Supplementary Material

Cell treatments

Vitiligo and control fibroblast-conditioned media were collected after 48 hours from cells maintained in DMEM plus antibiotics in the absence of FBS and were kept at -80°C until use. Primary cultures of melanocytes were treated with fibroblast-conditioned media either for 24 and 48 hours. For HGF treatment, cells were incubated with recombinant human HGF (10 and 50 ng/ml) (EMD Millipore Corp., Temecula, CA, USA) for 24 and 48 hours. To block HGF activity, the medium containing recombinant HGF and fibroblast-conditioned media was pre-incubated at 37 °C with a neutralizing antibody to HGF (1.2 µg/ml) (Sigma) for 1 hour, accordingly to Zhang et al. (2002). For H$_2$O$_2$ treatment, cells were exposed to H$_2$O$_2$ (25 and 50) µM for 72 hours, changing the treatment every 24 hours. Results represent the mean value ± from three different experiments.

Immunofluorescence

Cells were fixed in cold methanol for 4 min at -20°C or with 4% paraformaldehyde for 30 min followed by 0.1% Triton X-100 to allow permeabilization and were then incubated with the following primary antibodies: anti-p53 monoclonal antibody (1:100) (Zymed Laboratories, Inc., South San Francisco, CA, USA), anti-Lamin B1 rabbit antibody (AbCam, Cambridge, UK), anti-αSMA monoclonal antibody (1:300) (Sigma), anti-E cadherin monoclonal antibody (1:100) (Dako), anti-N cadherin rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 hour. Primary antibodies were visualized using anti-mouse Alexa fluor 488 (1:500) or anti-rabbit Alexa fluor 555 (1:500) (Cell Signalling Technology, MA, USA). Nuclei were visualized with 4’,6’-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Srl, Milan, Italy). For staining of actin cytoskeleton, cells were incubated with TRITC-phalloidin (1:1000) (Sigma Chemicals). Fluorescence signals were analyzed by recording stained images using a CCD camera (Zeiss). Percentage of α-SMA and p53 positive cells was evaluated counting for each condition, a total of 500 cells observed in 10 fields and results represent the mean values of positive cells/total cells±SD (%) from three different experiments. For the measurement of the cell area, control and vitiligo
fibroblast pairs were analyzed at the same culture passage. For each control and vitiligo fibroblast cultures the area of at least 100 cells was measured, and the results are expressed as cell area mean value ± SD (µm²). Three experiments were performed.

**Immunohistochemistry**

Serial sections (3 µm) derived from formalin-fixed and paraffin-embedded blocks were dewaxed in xylene and rehydrated through graded ethanol to PBS. Endogenous peroxidase activities were blocked by 0.03% hydrogen peroxide. Following antigen retrieval, tissue sections were incubated with the following primary antibodies: anti-MITF monoclonal antibody (1:20) Novocastra, Leica Biosystems, Newcastle Upon Tyne, UK), anti-MART1 antibody (1:50) (Melan A, monoclonal antibody (Dako Corp., Carpinteria, CA, USA) anti-αSMA mouse monoclonal antibody (1:300) (Sigma), anti N-cadherin monoclonal antibody (1:200) (Zymed Laboratories Inc.), anti-p53 monoclonal antibody (Dako Corp., Carpinteria, CA, U.S.A), anti-IL6 rabbit polyclonal antibody (1:800) (AbCam). The staining was visualized using the Thermo Ultravision Quanto Detection System HRP, employing 3-amino-9-ethyl-carbazole or 3,3’-diaminobenzidine as substrate chromogen. All the sections were counterstained with haematoxylin. Negative controls were obtained by omitting the primary antibodies. For immunofluorescence staining of fibronectin, tissue sections were dewaxed and processed for antigen retrieval and then incubated with anti-fibronectin mouse monoclonal antibody (1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Primary antibody was then visualized using anti-IgG-Alexa Fluor 546 (Invitrogen, Thermo Life Technologies Corp., Eugene, Oregon, USA Fischer). Nuclei were visualized with 4’,6’-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Srl). For double immunofluorescence, tissue sections were incubated with anti- E-cadherin monoclonal antibody (1:100) (Dako) and anti-c-kit (CD117, polyclonal antibody (1:300) (Dako). Primary antibodies were visualized by anti-mouse IgG-Alexa Fluor 488 and anti-rabbit Alexa Fluor 546 (Invitrogen, Thermo Fischer). Nuclei were visualized with DAPI.
Flow cytometry

Intracellular ROS production was detected by 2’,7’-dichlorofluorescein diacetate (DCFH-DA, Fluka AG, Switzerland). Cells were incubated at 37°C for 30 min with 2.5 µM DCFH-DA in phosphate-buffered saline with calcium and magnesium and 5 mM glucose (PBS) and then immediately analyzed by flow cytometer (FACSCalibur, Becton Dickinson, San Diego, CA; argon laser 488 nm/15mW and red diode 635 nm), and CellQuest and FloJo software). The MFI analysis was performed in a linear scale. In all, 5,000 total events were acquired. DCFH-DA fluorescence was analyzed on gated viable cells.

Gas chromatography-mass spectrometry analysis of cholesterol and cholesterol oxidation products

Cells were lysed in distilled water by repeated freezing in liquid nitrogen and thawing. Protein concentration was determined in the supernatants according to Bradford (Bradford, 1976), using BSA as a standard, by a Lambda 25 UV/Vis spectrophotometer (Perkin-Elmer, UK). Lipids were extracted from the cells using chloroform methanol 2:1 in the presence of butylated hydroxytoluene (BHT, Sigma-Aldrich, St. Louis, MO, USA) as antioxidant and 5α-cholestane and d6-cholesterol as internal standards. Organic phase was collected and dried under nitrogen stream. After the addition of 1 ml of freshly prepared 1 M KOH solution in ethanol, hydrolysis of oxysterols ester was performed for 1h at 60 °C in the darkness in a water bath. Samples were neutralized to pH 7 with phosphoric acid. Cholesterol and its oxidation products were extracted using chloroform methanol 2:1. Dried lipid extracts were derivatized to trimethylsilyl ethers (TMS). After 30 min at 60°C the samples were analysed by gas chromatography-mass spectrometry (Trace GC Ultra, Trace DSQ, Thermofinnigan) The separation was performed by capillary column HP-5MS (30 m x 0.25 mm x 0.25 µm, Restek Corporation, Bellefonte PA, USA) using helium as gas carrier. An oven temperature gradient from 180 to 250°C at 20°C/min and then from 250 to 300°C at 5°C/min was used. Mass spectra were recorded in Electronic Impact and in SIM modality. Selected ions were
217,2 for α-cholestane; 464,5 for d6-cholesterol; 458,5 for cholesterol; 456,4 for 7β-OH-cholesterol; 472,4 for 7-keto-cholesterol.

**Protein determination by sandwich enzyme-linked immunosorbent assay**

IL-6, HGF, SCF, TGF-β and ET-1 in the supernatants of control and vitiligo fibroblasts were quantified by ELISA assay (IL-6 and TGF-β kits from 4ABiotech, HGF and ET-1 kits from Cusabio, CliniSciences; SCF kit from AbCam) according to the manufacturer’s protocol. The results were normalized for the number of cells contained in each sample and were expressed as picograms per 1x10^6 cells. The measurement was performed in duplicate for each sample and the experiments were repeated twice.

**Western blot analysis**

Cells were lysed and processed as previously reported (Flori et al. 2011). Membranes were incubated with anti-p53 mouse monoclonal (Dako, Milan, Italy), anti αSMA mouse monoclonal (Sigma), anti-fibronectin mouse monoclonal (Santa Cruz Biotechnology), anti-vimentin rabbit polyclonal (Cell Signaling) and anti-E-cadherin mouse monoclonal (Dako, Milan, Italy) antibodies. Gapdh rabbit polyclonal (Santa Cruz Biotechnology) or βactin mouse monoclonal (Sigma) antibodies were used as loading control proteins. Protein levels were quantified by measuring the optical densities of specific bands using a GS-800 Calibrated Image Densitometer (Bio-Rad Laboratories Srl, Milan, Italy).

**Real-time RT-PCR**

RNA was isolated (Aurum Total RNA Mini kit, Bio-Rad) and reverse transcribed (RevertAid First Strand cDNA Synthesis kit, Fermentas, ThermoFisher Scientific), and transcripts were quantified using the Real-Time Detection System (iQ5 Bio-Rad) supplied with iCycler IQ5 optical system software version 2.0 (Bio-Rad). Levels of gene expression were quantified applying the 2^{\Delta \Delta CT} method, using GAPDH as an endogenous control, and are expressed relative to untreated control cells. Results represent the mean values ± SD from three different experiments. Sequences of the
primers used are indicated in the following table:

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<th>Target Gene</th>
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**Supplementary Reference:**

Supplementary Figure S1

Expression and release of TGF-β in non lesional vitiligo fibroblasts
(A) mRNA transcript levels of TGF-β evaluated by qRT–PCR in control and vitiligo fibroblasts. (B) TGF-β and (C) ET-1 quantitation by enzyme-linked immunosorbent assay on control and vitiligo fibroblasts supernatants.
**Supplementary Figure S2.**

The ROS scavenger N-acetyl-cysteine (NAC) down-modulates αSMA expression in non-lesional vitiligo fibroblasts.

(a) Western blot analysis and corresponding densitometric analysis of αSMA in vitiligo fibroblasts after treatment with NAC (2.5 and 5 mM) for 24 hours. (b) Immunofluorescence analysis and (c) corresponding quantitative analysis of the percentage of positive cells for αSMA on vitiligo fibroblasts following NAC treatment (2.5 and 5 mM) for 24 hours. Nuclei are stained with DAPI. Scale bar b: 50 μm. (d) ROS detection in vitiligo fibroblasts in response to NAC treatment (2.5 and 5 mM) for 12 and 24 hours, evaluated by FACS analysis using DCFH-DA labeling. A representative experiment is shown.
Supplementary Figure S3.
**Distribution of E-cadherin in melanocytes treated with fibroblast-conditioned medium.**
Right panel: Immunofluorescence analysis of E-cadherin expression in normal melanocytes kept untreated or treated for 48 hours with conditioned medium collected from normal and vitiligo fibroblasts. Arrows point at E-cadherin staining. Left panel: Cell shape of the same images is outlined with white dashed lines. Scale bars: 10 μm. Results are representative of three different experiments.
Supplementary Figure S4.

Upper panel: Influences of HGF activity on E-cadherin modifications in response to fibroblast-conditioned medium. Lower panel: Modulation of N-cadherin expression following the treatment with fibroblast-conditioned medium.

(a) Immunofluorescence analysis of E-cadherin expression (green signal) in normal human melanocytes treated with recombinant HGF (10 and 50 ng/ml) for 24 and 48 hours. Results are representative of three different experiments. (b) Immunofluorescence analysis of E-cadherin expression in normal human melanocytes treated for 24 hours with recombinant HGF (50 ng/ml) or with vitiligo fibroblast-conditioned medium in presence of anti-HGF neutralizing antibody (1.2 mg/ml). Results are representative of three different experiments. (c) Immunofluorescence analysis of N-cadherin expression (red signal) in normal human melanocytes treated with control and vitiligo fibroblast-conditioned media for 24 hours. Nuclei are stained with DAPI. (d) Quantitative analysis of N-cadherin expression. Results are expressed as fold change of mean fluorescence intensity ±SD relative to the untreated cell value, which was set as 1. Values are representative of three different experiments. Scale bars: a and b: 10 μm; c: 50 μm.
Supplementary Figure S5.

**Expression of E and N-cadherins in non lesional vitiligo skin biopsies.**

(a) Immunofluorescence analysis of the expression of E-cadherin (green signal, low magnification and enlarged view of the boxed areas) and c-kit (red signal, low magnification and enlarged view of the boxed areas) in a representative non lesional vitiligo skin biopsy. Arrows in the boxed areas point to a positive suprabasal cell. Nuclei are stained with DAPI. The basal membrane is outlined as white dashed line. (b) Serial sections of non lesional vitiligo skin biopsies stained with haematoxylin and eosin and analyzed for N-cadherin expression. Low magnification images and enlarged view of the boxed areas are presented. Black arrows point to melanocytes. Scale bars: a: 10 μm; enlarged view of the boxed areas: 10 μm. b: 50 μm; enlarged view of the boxed areas: 20 μm.