Vitiligo Skin: Exploring the Dermal Compartment

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There is an increasing interest in the apparently normal skin in vitiligo. Altered expression of the adhesion molecule E-cadherin and persistent deregulated intracellular redox status that promotes the acquisition of a stress-induced senescent phenotype in melanocytes of normally pigmented skin from patients with vitiligo have been described. Growing evidence has shown that such cellular and functional alterations are not necessarily restricted to melanocytes but may be extended to other cutaneous cell populations in both lesional and nonlesional areas. However, whether dermal fibroblasts exhibit related alterations that may contribute to the defects associated with melanocytes in vitiligo is not known. Here we reveal within the dermal compartment cells a myofibroblast phenotype and a predisposition to premature senescence, indicating the existence of altered cross-talk between dermal and epidermal components that may affect melanocyte functionality even in the apparently normal skin of patients with vitiligo.

INTRODUCTION

Several mechanisms have been considered to be responsible for melanocyte loss in vitiligo, including genetic, inflammatory, autoimmune, oxidative, and metabolic alterations, but the individual contribution of each of these alterations is still unclear. A deregulated redox state associated with increased cellular vulnerability to oxidative insults due to intrinsic metabolic abnormalities is a major factor able to trigger immune responses leading to melanocyte degeneration and disappearance (Picardo et al., 2015). Alterations in the distribution of the cell-cell adhesion molecule E-cadherin, as well as a deregulated intracellular redox status that, when persistent, promotes the acquisition of a stress-induced premature senescent-like phenotype, have been highlighted in melanocytes of nonlesional skin (Bellei et al., 2013; Wagner et al., 2015). Increasing evidence suggests that the presence of cellular and functional abnormalities extends to other cutaneous cell populations in both lesional and nonlesional skin. Immunohistochemical analysis on biopsies showed the overexpression of senescence-associated markers distributed to the entire nonlesional epidermis (Bellei et al., 2013), and redox imbalance and modifications in the expression of proliferation and senescence markers have been highlighted in cultured

lesional keratinocytes (Bondanza et al., 2007; Kostyuk et al., 2010). Dermal components, via extracellular matrix (ECM) proteins and fibroblasts, exert an important role in the regulation of melanocyte homeostasis. ECM proteins contribute to melanocyte adhesion to the basement membrane. Fibroblasts release growth factors and messengers that are part of a paracrine signaling network that controls melanocyte function. Most of these mediators such as hepatocyte growth factor (HGF), stem cell factor (SCF), keratinocyte growth factor, and neuregulin-1 act as promelanogenic factors favoring melanocyte growth, differentiation, migration, and survival. Others, such as Dickkopf1 (DKK1), function as negative regulators of pigmentation and melanocyte growth (Bastonini et al., 2016). An altered expression of growth factors controlling melanocyte homeostasis has been observed in several pigmentary disorders including vitiligo (Kitamura et al., 2004; Kovacs et al. 2010; Lee et al., 2005; Moretti et al., 2002). However, only few reports point to a specific involvement of the dermis in vitiligo, and most of the data are focused on lesional skin. Uregulation of the antiadhesive ECM protein tenascin and DKK1 has been demonstrated in lesional dermis (LePoole et al., 1997; Oh et al., 2012). By contrast, the expression and release of keratinocyte growth factor by lesional fibroblasts is reduced (Purpura et al., 2014). Because of the decisive role of the dermal compartment in regulating melanocyte activity and survival, we aimed to investigate the involvement of the dermis in vitiligo, focusing on the analysis of the features of fibroblasts to: (i) investigate whether the degeneration/senescence prone phenotype detected in vitiligo epidermis also features resembling a senescent phenotype (Kovacs et al., 2010),
we measured cell areas and actin cytoskeleton organization, demonstrating a significant enlargement in the size with respect to control (Figure 1a and b). Vitiligo-associated fibroblasts also exhibited an increase in actin stress fibers detected using phalloidin (Figure 1a). A significant increase in reactive oxygen species (ROS) content was also observed in vitiligo fibroblasts (Figure 1c), similar to that reported in melanocytes (Bellei et al., 2013). Consequently, we analyzed the expression of the stress-induced cell cycle regulator p53, which is upregulated in vitiligo melanocytes and keratinocytes (Bellei et al., 2013; Salem et al., 2009). Vitiligo fibroblasts showed a marked increase in the levels of the p53 protein in most cultures by western blot analysis (Figure 1d). Immunofluorescence analysis confirmed a higher number of cells displaying p53-positive nuclei in vitiligo with respect to control, as assessed by the nuclear counterstaining with DAPI and the double staining with the nuclear membrane marker lamin B1 (Figure 1e and f). As a positive control of oxidative stress, control fibroblasts were exposed to H2O2. An increased number of p53-stained cells were detected (Figure 1g), which appeared comparable to that observed in vitiligo fibroblasts (Figure 1h). The p53-responsive stress gene GADD45 was evaluated by quantitative real time reverse transcriptase-PCR (qRT-PCR) was also significantly induced in vitiligo (Figure 1i).

Nonlesional vitiligo fibroblasts exhibit a myofibroblast-like phenotype, which is correlated with the increased intrinsic oxidative stress

As high levels of intracellular ROS favor the conversion of fibroblasts into transdifferentiated myofibroblasts (Cat et al., 2006), we analyzed the expression of the alpha-smooth muscle actin (α-SMA) marker in our cell cultures. A significantly higher α-SMA mRNA level was detected in vitiligo fibroblasts compared with control as demonstrated by qRT-PCR (Figure 2a), which was reflected in a clear increase at the protein level detected by western blot (Figure 2b). Immunofluorescence analysis revealed the staining pattern for α-SMA as positive stress fibers distributed throughout the cytoplasm, whereas only a weak cytosolic reactivity was evident in a few normal fibroblasts (Figure 2c and d). The treatment of control fibroblasts with H2O2 was able to induce an increase in the number of α-SMA-positive cells comparable to that measured in vitiligo (Figure 2d and e). The mRNA transcript of the splice variant of extra domain A-fibronectin, typically expressed by myofibroblasts (Serini et al., 1998), also showed a clear increase in vitiligo (Figure 2f). α-SMA has been reported to be a direct transcriptional target of p53 (Comer et al., 1998). Consistent with this, we found a positive correlation between the percentage of stained α-SMA and those positive for p53 ($r = +0.88$).

On the basis of these results, we deepened the analysis of the features of fibroblasts by evaluating the expression of growth factors and cytokines, as well as collagen isoforms and ECM molecules associated with the myofibroblast phenotype (Desmoülière et al., 2004; Powell et al., 1999). We analyzed the inflammatory cytokines IL-1β, IL-6, and the growth factors HGF and SCF, for their role in controlling melanocyte homeostasis and for their known upmodulation in stress-induced senescent cells (Kovacs et al., 2010; Miyazaki et al., 1998; Waldera Lupa et al., 2015). A significant induction of expression of the IL-1β, IL-6, and HGF genes was observed in vitiligo fibroblasts with respect to normal control cells as demonstrated by qRT-PCR, whereas SCF transcripts showed no difference (Figure 3a). An enhanced release of IL-6 and HGF was also observed at the protein level by ELISA on culture supernatants (Figure 3b and c), whereas the secretion of SCF showed a tendency to decrease, although not significant (Figure 3d). Moreover, IL-6 and HGF levels correlated with the expression of α-SMA ($r = +0.85$ and $+0.82$ for IL-6 and HGF, respectively). The ECM glycoprotein fibronectin and the intermediate filament- associated protein vimentin, both associated with myofibroblast differentiation and known to be induced by ROS and in senescent cells, were also upmodulated in vitiligo (Figure 3e–g), as well as the fibroblast-derived factor DKK1 (Figure 3h). Among the different collagens produced by myofibroblasts (Powell et al., 1999), the expression of collagen IV, which represents the major molecule of the basement membrane and contributes to the maintenance of the correct location of melanocytes, was significantly upregulated in vitiligo, as assessed by qRT-PCR (Figure 3i). Because dermal fibroblasts subjected to stress-induced premature senescence display an increased amount of cholesterol and its oxidative products oxysterols (Briganti et al., 2014), we evaluated the levels of cholesterol and of the cholesterol oxidation derivatives 7-beta-hydroxycholesterol and 7-ketocholesterol. We found a higher amount of cholesterol (128.36 ± 36; $P < 0.05$) and of the two oxysterols in vitiligo compared with normal fibroblasts (230 ± 97% and 148 ± 33%, respectively; $P < 0.05$). Finally, we analyzed the expression of the transforming growth factor-β and of endothelin-1 (ET-1), as they represent important mediators in promoting myofibroblast induction (Desmoulie`re et al., 1993; Shi-Wen et al., 2004). The results showed no significant differences either at the mRNA (Supplementary Figure S1a online) or protein levels (Supplementary Figure S1b) for transforming growth factor-β between vitiligo and control fibroblasts. The overall amount of ET-1 released in vitiligo culture supernatant tended to be higher in comparison to control (Supplementary Figure S1c). However, the difference was not statistically significant.

To evaluate whether the myofibroblast phenotype could be ascribed to the augmented intracellular ROS content, the expression of α-SMA was analyzed after the treatment with the ROS scavenger N-acetyl-L-cysteine. Western blot and immunofluorescence analyses demonstrated that α-SMA protein levels and the number of positive cells were significantly reduced in the presence of N-acetyl-L-cysteine in a dose-dependent manner (Supplementary Figure S2a–c online). The reduction is preceded and accompanied by the decrease of the intracellular ROS production ($P < 0.05$) (Supplementary Figure S2d).

Conditioned medium from vitiligo fibroblasts downregulates E-cadherin expression on melanocytes

Dermal end epidermal constituents release growth factors and cytokines, which regulate melanocyte functionality both in an autocrine and a paracrine manner (Bastonini et al., 2016). We therefore investigated the possible
Figure 1. Vitiligo fibroblasts display increased ROS levels associated with upmodulation of stress-induced markers. (a) Phase-contrast and TRITC-phalloidin staining. (b) Cell area measurement (mean value ± SD, μm²). (c) ROS detection by FACS analysis. (d) Western blot of p53 expression and corresponding densitometric analysis. (e) Immunofluorescence of p53 expression. (f) Magnified image of p53 staining in vitiligo fibroblasts and double immunofluorescence with anti-p53 and anti-lamin B1 antibodies. (g) Immunofluorescence of p53 in H₂O₂-treated control fibroblasts. (h) Percentage ± SD of p53-positive cells on control, vitiligo, and control fibroblasts treated with H₂O₂. (i) mRNA transcripts of GADD45. Nuclei are stained with DAPI. The arrows point at positive cells. The cell shape is outlined. Scale bars: a, e, and g: 50 μm; f: 20 μm. DCFH-DA, 2',7'-dichlorofluorescein diacetate; NHF, normal human fibroblast; ROS, reactive oxygen species; SD, standard deviation; TRITC, tetramethylrhodamine; VHF, vitiligo fibroblast.
influences of vitiligo fibroblasts on primary melanocytes by treating them with conditioned medium obtained from normal and vitiligo fibroblasts. Emerging data point on an altered expression of the adhesion molecule E-cadherin in vitiligo melanocytes, which occurs even earlier than the appearance of clinical lesions (Wagner et al., 2015). We first evaluated the basal expression level of the protein on a panel of normal and vitiligo melanocytes collected from nonlesional areas. Most vitiligo melanocytes demonstrated a significant reduction of E-cadherin expression with respect to control by western blot and immunofluorescence analyses (Figure 4a and b), confirming in vitro the altered expression pattern demonstrated ex vivo (Wagner et al., 2015). We then treated melanocytes with conditioned medium collected from control and vitiligo fibroblasts with the aim of simulating in vitro the dermo-epidermal cross-talk existing in the cutaneous microenvironment. The treatment with normal fibroblast-conditioned medium did not significantly modify the expression of E-cadherin in normal melanocytes, whereas it induced a decrease in the E-cadherin level in vitiligo melanocytes, as assessed by western blot and immunofluorescence (Figure 4c and d). Vitiligo fibroblast-conditioned medium induced a reduction in E-cadherin expression in both vitiligo and normal melanocytes that was more pronounced in vitiligo cells, as assessed by densitometric analysis (Figure 4c). Although most untreated melanocytes displayed a regular and constant membranous distribution of E-cadherin, cells treated with conditioned medium from vitiligo fibroblasts showed a reduction associated with a discontinuous expression pattern of the adhesion molecule (Supplementary Figure S3 online). We next evaluated whether the reduction of E-cadherin expression observed after the treatment with vitiligo fibroblast-conditioned medium could be ascribed to the higher production of HGF observed in vitiligo. To this aim, we first treated normal melanocytes with recombinant HGF and, as expected (Li et al. 2001; Soong et al. 2012), the results showed that the growth factor induced a reduction in E-cadherin expression (Supplementary Figure S4a online). The specific involvement of HGF in modulating this adhesion molecule was ascertained by neutralizing the growth factor activity with an anti-HGF antibody, which led to the inhibition of E-cadherin downregulation (Supplementary Figure S4b). The pretreatment of vitiligo fibroblast-conditioned medium with the neutralizing antibody to HGF resulted in a partial reduction of E-cadherin downmodulation in response to vitiligo culture medium, confirming the relevant role of HGF in mediating such effect (Supplementary Figure S4b).
To analyze whether the reduction of E-cadherin in response to the treatment with vitiligo fibroblast-conditioned medium could be associated with altered expression of other cadherin adhesion molecules, we analyzed the expression of N-cadherin. Immunofluorescence analysis showed that normal melanocytes...
displayed basal low levels of the adhesion molecule, which were not modified by the treatment with conditioned medium either from control or vitiligo fibroblasts (Supplementary Figure S4c and d).

** Modifications in the dermal compartment are detectable ex vivo **

To validate the in vitro results, we performed the immunohistochemical evaluation of some markers detected in vitro on skin biopsies collected from the same patients from whom the cells were derived. Vitiligo specimens were first analyzed for the number and distribution of melanocytes. Positive cells for the melanocyte markers MITF and MART1 were detected in nonlesional vitiligo skin with no significant differences in the overall number of cells in comparison to control. However, some vitiligo melanocytes appeared both to extend into the dermis and to be localized suprabasally. The melanocytes detected in the dermis were higher in number but not significantly different with respect to control, whereas the melanocytes found in the suprabasal layers were significantly more abundant than those in control skin ($P < 0.05$) (Figure 5a and b). To evaluate whether the melanocytes located suprabasally could show alterations in the E-cadherin expression, double immunofluorescence with anti-c-kit and anti-E-cadherin antibodies was performed. Immunostaining revealed the presence of suprabasal c-kit positive melanocytes characterized by a discontinued and low expression of E-cadherin (Supplementary Figure S5a online). Parallel immunohistochemical analysis of the expression of N-cadherin did not show melanocyte positive reactivity in any vitiligo sample (Supplementary Figure S5b). Immunohistochemical analysis of p53 revealed the presence of positive cells in the dermis from nonlesional areas and, although a variable degree of reactivity was observed among the samples, the number of labeled cells was higher in vitiligo with respect to control (Figure 5c).
expression revealed, as expected, positive staining of smooth muscle cells in vessel walls in both normal and vitiligo skin. Some positive cells were detected in the dermis of vitiligo, whereas no immunoreactivity was observed in control dermis (Figure 5d). Immunohistochemical analysis for the expression of IL-6 showed an increased immunoreactivity for the
cytokine in vitiligo in comparison to control skin (Figure 5e). Also, immunofluorescence analysis of fibronectin expression showed a higher reactivity in vitiligo dermis compared with control (Figure 5f).

DISCUSSION
Despite the important role exerted by fibroblasts and ECM proteins in regulating melanocyte functionality, few studies examine the involvement of the dermal constituents in vitiligo. Our data show that changes linked to redox imbalance previously detected in vitiligo melanocytes (Bellei et al., 2013) also extend to the dermal fibroblasts of normally pigmented areas. The generation of oxidative stress detected in vitiligo fibroblasts was correlated with morphological and functional modifications. We found a higher number of cells with biological characteristics resembling a myofibroblast phenotype. Myofibroblasts may arise from different precursors. Besides their origin from adjacent local tissue fibroblasts, they may derive from other resident cells including smooth muscle, endothelial and epithelial cells, tissue-derived mesenchymal stem/stromal cells, as well as bone-marrow-derived cells such as fibrocytes (Kendall and Feghali-Bostwick, 2014; Leask, 2010). As the origin of myofibroblasts may be heterogeneous, even the factors controlling their induction and persistence are under the control of a complex interplay of promoting and suppressing mediators (Hinz, 2016). Although the specific contribution of the different cell sources in the induction of myofibroblasts in vitiligo remains to be characterized, the presence of myofibroblasts may be the consequence of a deregulation in the interplay between mediators and messengers released in vivo in the entire skin. The elevated basal ROS levels detected in vitiligo may represent a pivotal player because it has been shown that ROS represent positive mediators in the induction of myofibroblasts. Mitochondria have been proposed as a possible site of the increased production of ROS in vitiligo, and an altered expression and activity of complex I has been demonstrated both in peripheral blood mononuclear vitiligo cells and in melanocytes from nonlesional skin (Dell’Anna et al., 2003, 2007). Impairment of mitochondrial function leading to high basal intracellular ROS production may also be present as an intrinsic defect in dermal fibroblasts and encourage, in turn, myofibroblast differentiation. In fact, it has been observed that fibroblasts carrying dysfunctional mitochondrial complex I produce high levels of ROS that correlate with elevated α-SMA expression and consequently with their transdifferentiation into myofibroblasts (Taddei et al., 2012). Dysfunction in the mitochondrial complex I associated with increased ROS levels also drives irradiation-induced myofibroblast differentiation of lung fibroblasts (Yang et al., 2017). Accordingly, strong production of ROS has been found in nonlesional vitiligo skin biopsies in comparison to control (Wagner et al., 2015). At the same time, we cannot exclude a combined effect guided by local inflammation, as the presence of positive CD3 in the dermis has been detected in clinically normal pigmented skin areas (Wagner et al., 2015).

The upregulation of p53 observed in our cell cultures was also previously demonstrated in vitiligo melanocytes and keratinocytes (Bellei et al., 2013; Salem et al., 2009), and could be an additional element involved in the induction of the myofibroblast phenotype, because α-SMA is a direct transcriptions target of p53 (Comer et al., 1998). Accordingly, the treatment of control fibroblasts with H2O2 resulted in an increased expression of both p53 and α-SMA proteins, supporting the involvement of oxidative stress in mediating such an effect.

Consistent with the idea that the acquisition of stress-related modifications could be extended to the entire skin, we observed that vitiligo fibroblasts display an increased content of cholesterol and oxysterols, which are associated with the occurrence of a senescence-like phenotype in a stress-induced premature senescence model caused by the exposure of fibroblasts to 8-methoxypsoralen plus UVA irradiation (Briganti et al., 2014). Oxysterols may be involved in the induction of the myofibroblast phenotype by contributing to the unbalanced redox status in the dermis and favoring over time the induction of premature senescence. Interestingly, an increase in the number of α-SMA expressing cells has been observed in senescent fibroblasts with respect to young cells (Cáceres et al., 2014; Yanai et al., 2015). Vitiligo cells also display an enlarged shape associated with a higher expression of fibronectin, vimentin, and stress fibers, all features exhibited by senescent fibroblasts (Kim et al., 2009; Nishio et al., 2001). As previously demonstrated in melanocytes, vitiligo fibroblasts showed an increased synthesis and production of IL-6, a contributing factor to aging-associated secreted proteins released by prolonged damaged and senescent cells in skin (Waldera Lupa et al., 2015), further supporting the presence of a stress-mediated senescence phenotype extended to the entire skin. Interestingly, some features observed in vitiligo fibroblasts resemble the phenotype described in fibroblasts of scleroderma/systemic sclerosis (Hinz et al., 2012). Skin fibroblasts from patients with systemic sclerosis generate excessive ROS and show increased expression of α-SMA (Spadoni et al., 2015). Transforming growth factor-β represents a pivotal factor in the accumulation of myofibroblasts in scleroderma (Lafyatis, 2014). In vitiligo skin, we did not find a significant increase in the expression of transforming growth factor-β, suggesting that myofibroblast transdifferentiation is not directly mediated by this growth factor, at least not via autocrine production. However, we cannot exclude the action of other factors, for example, IL-6 and ET-1, whose production is also increased in scleroderma fibroblasts (Feghali et al., 1992; Kawaguchi et al., 1994; Shi-Wen et al., 2004). In addition, some reports have shown a similar gene expression profile in both affected and unaffected fibroblasts (Fuzii et al., 2008; Whitfield et al., 2003) and higher levels of ROS in both fibrotic and nonfibrotic skin of patients with scleroderma (Bourji et al., 2015). Despite the occurrence of these alterations, the absence of clinical manifestations related to fibrosis in uninvolved scleroderma skin suggests that other additional factors are necessary to develop a macroscopically detectable fibrotic phenotype. A similar phenomenon may occur in vitiligo skin, in which the myofibroblast phenotype does not appear to be adequate to develop a clinically manifested fibrosis. In fact, although only few data are available in the literature on the histological examination of vitiligo normal appearing skin, fibrosis does not appear to have a disclosed characteristic.
Most of our vitiligo melanocytes showed a reduction of the E-cadherin level with respect to control cells, confirming in vitro the altered expression of E-cadherin demonstrated ex vivo (Wagner et al., 2015). We observed E-cadherin down-modulation after treatment with conditioned-medium derived from fibroblasts, which appeared significant only for vitiligo melanocytes when treated either with normal or nonlesional vitiligo fibroblast-conditioned media. By contrast, in control melanocytes, E-cadherin expression was diminished only when treated with conditioned medium derived from vitiligo cells. In the latter condition, the distribution of E-cadherin appeared discontinuous and interrupted, features resembling the expression pattern described by Wagner et al. (2015) ex vivo. Although these results indicate a higher intrinsic susceptibility of vitiligo melanocytes to external influences, it also appears that vitiligo fibroblasts release an elevated amount of biologically active messengers able to affect E-cadherin expression.

Among them, both HGF and ET-1 downmodulate E-cadherin in melanocytes (Haass and Herlyn, 2005). The increased secretion of HGF by vitiligo cells may therefore affect basal E-cadherin expression as detected in normal appearing skin. The partial abrogation of such a reduction after the treatment of vitiligo fibroblast-conditioned medium with an HGF neutralizing antibody confirms the involvement of HGF in modulating E-cadherin expression in vitiligo skin. However, additional and often synergistic effects can be caused by other factors released in the surrounding microenvironment, for example ET-1 itself and/or IL-6, which are both able to affect E-cadherin expression. Among the bioactive messengers involved in the intricate dermal-epidermal cross-talk, also the fibroblast-derived factor DKK1 reduces E-cadherin in HEK293 cells (Kuang et al., 2009). Previous work showed a higher DKK1 level in lesional skin compared with nonlesional vitiligo biopsies (Oh et al., 2012).

Our data demonstrate increased expression of DKK1 in fibroblasts cultures from nonlesional areas, indicating a deregulation of this mediator in the entire skin. DKK1 may act dually on melanocytes both by negatively regulating their proliferation and melanogenesis (Yamaguchi et al., 2004) and by reducing E-cadherin expression. As a consequence, melanocytes may be more likely to dissociate themselves from the neighboring keratinocytes, relocate suprabasally, and progressively disappear. In some conditions, such as melanoma progression, the downregulation of E-cadherin expression is often associated with the parallel induction of N-cadherin (Hsu et al., 2000). The absence of positive N-cadherin immunoreactivity in vitiligo suggests that the dermal protrusion of melanocytes does not appear to be mediated by cadherin switching. Rather, since the presence of focal gaps in the basal membrane of vitiligo skin has been described (Bose and Ortonne, 1994; Panuncio and Vignale, 2003), melanocytes may drop into the dermis through these basement membrane abnormalities.

The higher expression of HGF, as well as of IL-1β, detected in vitiligo fibroblasts further supports that these cells, similar to melanocytes, display a predisposition to a premature senescence phenotype. In fact, the production of HGF in dermal fibroblasts increases with aging, mainly due to autocrine stimulation by IL-1 (Miyazaki et al., 1998). Moreover, although in aged fibroblasts fibronectin is increased, it results in defective cell adhesion (Chandrasekhar and Millis, 1980). We can speculate that despite its higher production, in vitiligo fibronectin fails to properly exert adhesion activity because of the senescence-prone phenotype of fibroblasts.

Collectively, our results point to the involvement of the entire skin in vitiligo, even in normal appearing skin, showing the presence in the dermal compartment of cells with a myofibroblast and a premature senescence phenotype. These cells, producing skin aging-associated secreted proteins, can in turn affect melanocyte functionality favoring their loss. Focusing on normal-appearing skin may allow us to recognize the occurrence of cellular phenomena before the clinically manifested onset of the disease and possibly restrict the spread of the lesions.

### MATERIAL AND METHODS

#### Skin biopsies and cell cultures

Specimens were collected from nonlesional gluteal skin areas of eight vitiligo subjects (two men and six women, age range: 26–66 years) with active nonsegmental disease (on the basis of the progression or appearance of lesions in the last 6 months) observed in the San Gallicano Dermatologic Institute. At the time of patient enrollment, none of the subjects had received either local or systemic therapy for at least 5 months. Normal human skin samples matched for gender, age, and anatomic site were taken from eight healthy volunteers subjected to plastic surgery. The study was approved by the Medical Ethical Committee of the San Gallicano Dermatologic Institute and was conducted according to the Declaration of Helsinki principles. Participants gave their written informed consent. Primary cultures of dermal fibroblasts and melanocytes were isolated and grown as previously described (Flori et al., 2011; Kovacs et al., 2010). All the experiments were performed employing cells from short-term cultures (2–10 cell culture passages).

#### Statistical analysis

Student’s *t*-test was used to assess statistical significance with thresholds of *P* ≤ 0.05 and **P** ≤ 0.01. The correlation analysis was determined by the coefficient of Pearson’s test (*r*).

For immunofluorescence, western blot analysis, flow cytometry, immunohistochemical, real-time RT-PCR, sandwich ELISA, and gas chromatography-mass spectrometry analyses methods, see Supplementary Material online.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

### ACKNOWLEDGMENTS

We thank Marco Zaccarini for technical assistance. This work was partially supported by the grant RF-2013-02359621 from Ministero della Salute, Italy.

### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at [https://doi.org/10.1016/j.jid.2017.06.033](https://doi.org/10.1016/j.jid.2017.06.033).

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