rmCCL20 where cells were either treated with rmCCL20 (mtB6 + rmCCL20) or rmCCL20 (mtB6/CCL20) or mtBALB cybrids expressing high levels of CCL20 (mtB6/mtBALB) were added to the bottom well as chemo-attractants. Three individual cybrid clones were assayed in triplicate transwells for each experiment. At least three independent experiments were executed. The data are graphed as a mean ± SD with * $P \leq 0.005$, ** $P \leq 0.0005$, *** $P \leq 0.05$.

(Fig. 6B). There was not found statistically significant difference in migration capabilities between mtB6 cybrids co-cultured with mtBALB cybrids and the same cybrids treated with rmCCL20 as chemo-attractant. Thus, these observations confirm a critical role of CCL20 secreted soluble factor on cellular migration.

3.7. Migratory capabilities of mtBALB cybrids containing the 9821insA in mitochondrial DNA were significantly decreased by specific NF-κB and MAPK inhibitors

Furthermore, we tested if specific inhibitors of NF-κB and/or MAPK signaling will affect the migratory abilities of mtBALB cybrids expressing high levels of CCL20 and CCR6. One hour pre-treatment of these cybrids with either 5 μM BAY11-7082 or 50 μM PD98059 for a total of four days was able to significantly decrease their migration with PD98059 showing the more profound effect (Fig. 7A and B). However, the treatment of cybrids with combination of both inhibitors further reduced the migratory abilities of both cybrid lines (Fig. 7C). To assess the relative contribution of CCL20 on migration of cybrids, the mouse functional blocking antibody against CCL20 was added into migrating media. CCL20 neutralizing antibody was able to significantly block the migration of mtBALB cybrids expressing high levels of CCL20, while having no significant effect on mtB6 cybrids expressing low levels of CCL20 (Fig. 7D).

3.8. Functional blocking anti-CCL20 antibody decreased the in vivo tumor growth of mtBALB haplotype compared to mtB6 haplotype

To establish the role of CCL20 in tumor development, we evaluated the effect of neutralizing anti-CCL20 antibody on *in vivo* growth of fibroblast cybrids. We observed increased tumorigenicity in IgG1 treated mice injected with mtBALB cybrids expressing high levels of CCL20 compared to IgG1 treated mice injected with mtB6 cybrids expressing low levels of CCL20. In the group of anti-CCL20 treated mice that were injected with mtB6 cybrids, the tumor

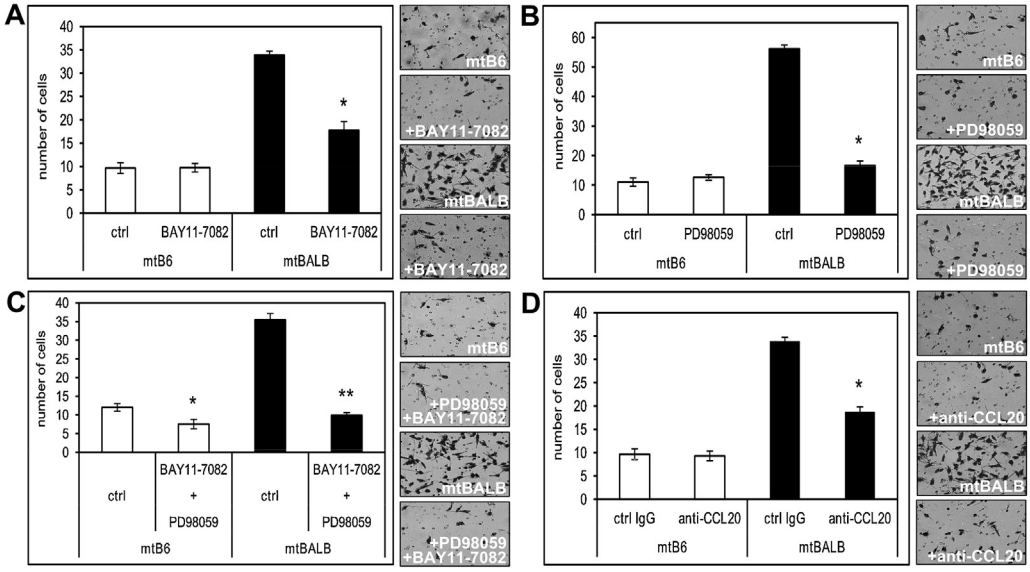

Fig. 7. Migratory capabilities of fibroblast cybrid cells in the presence of MAPK inhibitor PD98059, NF-κB inhibitor BAY11-7082 and functional blocking antibody against CCL20. Migratory experiments were performed using uncoated inserts in transwell system. The bar graphs represent the average numbers of migrated cells pre-treated with (A) 5 μM BAY11-7082 inhibitor alone; (B) 50 μM PD98059 inhibitor alone; (C) in combination with 5 μM BAY11-7082 and 50 μM PD98059 inhibitor; and (D) 50 μM anti-CCL20 blocking antibody or control IgG. Representative microscopic pictures of fibroblast cybrids that migrated through the membrane pores are also shown for each experimental condition. Three individual cybrid clones were assayed in triplicate transwells for each experiment. At least three independent transwell experiments were executed. The data are graphed as a mean ± SD with $^{*}P \leq 0.05$.

size was significantly decreased only at day eight of anti-CCL20 treatment compared to the same group of mice but treated with isotype control antibody (Fig. 8A and C). This observation might suggest a role for CCL20 in the establishment and formation of primary tumors. However, significant decrease in tumor growth was observed in the group of anti-CCL20 treated mice injected with mutant mtBALB cybrids compared to the control group of IgG1-treated mice injected with these cells (Fig. 8B and C) suggesting the role of CCL20 in tumor progression. Moreover, the tumors from the group of anti-CCL20 treated mice injected with mtBALB cybrids showed significantly lower mRNA expression of CCL20 compared to control group tumors (Fig. 8D).

4. Discussion

This study presents the up-regulation of chemokine CCL20 and its receptor CCR6 resulting from mtDNA changes at the *mt-Tr* locus encoding the mitochondrial tRNA for arginine. This particular mutation (9821insA) appears to be a hotspot for UV-induced mutations that was discovered in approximately one third of premalignant and malignant skin tumors. To determine the potential role of this mtDNA alteration in tumor progression, cybrid cells containing different *mt-Tr* ($tRNA^{Arg}$) alleles were generated: mtB6 (wild type) and mtBALB (mutant type). Specifically, there are only three nucleotide changes between these two mtDNA types, the 9821insA in *mt-Tr* locus together with 9348G to A base change and the 9461T to C change which is a neutral polymorphism [23]. mtBALB cybrids with genetic 9821insA alteration in *mt-Tr* gene showed significant biochemical changes such as diminished levels of complex I protein, less cellular oxygen consumption, and lower ATP levels [23] and increased levels of ROS, resistance to UV-induced apoptosis and enhanced cellular growth and motility [24]. Previously, it was suggested that accumulation of mtDNA mutations induced by ROS exposure leads to errors in mtDNA-encoded polypeptides. Such alterations can affect all four mitochondrial complexes (complex I appears to be mostly affected since 7 out of 13 polypeptides encoded by mtDNA belong to this complex) that results in defective electron transfer and oxidative phosphorylation. Respiratory chain defects may further increase the production of ROS [23,27]. Our findings of increased ROS levels along with diminished complex I in mtBALB cybrids correlate with these suggestions [23].

We recently published that nucleotide changes in mtDNA can trigger the differences in expression levels of some nuclear encoded genes associated with malignancy. Microarray analysis of cybrids harboring the 9821insA mutation in *mt-Tr* locus revealed hundreds of differentially expressed nuclear encoded genes compared to wild type cybrids, including the chemokine CCL20 [25]. These observations are similar to those published by Ishikawa et al. [27] who also showed that cybrids containing mtDNA mutations (G13997A and 13885insC) in the gene encoding ND6 produced a deficiency in respiratory complex I activity that is consequently associated with over-production of ROS ultimately leading to the up-regulation of specific nuclear encoded genes. Indeed, altered levels of ROS as a result of impaired respiratory chain in mitochondria seem to be a key factor responsible for modulating expression of some nuclear encoded genes [28]. ROS as natural byproducts of oxidative phosphorylation are responsible for damaging of cellular compounds such as lipids, proteins and DNA and can consequently lead to the development of malignant tumors [29]. It is known that ROS are accumulated in higher levels in cells as a result of altered mitochondrial function [24]. ROS directly affect all types of nucleic acids including mtDNA by the induction of strand breaks and base damage, and by inducing point mutation and deletions [30–32]. In addition to direct damaging effect of ROS on nucleic acids, Marin-Garcia et al. [28] suggested the mechanism in which ROS can modulate expression of nuclear encoded genes through affecting nuclear transcriptional factors such as NF-κB to further alter gene expression, cellular growth and apoptosis. Furthermore, it was suggested that in addition to a direct ROS influence on gene expression, ROS can also alter expression of some genes through the epigenetic regulations by mechanisms involving DNA methylation, chromatin phospohorylation [33] and acetylation [34].

One of the up-regulated genes in mtBALB haplotype is the chemokine CCL20, which plays a pivotal role in various tumors

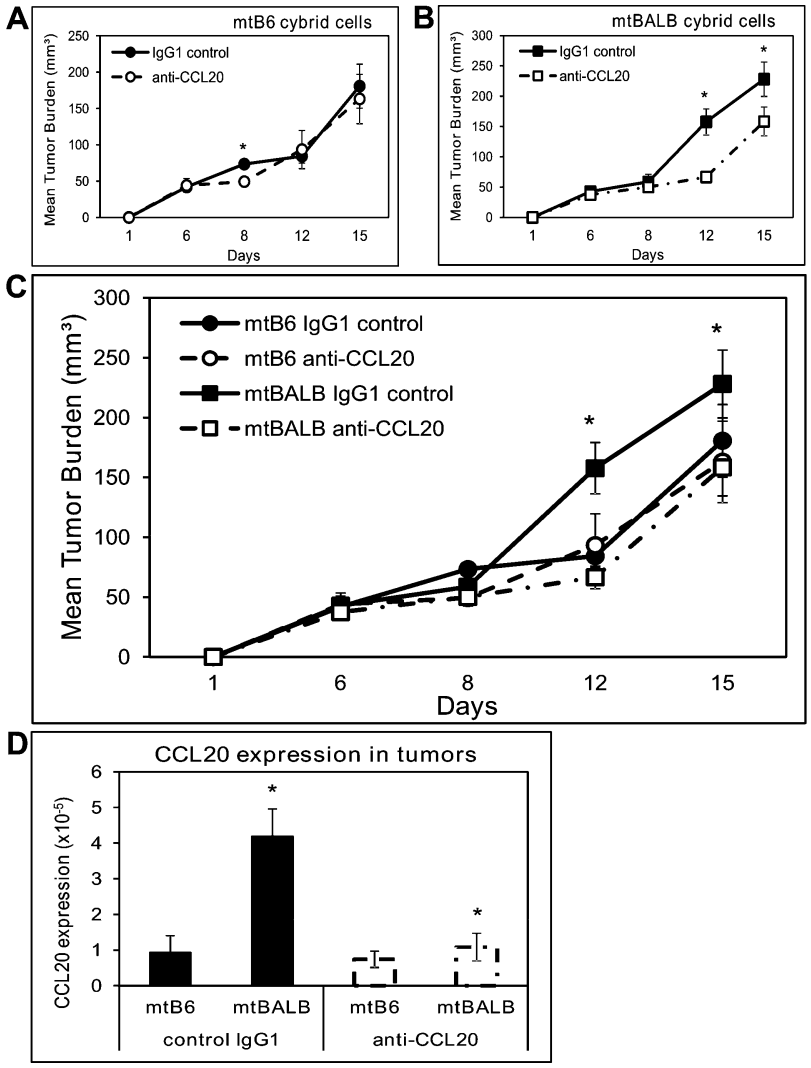

Fig. 8. Chemokine CCL20 regulates the growth of mutant fibroblast mtBALB cybrid cells. Mutant mtBALB cybrid cells with genetic alteration in *mt-Tr* gene (9821insA) with previously reported tumorigenic phenotypes [23,24] and wild type mtB6 cybrid cells were injected into the SCID mice (10×10^6 cells/mouse). 24 h after the cell injection, mice started to get the subcutaneous injections of anti-CCL20 or control IgG1 antibodies (20 μg/injection) every other day for 15 days. Individual graphs are showing the ability of neutralizing anti-CCL20 antibody to significantly decrease the growth of (A) mtB6 and (B) mtBALB tumors. (C) Summarized graph showing the growth of mtB6 and mtBALB cells in either anti-CCL20 or IgG1-treated mice ($n = 8$). (D) Quantitative RT-PCR histogram showing the mRNA expression levels of chemokine CCL20 in tumors ($n = 4$). The data are presented as a mean ± SEM with * $P \leq 0.05$.

including colorectal cancer [9], pancreatic carcinoma [10], breast cancer [14], renal cell carcinoma [16], and melanoma [17]. Thus, we hypothesized that hyper-proliferation seen in mtBALB cybrids might be caused by a higher CCL20 expression triggered by alterations in their mtDNA. To test the role of CCL20 on cellular growth of murine cybrids, we used rmCCL20 protein as a growth stimulus. mtB6 cybrids showed significant increase in proliferation after rmCCL20 was added to the cultured media. Moreover, the expression of CCL20 in mtB6 cybrids was increased to the level of CCL20 expression in mtBALB cybrids after the treatment with rmCCL20 protein likely caused by the ability of CCL20 for autocrine stimulation [35]. Furthermore, rmCCL20 treatment not only significantly increased expression level of CCL20 and its CCR6 receptor, but also the expression of MMP-9 which plays important role in tumor progression. Conti and Rollins [36] have made a similar observation with chemokine CCL5 which induced gene expression of various MMPs including MMP-9.

Another characteristic of malignant cells is enhanced motility. We have previously observed increased motility of mtBALB haplotype that has elevated expression of CCL20 compared to mtB6 cybrids [24]. Fleming et al. also demonstrated that chemokine CCL20 can enhance migratory capabilities of cutaneous keratinocytes [37] through up-regulating of CCL20 by inflammation [38]. Moreover, Calvayrac et al. [39] showed that increased release of CCL20 significantly induced human lymphocyte migration in transwell assay. Thus, we assumed that increased migration of mtBALB cybrids might be caused by higher expression of CCL20 in these cells. Transwell migration assays were performed to test the role of CCL20 on cellular migration. We confirmed that mtB6 cells expressing very low levels of CCL20/CCR6 migrated at lower rate than mtBALB cybrids expressing high levels of CCL20/CCR6. However, mtB6 cybrids migrated at significantly higher rate when rmCCL20 protein was either used as a chemo-attractant (added to the bottom well) or these cells were treated with rmCCL20 (added to the top well). Moreover, we observed that mtB6 cybrids tested for migration in co-culture system with chemo-attractant mtBALB cells expressing high levels of CCL20 migrated at significantly higher rate compared to control cells without any chemotactic stimuli. Furthermore, treatment of mtB6 cybrids with rmCCL20 also caused increase in expression of MMP-9 which is known to be a key player

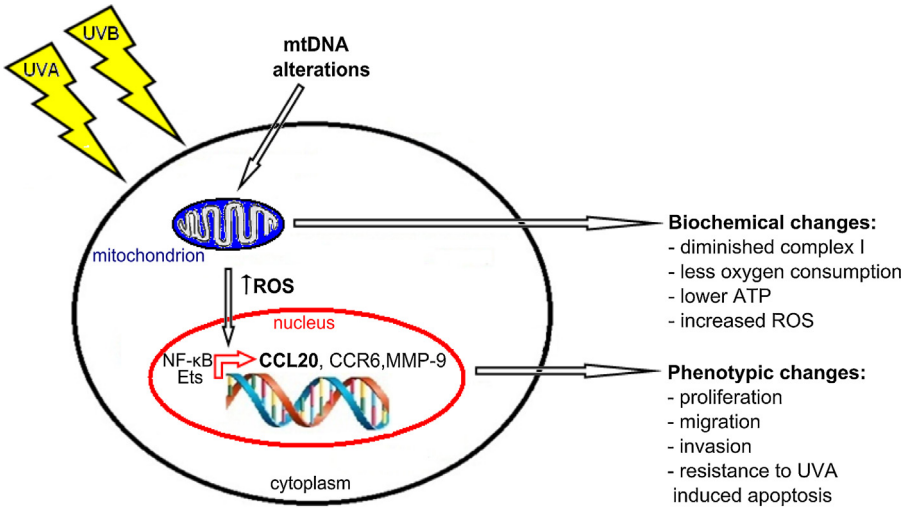

Fig. 9. mtDNA alterations seen in UV-induced mouse models of skin cancer can alter biochemical characteristics of cells, trigger changes in expression of nuclear encoded genes associated with tumorigenesis and subsequently lead to significant phenotypic changes of cells. mtBALB cybrid cells with genetic alteration in mt-Tr gene (9821insA) showed significant biochemical changes such as diminished levels of complex I protein, less cellular oxygen consumption, and lower ATP levels [23] and increased levels of ROS, resistance to UV-induced apoptosis and enhanced cellular growth, migratory and invasive capabilities [24]. Moreover, nucleotide changes in mtDNA can trigger the differences in expression levels of some nuclear encoded genes associated with malignancy such as MMP-9 and CCL20 [25].

in motility of malignant cells. These findings suggest important role of CCL20 as an autocrine stimulator of cellular migration and thus strongly support the role of mtDNA alterations in the process of tumorigenesis.

Previously, it was suggested that NF-κB and MAPK signaling might be involved in the regulation of inducible chemokines including CCL20 [20,22]. We demonstrate here, that inhibitors of NF-κB activation (BAY11-7082) and MAPK signaling (PD98059) were able to significantly decrease CCL20/CCR6 expression levels in mtBALB cybrids suggesting that expression of CCL20/CCR6 can be partially regulated by inhibition of NF-κB and/or MAPK signaling. This is in correlation with Calvayrac et al. [39] and with Wang et al. [40] who prevented the up-regulation of CCL20 by inhibiting of NF-κB. Additionally, it was indicated that NF-κB is involved in modulation of CCL20 by other mediators such as TNFα in some human cancer cells [41], prolactin in keratinocytes [42] or hypoxia in primary human monocytes [43]. Moreover, Kwon et al. previously suggested a role of the Ras-MAPK pathway signaling in MIP-3alpha (CCL20) expression in Caco-2 human colonic epithelial cells through the Ets transcription factor activation [22].

Our *in vitro* data indicate that mtDNA alterations can trigger the changes in expression of some nuclear encoded genes associated with malignancy and subsequently induce biochemical and phenotypic changes in cells with altered mtDNA. The role of CCL20 in tumor growth was previously described [44]. Thus, we have evaluated whether the up-regulation of chemokine CCL20 induced by mtDNA alterations plays a critical role in tumor development. Our *in vivo* data indicate increased tumor growth in control IgG1-treated group of mice injected with mtBALB cells compared to the control IgG1-treated group of mice injected with mtB6 cybrids. Importantly, we observed the ability of anti-CCL20 neutralizing antibody to significantly inhibit the *in vivo* tumor growth of tumorigenic mtBALB cybrids expressing high levels of CCL20 compared to isotype control IgG1 antibody. These data correlate with the observations of Beider et al. who showed that CCL20 neutralization inhibits the CCL-20 and CXCR4-dependent growth of human prostate and colon tumor cells *in vivo* [45].

In conclusion, this study reports the evidence that nucleotide changes in mtDNA can cause the up-regulation of chemokine CCL20 through the increased ROS levels contributing to the development of tumorigenic phenotypes seen in our mutant mtBALB cybrids (Fig. 9). These findings strongly support the role of mtDNA alterations in tumorigenesis and further understanding of the role of mtDNA mutations in various human malignancies may help to identify new molecular targets for cancer therapies. Specifically, CCL20/CCR6 inhibition may serve as a potential tool for novel treatment strategies to halt skin cancer progression.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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