these genes and *BCL2*, even at the concentration of 1 ng/ml, suggesting that TNF-α likely had the strongest suppressive effect on gene expression. BCL2 expression was decreased following treatment with IL-1β, IL-6, and IL-17A in a dose-dependent manner. Overall, there was a tendency for molecules related to melanocyte function to be downregulated following treatment with exogeneous cytokines (Figure 4B, C).

To determine the direct effects of Th17 cells on melanocytes, we performed coculture of the Th17 cells induced from peripheral blood mononuclear cells by an in vitro protocol with and without TGF-β treatment (Wilson et al., 2007) with melanocytes and real-time PCR. Th17-polarized cells without TGF-β decreased the expression of *MITF* and its downstream melanogenic molecules more than Th2-polarized cells did (Figure S1).

Furthermore, melanin production was measured after continuous treatment with exogenous cytokines including IL-1β, IL-6, IL-17A, and TNF-α. The percentage of melanin production was significantly lower in melanocytes treated with 1 and/or 10 ng/ml of exogenous cytokines than in untreated cells. In contrast, no reduction in total protein was observed after the addition of any of the cytokines (Figure 5). Because these cytokines are critical for the maintenance and development of Th17 cells from naïve $CD4^+$ T cells, we suggest that the presence of a specific local cytokine environment might be indispensable for Th17 cell recruitment and activation in vitiligo lesions, thereby indicating that they contribute significantly to depigmentation in addition to CTL (cytotoxic T cell) activation.

Production of cytokines by skin-resident cells

We have shown infiltration of Th17 cells in vitiligo skin and have demonstrated the inhibitory effects of Th17 cell-related cytokines on melanocyte function. As the cytokines examined in this study are produced not only by inflammatory cells but also by the surrounding cells, such as keratinocytes and fibroblasts, the source of the cytokine production was examined. We treated normal human epidermal keratinocyte (NHEK) and normal human dermal fibroblast (NHDF) cells with recombinant IL-17A and measured IL-1β, IL-6, and TNF-α production (Figure 6A, B). IL-17A exponentially increased the pro-

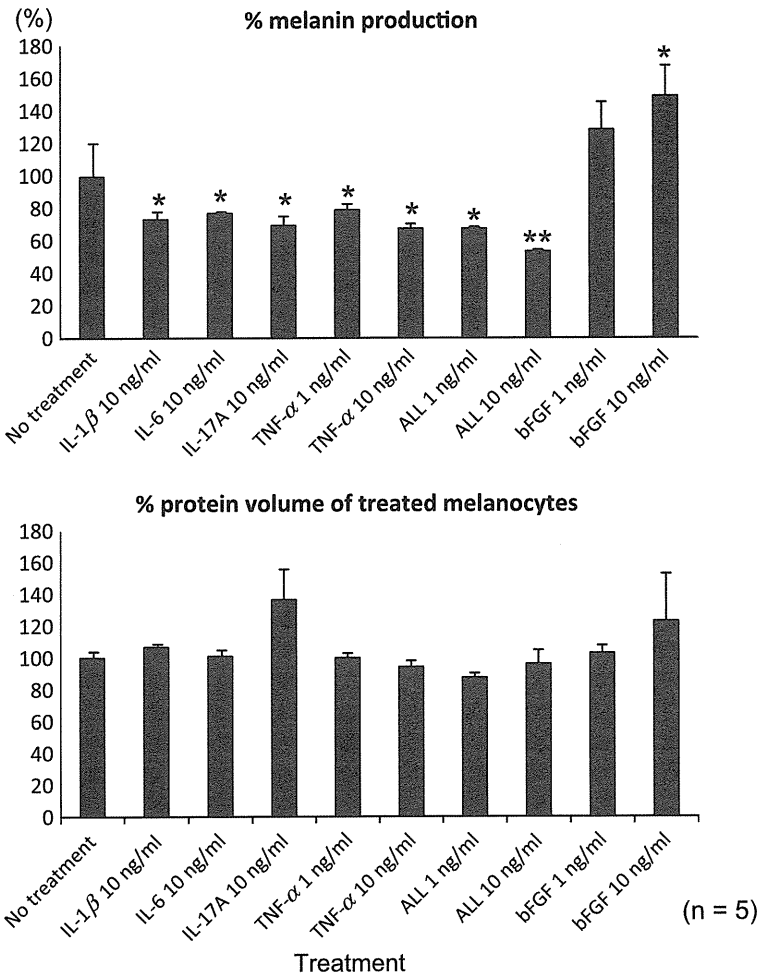

Figure 5. There is a decrease in melanin production after treatment with cytokines. Human melanocytes were incubated with 1 ng and/or 10 ng/ml of recombinant cytokines for 5 days in the culture medium (n = 5). Recombinant cytokines were added everyday. Cultured melanocytes were treated with 1 N NaOH and processed for absorbance at 450 nm to quantify the melanin volume. The protein volume of the cell extracts was measured to demonstrate whether the cytokines exerted the reduction of whole cell protein. *P < 0.05; **P < 0.01; ***P < 0.001 compared with the expression level of the untreated controls.

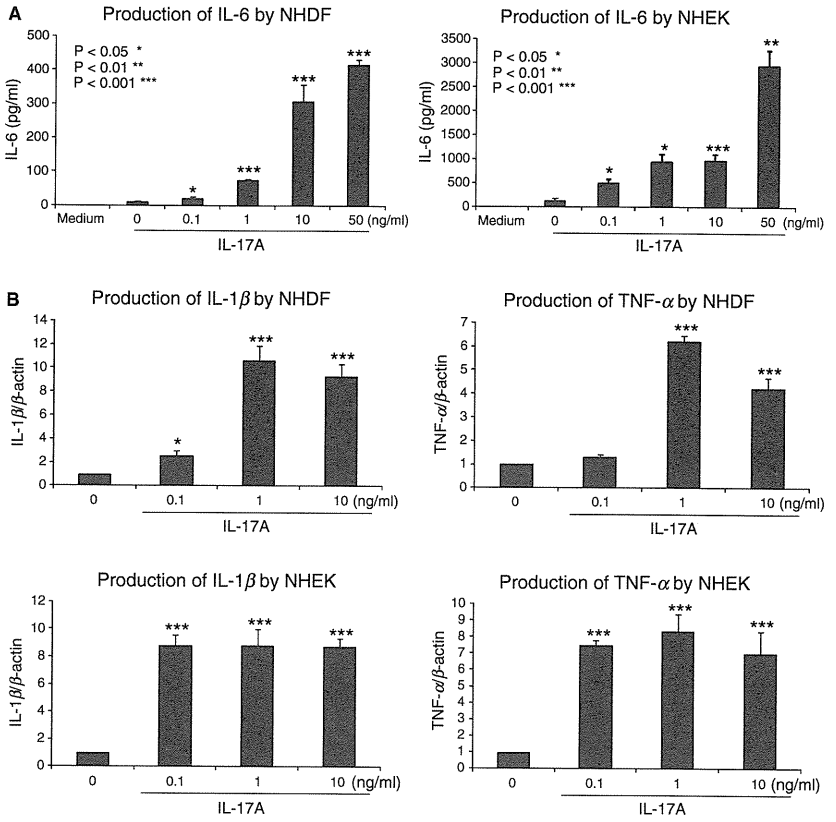

Figure 6. IL-17A induces the release of other Th17 cell-related cytokines from dermal fibroblasts and keratinocytes. (A) Human dermal fibroblasts and keratinocytes were incubated with recombinant IL-17A for 1 day at concentrations of 0.1, 1, 10, and 50 ng/ml in the culture medium, and the IL-6 secreted in the medium was measured by an ELISA. (B) After cells were incubated as in (A), the IL-1β and TNFα mRNA expression levels were measured by RT-PCR. β-actin was used as a housekeeping gene. *P < 0.05; **P < 0.01; ***P < 0.001 compared with the expression level of the untreated controls.

duction of these cytokines in a dose-dependent manner using both of these cell lines.

Cytokine-induced melanocyte dysfunction

Finally, we examined whether proinflammatory cytokines could directly induce melanocyte apoptosis and/or destruction in vitro. The cultured melanocytes were incubated with 1 and 10 ng/ml of recombinant IL-1β, IL-6, IL-17A, TNF-α, or all of the factors for 5 days, and then the melanocytes were observed microscopically under polarized light (Figure 7A). The cells were obviously aggregated and varied in shape after treatment with both the single cytokines and the cytokine cocktail, whereas the untreated cells and those treated with bFGF (basic fibroblast growth factor) grew with a spindle-shaped morphology. Staurosporine, a chemical that induces apoptosis by activating caspase-3, increased the number of round-shaped apoptotic melanocytes. TNF-α induced the greatest extent of melanocyte destruction compared with the other cytokines.

Next, melanocyte apoptosis was assessed by measuring caspase-3 activity after continuous treatment with 10 ng/ml of each of the individual cytokines and the cytokine cocktail. Staurosporine led to an increase in caspase-3 activity (Figure 7B), whereas there was no induction of caspase-3 activity following treatment with any of the cytokines. These results indicate that there appears to be direct inhibition of melanocyte activity by cytokines, rather than induction of cell apoptosis.

Discussion

In the present study, we identified a significant number of Th17 cells that had infiltrated vitiligo skin, and demonstrated the inhibitory effect of Th17 cell-related proinflammatory cytokines on melanocyte activity. We therefore hypothesize that the functional Th17 cell involvement in the initiation of psoriasis and atopic dermatitis may also play an important role in the pathogenesis of vitiligo. Although the precise pathogenic mechanisms underlying the induction of depigmentation in an immunological manner (Ongenae et al., 2003) still remain unknown, non-segmental vitiligo has been thought to be an autoimmune disease because of the high frequency of associated Hashimoto's thyroid disease (Daneshpazhooh et al., 2006; Hegedus et al., 1994; Schallreuter et al., 1994a), type I diabetes (Gould et al., 1985), collagen diseases with antinuclear antibodies (Mihailova et al., 1999), etc. Pathogenic antibodies were also detected in approximately 50% of vitiligo patients (Cui et al., 1992; Ruiz-Arguelles et al., 2007). With respect to the cellular immune condition, the infiltration of cytotoxic T cells targeting melanocyte-specific antigens in vitiligo lesions has been thought to play a critical role in hypopigmentation (Lang et al., 2001; Norris et al., 1994; Ogg et al., 1998). Recent reports have also suggested that there is the local environment of proinflammatory cytokines such as IL-1, IL-6, and TNF-α also contributes to the inhibition of melano-

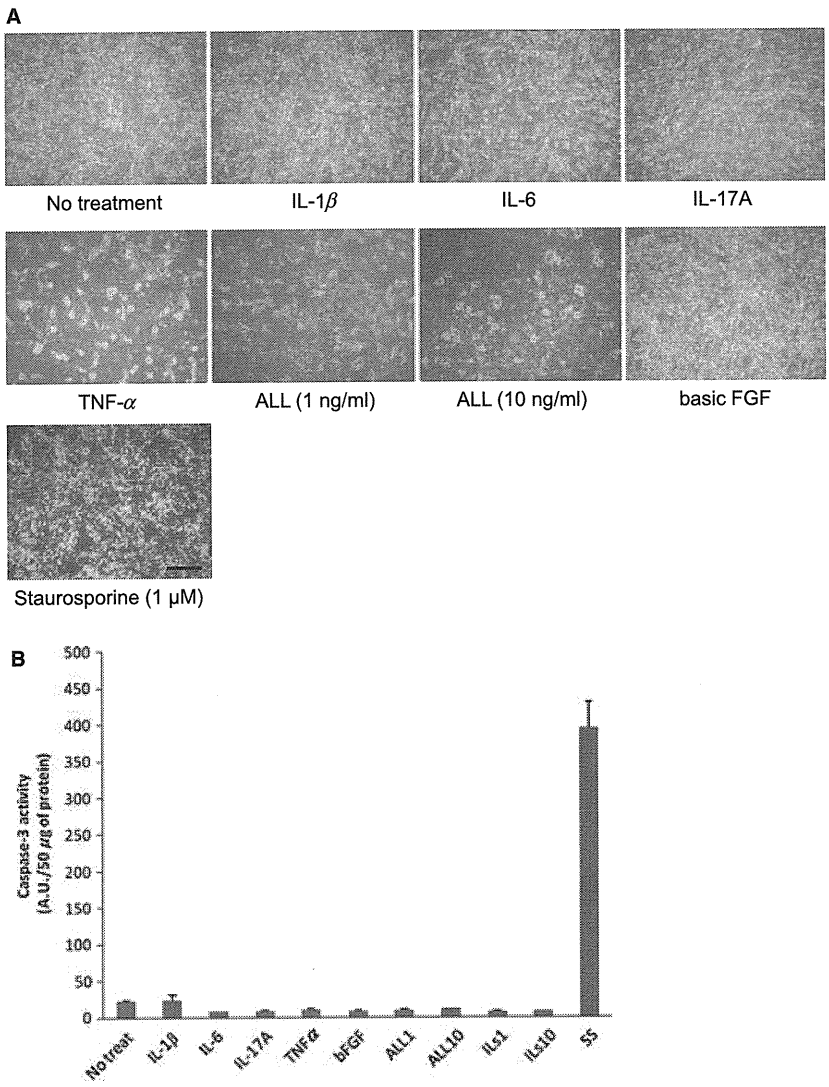

Figure 7. Proinflammatory cyotokines induce melanocyte cell destruction, but not apoptosis. (A) Human melanocytes were incubated with recombinant proinflammatory cytokines, including IL-1β, IL-6, IL-17A, and TNF-α continuously for 5 days at concentrations of 1 or 10 ng/ml in the culture medium. These cytokines were used either alone or in combination. Staurosporine was used as a positive control for cell apoptosis. The photographs were taken by a polarized microscope. The bar indicates 50 μm. (B) The absorbance at 450 nm was measured to determine the caspase-3 activity of the cells treated with cytokines. ILs indicates treatment together with IL-1β, IL-6 and IL-17A.

genesis and melanocyte survival (Moretti et al., 2002, 2009).

Direct regulation of melangenic factors by cytokines

The tyrosinase mRNA levels are generally correlated with tyrosinase activity (Ando et al., 1995). In our study, cytokine treatment decreased the mRNA levels of MITF, a transcription factor implicated in regulating melanogenic and antiapoptotic genes, and decreased the expression of genes encoding melanogenic enzymes such as tyrosinase, TYRP-1, and DCT (TYRP-2) in a dose-dependent manner. These results indicate that proinflammatory cytokines can play a pivotal role in the regulation of melanocyte fate through the downregulation of gene expression.

There have been several reports providing evidence that melanocyte functions are regulated by cytokines through several cellular signaling pathways (Kamaraju et al., 2002; Kholmanskikh et al., 2010). For example, IL-1β and 1α were found to direct the downregulation of MITF-M expression through the NF-κB and JNK pathways in two different melanoma cell lines (Kholmanskikh et al., 2010). IL-6/IL-6R signaling silenced the MITF promoter activity and this was mediated by Pax3 downregulation (Kamaraju et al., 2002). IL-6 is a pleiotropic cytokine involved in a variety of inflammatory responses. With regard to the relationship to Th17 cells, IL-6 is essential for induction of Th17 cell development and maintenance (Diveu et al., 2008). As the proinflammatory cytokines involved in Th17 cell fate include IL-1β and TGF-β in addition to IL-6, we examined the expression and activity of some of these cytokines in melanocytes.

Although there is no doubt that cellular and antibody-mediated immune reactions are related to melanocyte destruction, our data suggest that Th17 cells and skin-resident cells, particularly epidermal keratinocytes and dermal fibroblasts, might orchestrate a response that inhibits the stability of melanocytes in some vitiligo skin

through the production of proinflammatory cytokines. In addition, there might be an initial trigger attracting Th17 cells to vitiligo (or pre-vitiligo) skin.

A recent study using several skin samples showed greater numbers of Th17 cells, especially on the leading edge of vitiligo skin (Wang et al., 2011). In the present study, we confirm the presence of Th17 cell infiltration in vitiligo skin and suggest that there was a pathogenic function not only because of cytotoxic T cells but also because of Th17 cells and Th17 cell-related cytokines. Although we expected that there would be more infiltration of Th17 cells in the generalized type and progressive vitiligo compared with other clinical types, there was no significant correlation between the Th17 cell number and the clinical type and disease duration. It is possible that the small sample number, biopsy site, and preceding treatments, including the use of topical steroids, may have affected the status of inflammatory cell infiltration.

We observed that Th17 cells diffusely infiltrated the upper dermis, whereas $CD8^+$ cells were present beneath the basal membrane of the epidermis. In psoriatic skin, Th17 cells mainly infiltrate into the papillary dermis and epidermis. We therefore speculated that Th17 cells might be able to act on melanocytes by producing cytokines, rather than exerting a direct effect on the cells. To address this point, we stimulated dermal fibroblasts and keratinocytes using a characteristic pro-Th17 cytokine, IL-17A. IL-17A robustly upregulated the production of IL-1β and TNF-α by these skin-resident cells, suggesting the presence of mutual cytokine signaling between skin-resident cells and accumulating inflammatory cells. The melanocytes themselves can also synthesize IL-1α and β (Swope et al., 1994).

Previous studies have shown that cytokines associated with skin inflammation, such as IL-1β, IL-6, and TNF-α, inhibited melanin production in vitro (Englaro et al., 1999; Kamaraju et al., 2002; Kholmanskikh et al., 2010). We found that there were significant changes in the expression of epidermal cytokines in vitiligo lesions, where no melanocytes are present, compared with perilesional, non-lesional and healthy skin, where melanocytes are normally present. Therefore, it is conceivable that the cytokines derived from infiltrating cells, as well as the lesional epidermis, would be implicated in depigmented skin disorders. In the present study, treatment with a physiologically relevant concentration of IL-17A, in addition to IL-1β and IL-6, could directly regulate the expression of MITF and downstream molecules, and subsequently melanin synthesis, in human melanocytes.

Putative involvement of proinflammatory cytokines in vitiligo

Based on these experimental results, we propose the putative involvement of proinflammatory cytokines in the pathogenesis of vitiligo (Figure 8). Previous studies have shown that the perforin produced from CD8-positive cytotoxic T cells (Lang et al., 2001; Norris et al., 1994; Ogg et al., 1998), antimelanocyte antibodies (Baharav et al., 1996; Cui et al., 1992; Ruiz-Arguelles et al., 2007), and reactive oxygen species were related to the injury of melanocytes and were triggers for vitiligo (Schallreuter et al., 1994b). In the present study, we found a significant infiltration of Th17 cells in vitiligo skin, and demonstrated that Th17-related cytokines such as IL-1β, IL-6, and IL-17A directly or indirectly regulated melanin production and the expression of melanogenic and antiapoptotic molecules. The presence of a cytokine network and the secretion of IL-17A from Th17 cells may therefore represent a new mechanism underlying the pathogenesis of vitiligo concerning the downregulation of melanocyte activity. Indeed, the activation of the innate immune system may lead to the accumulation of Th17 cells in the vitiligo lesion as they do in psoriasis. In fact, the IL-1β released from lesional keratinocytes and melanocytes (Moretti et al., 2002, 2009; Swope et al., 1994) may act as the first inducer of the differentiation of naïve helper T cells into Th17 cells in vitiligo lesions. Thereafter, antimicrobial peptides derived from the lesional epidermis might induce the production of IL-17A by Th17 cells (Infante-Duarte et al., 2000).

Although psoriasis is one of representative skin disorders characterized by pathogenic Th17 cell infiltration, the phenotypic change in this disorder is not akin to that in vitiligo vulgaris. Because the final targets of IL-17A in

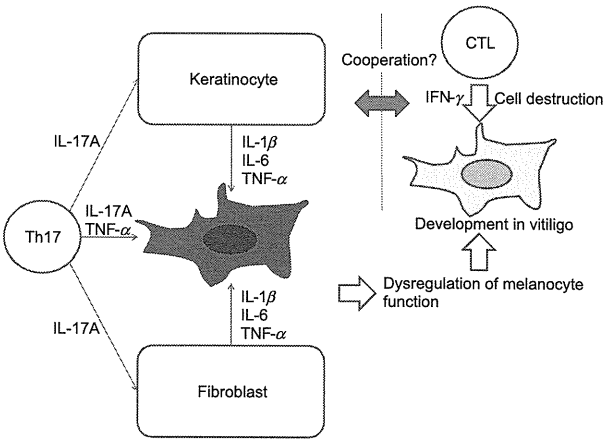

Figure 8. The proposed cues provided by Th17 cells and Th17 cell-related cytokines during the pathogenesis of autoimmune vitiligo. Previous known pathogenic mechanisms of melanocyte destruction in vitiligo include the presence of cytotoxic T cells attacking the melanocytes, local oxidative stress, and downregulation of melanogenesis-inducing factors in the vitiligo epidermis. The newly proposed phenomenon is that an imbalance in the local cytokine network is involved in the downregulation of melanocyte activity found in the present study. The IL-17A secreted from the Th17 cells in vitiligo skin can trigger the production of inhibitory cytokines from dermal fibroblasts and keratinocytes. IL-1β, IL-6, and TNF-α as well as IL17-A also repress melanocyte activity and induce melanocyte destruction.

vitiligo vulgaris and psoriasis are different, that is, melanocyte dysfunction in vitiligo vulgaris and abnormal keratinocyte turnover in psoriasis, Th17 cells may augment vitiliginous skin lesion formation in cooperation with skin-resident cells such as dermal fibroblasts and keratinocytes, as described previously. Microbial lipopeptides may then induce the cell polarization to Th17 cells, producing IL-17 and TNF-α as a result of the stimulation of the innate immune system in vitiligo (Infante-Duarte et al., 2000). Tip dendritic cell (tipCD)-like cells might also be involved in vitiligo formation, as previous reports demonstrated that the number of αDCs was increased in vitiligo vulgaris lesions or there was a unique distribution pattern of Langerhans cells present in such lesions (Mishima et al., 1972). Moreover, recent reports suggest that an altered innate immune response is observed in autoimmune vitiligo in concert with frequent *NALP1* gene mutations (Jin et al., 2007, 2010). An unrecognized micro-organism might stimulate the attending inflammatory cells through antimicrobial peptides and sequentially trigger vitiligo vulgaris. These issues should be clarified in further experiments.

Methods

Cell lines

HeMnMP, a moderately pigmented human melanocyte cell line, was obtained from Cascade Biologics and cultured in Medium 254 with human melanocyte growth supplement (Gibco Inc., Tokyo, Japan), and maintained at 37°C with 5% CO_2 in a humidified incubator. The cells were used for this study by the 5th passage to ensure melanin production. Dermal fibroblasts and epidermal keratinocytes were purchased from TAKARA BIO Inc., (Shiga, Japan) and maintained in DMEM containing 10% FCS and Medium 154 (Gibco Inc.), respectively.

Reagents

Human recombinant cytokines were purchased from Cell Signaling Technology (Tokyo, Japan) and synthetic melanin was from Sigma-Aldrich, Japan. The antibodies used for this study were as follows: anti-MITF mouse monoclonal Ab (D5) from Abcam (San Francisco, CA, USA), horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG from Cell Signaling Technology, anti-CD4 mouse monoclonal antibody from Novocastra Reagents (Tokyo, Japan), anti-CD8, -Foxp3, -CD20 and -Melan A mouse monoclonal antibodies from Dako (Tokyo, Japan), anti-IL17A goat monoclonal antibody from R&D (Minneapolis, MN, USA), Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 555-conjugated anti-goat IgG from Invitrogen (Tokyo, Japan).

Tissue specimens

Approval for the use of human skin tissue samples was obtained from the local Ethical Committee of Osaka University Hospital and written informed consent was received from each patient after appropriate explanation of this study. Spindle-shaped skin biopsy specimens on the leading edge of vitiligo lesions were taken from 23 vitiligo patients. Twenty-three skin specimens were fixed in buffered 10% formalin and embedded in paraffin and processed for an immunohistochemical analysis as described below. Non-lesional skin from the matched vitiligo patients and normal skin from normal donors were processed as well.

RNA isolation and PCR assay for cytokine and melanocyte markers expression

Total RNA was extracted from HeMnMP cells using the TRIZOL reagent according to the manufacturer's instructions. Reverse transcription (RT) reactions were performed with Moloney murine leukemia virus reverse transcriptase (Promega, Tokyo, Japan) with oligo (dT) primers. For RT reaction of tissue RNA, total RNA was extracted from frozen vilitigo skin tissue using the Sepasol-RNA I reagent (NACALAI TESQUE, INC., Kyoto, Japan) according to the manufacturer's instructions. Genomic DNA contamination was removed by DNase I (TAKARA BIO INC.). The qRT assay was performed using an ABI prism 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's specifications. Briefly, the reaction mixture totaling 10 μl for each qRT consisted of 1 μl of cDNA generated from 250 ng of total RNA, 0.5 μM of Taqman probe labeled with FAM, the master mix for melanogenic markers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or the Power SYBR green PCR master mix for cytokines. The mixture was processed by a two-step PCR method with an initial heating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 60 s for all genes. The obtained PCR amplification curves were analyzed using SDS software program, version 2.1 (Applied Biosystems). GAPDH was used as a control housekeeping gene, and the relative mRNA copy numbers were obtained as the ratio of the mRNA copies of each gene/copies of GAPDH. Each assay was performed at least three times. The specific probe and primers sequences used for this study were as follows:

MITF-M: 5′-AGCTCACAGCGTGTATTTTTCCCAC-3′
TYR: 5′-TCTCCTCTTGGCAGATTGTCTGTAG-3′
TRP-1: 5′-CTTTGTAACAGCACCGAGGATGGGC-3′
DCT: 5′-TGCAAGTGCACAGGAAACTTTGCCG-3′
BCL2: 5′-AACGGAGGCTGGGATGCCTTTGTGG-3′
GAPDH: 5′-GGGCGCCTGGTCACCAGGGCTGCTT-3′
IL-1β: Forward 5′- TGCACGCTCCGGGACTCACA-3′
IL-1 β: Reverse 5′- CGCCTTTGGTCCCTCCCAGG-3′
TNF-α: Forward 5′- CCCCTGACAAGCTGCCAGGC-3′
TNF-α: Reverse 5′- CAGCTCCACGCCATTGGCCA-3′

Reverse transcriptase PCR (RT-PCR) for IL-17A and cytokine receptor expression

To confirm IL-17A expression in vitiligo tissue and determine the expression levels of the cytokines and their receptors, we performed RT reactions with the above-mentioned procedure and PCR with an initial heating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at appropriate temperatures for 60 s for all genes. Samples were then processed for electrophoresis. The following primer sets were used:

IL-17A: Forward 5′- ACAAACTCATCCATCCCCAG-3′
IL-17A: Reverse 5′- GTGAGGTGGATCGGTTGTAG-3′
IL-1R1: Forward 5′-CCCGTTGCAGGAGACGGAGG-3′
IL-1R1: Reverse 5′-CCACCCAGCCAGCTGAAGCC-3′
IL-1R2: Forward 5′-CTTTAAAGCTGCTTCTGCCACGTG-3′
IL-1R2: Reverse 5′-CATTGCCCGTCCACCACAGCA-3′
IL-6R: Forward 5′-GAGTTCGGGCAAGGCGAGTGG-3′
IL-6R: Reverse 5′-AGGCTCCCTCCAGCAACCAGGAA-3′
IL-17RA: Forward 5′-AAGCCTCAGAACGTTCGTTCGCT-3′
IL-17RA: Reverse 5′-TTGGGCAGGTGGTGAACGGT-3′

Melanin content assay

Melanin production was determined as described previously (Virador et al., 1999). Briefly, 2 days after the plating of 1×10^5 melanocytes into a 6-well culture dish, we performed 5 days of

sequential treatment with 1–10 ng/ml of recombinant cytokines. To determine the melanin content, the pellets of treated cells were dissolved in 200 μl of 1 N NaOH for 30 min, and the concentrations of melanin were calculated by measuring the absorbance at 450 nm. Synthetic melanin was used to generate a standard curve. The melanin content was expressed as nanograms of melanin per microgram of total protein, and the ratio was compared among the samples.

Immunostaining

Vitiliginous skin specimens were processed after receiving written informed consent from vitiligo patients (n = 23). Paraffin-embedded archival tissues were deparaffinized with absolute xylene and dehydrated in a sequential ethanol dilution series. The deparaffinized sections were boiled in an oil bath for 15 min in 10 mM Tris-1 mM EDTA buffer (pH 9.0) for antigen retrieval. The slides were blocked by the Protein Block Serum-Free solution (Dako) for 15 min and incubated with an anti-IL-17A goat monoclonal Ab (×200) at 4°C overnight. After being washed with TBS (pH 7.6), the slides were incubated with Alexa Fluor 555-conjugated anti-goat IgG Ab (×200) and then incubated with anti-CD4 mouse monoclonal Ab (×25) at RT for 1 h, followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG Ab (×200). The following primary antibodies were used to assess the expression of melanocyte markers and the melanosomal protein MART1: anti-CD8 mouse monoclonal Ab (×25), anti-CD20 mouse monoclonal Ab (×25), anti-Foxp3 mouse monoclonal Ab (×50), and anti-Melan A (recognizing the MART-1 antigen) mouse monoclonal Ab (×50). These antibodies were provided by DAKO Inc.

For the immunocytochemistry analyses, the HeMnMP cells cultured in two-well Lab-Tek chamber slides (Nunc, Tokyo, Japan) were incubated with an anti-MITF mouse monoclonal antibody (×25) at 4°C overnight, followed by incubation with Alexa Fluor 555-conjugated anti-mouse IgG (×500) as the secondary antibody. The mouse isotype IgG was used as a negative control for staining. Nuclei were counterstained after DAPI staining (×1000).

Quantitative analysis of proinflammatory cytokines after the treatment with Th17-related cytokines

To assess the cell–cell interactions between the cells in the skin occurring as a result of paracrine cytokine production, the concentrations of proinflammatory cytokines such as IL-6 and IL-1β in the culture supernatant from dermal fibroblasts was measured 24 h after treatment with recombinant IL-17A using an ELISA kit from R&D.

Apoptosis assay

We determined the cleaved caspase-3 activity following treatment with recombinant cytokines using an apoptosis detection kit (R&D Systems). Briefly, cultured melanocytes were treated with cytokines (1 or 10 ng/ml) for 5 days. The culture medium was not changed until cell extraction, and cytokines were added in the culture medium every day. Thereafter, in addition to observation of melanocyte morphology under a polarized microscope, the melanocytes were processed for measurement of cleaved caspase-3 activity according to manufacturer's instructions.

Statistical analysis

The unpaired *t*-test was used for the analysis of differences in gene and protein expression. The results are shown as the means + SD. A value of $P < 0.05$ (two-tailed) was considered significant. All statistical analyses were performed using the PRISM software program, version 5 (GraphPad Software Inc., La Jolla, CA, USA).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Coculture of helper T cells and melanocytes, and measurement of the expression of melanogenic markers.

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REVIEW Open Access

Therapeutic strategies in epithelial ovarian cancer

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Abstract

Ovarian cancer is the most lethal gynecologic malignancy. It appears that the vast majority of what seem to be primary epithelial ovarian and primary peritoneal carcinomas is, in fact, secondary from the fimbria, the most distal part of the fallopian tube.

Treatment of epithelial ovarian cancer is based on the combination of cytoreductive surgery and combination chemotherapy using taxane and platinum. Although clear cell type is categorized in indolent type, it is known to show relatively strong resistance to carboplatin and paclitaxel regimen and thus poor prognosis compared to serous adenocarcinoma, especially in advanced stages. Irinotecan plus cisplatin therapy may effective for the clear cell adenocarcinoma.

The larger expectation for improved prognosis in ovarian carcinoma is related to the use of the new biological agents. One of the most investigated and promising molecular targeted drugs in ovarian cancer is bevacizumab, a monoclonal antibody directed against VEGF. PARP inhibitor is another one. A few recent studies demonstrated positive results of bevacizumab on progression-free survival in ovarian cancer patients, however, investigation of molecular targeting drugs in patients with ovarian cancer are still underway.

Keywords: Review, ovarian cancer, conventional treatment, novel treatment, clear cell carcinoma, bevacizumab, PARP inhibitor

Background

Ovarian cancer is the most lethal gynecologic malignancy. The origin and pathogenesis of epithelial ovarian cancer (EOC) have long been investigated but still poorly understood. Studies have shown that epithelial ovarian cancer is not a single disease but is composed of a diverse group of tumors that can be classified based on distinctive morphologic and molecular genetic features [1].

Treatment of epithelial ovarian cancer (EOC) is based on the combination of surgery and chemotherapy. Over the past three decades, surgical tumor debulking, followed by platinum-based chemotherapy is the standard treatment for advanced ovarian cancer. Although response rates and complete responses in advanced disease are >80% and 40-60%, respectively, after first-line treatment with carboplatin and paclitaxel, most patients will eventually relapse with a median progression-free survival of 18 months [2]. Intraperitoneal chemotherapy possibly improve progression-free and overall survivals (PFS and OS), however, intraperitoneal chemotherapy has not been universally accepted for at least three reasons: toxic effects, intraperitoneal treatment delivery issues and complications [3].

In this review, we first focus on the origin and pathogenesis of EOC, introducing emerging concepts of a unifying theory. Next we look at the history of treatment of EOC as well as novel treatment strategies (e.g. molecular targeted treatment).

Classification of epithelial ovarian cancer

Kurman et al. have proposed a dualistic model that categorizes various types of epithelial ovarian cancer into two groups designated type I and type II [1,4,5]. Type I tumors are clinically indolent and usually present at a low stage, while type II tumors exhibit papillary, grandular, and solid patterns and are highly aggressive and almost always present in advanced stage (Table 1). Type I tumors include low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas and type II include high-grade serous, high-grade endometrioid and undifferentiated carcinomas. Malignant mixed mesodermal tumors (carcinosarcomas) are included in the type

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Table 1 Characteristics of type I and type II tumors

	Type I	Type II
Clinical features	indolent	aggressive
Histological features	low-grade serous	high-grade serous
	low-grade endometrioid	high-grade endometrioid
	clear cell	undifferentiated
	mucinous	carcinosarcoma
Molecular features	K-Ras	TP53CCNE1
	BRAF	
	ERBB2	
	PTEN	
	CTNNB1	
	PIK3CA	

II category because their epithelial components are identical to the pure type II carcinomas.

Type I and type II tumors have remarkably different molecular genetic features as well as morphologic differences. For example, high-grade serous carcinoma (type II tumor) is characterized by very frequent *TP53* mutations (> 80% of cases) and *CCNE1* (encoding cyclin E1) amplification but rarely has mutations that characterize most type 1 I tumors such as *KRAS, BRAF, ERBB2, PTEN, CTNNB1, and PIK3CA* [6]. In general, type I tumors are genetically more stable than type II tumors and display a distinctive pattern of mutations that occur in specific cell types. Type II tumors which show greater morphologic and molecular homogeneity are genetically unstable and have a very high frequency of *TP53* mutations. These findings suggest that these two different types of ovarian cancers develop along different molecular pathways.

In terms of origin of ovarian cancer, many of researchers and gynecologic oncologists have traditionally understood that the various different ovarian tumors are all derived from the ovarian surface epithelium (mesothelium) and that subsequent metaplastic changes lead to the development of the different cell types (Table 2). It is well known that serous, endometrioid, clear cell, mucinous and transitional cell (Brenner) carcinomas morphologically resemble the epithelia of the fallopian tube, endometrium, gastrointestinal tract or endocervix and urinary bladder, respectively. The normal epithelial cells of the ovary, however, do not show any resemblance with these tumors. An alternate theory proposes that tumors with a mullerian phenotype (serous, endometrioid and clear cell) are derived from mullerian-type tissue not mesothelium. It has been suggested that they could arise from tissues ovarian epithelial tumors are embryologically derived from the mullerian duct [7]. This mullerian-type tissue (columnar epithelium, often ciliated) forms cysts located in paratubal and paraovarian locations. According to this theory, ovarian tumors develop from these cysts, not the ovarian surface epithelium. As the tumor enlarges, it compresses and eventually obliterates ovarian tissue resulting in an adnexal tumor that appears to have arisen in the ovary.

In summary, it appears that the vast majority of what seem to be primary epithelial ovarian and primary peritoneal carcinomas are, in fact, secondary. Previous data support the view that serous tumors develop from the fimbria, the most distal part of the fallopian tube, endometrioid and clear cell tumors from endometrial tissue passing through the fallopian tube resulting in endometriosis and mucinous and Brenner tumors from transitional-type epithelium located at the tubal-mesothelial junction where the fimbria makes contact to the peritoneum.

Although the data suggesting that epithelial ovarian carcinoma arises in extra-ovarian sites and involves the ovaries secondarily are compelling, low- and high-grade serous carcinomas involve the ovaries and other pelvic and abdominal organs, such as the omentum and mesentery, much more extensively than the fallopian tubes. Similarly, although endometrioid carcinomas develop from endometriosis, which frequently involves multiple sites in the pelvis, these tumors are usually confined to the ovaries. It is likely that the predisposition for growth in the ovary is multifactorial but the precise reasons for this are unknown.

The proposed model by assigning different epithelial ovarian tumors into two categories based on clinical, morphological, and molecular genetic characteristics could serve as a framework for studying ovarian cancer pathogenesis, but this model is not complete and does not resolve all the issues. For example, clear cell carcinoma and mucinous cadenocarcinoma are classified as type I tumors, but unlike the other type I tumors clear cell and mucinous cell types are often high-grade at presentation and show relatively strong resistance to platinum-based chemotherapy. This model does not replace traditional histopathologic classification but can be

Table 2 Origin of ovarian carcinoma

	Serous	Endometrioid/Clear	Mucinous/Brenner
Traditional theory	ovarian surface epithelium (mesothelim)	ovarian surface epithelium (mesothelim)	ovarian surface epithelium (mesothelim)
Recent theory	fimbria	endometrial tissue (endometriosis)	tubal-mesothelial junction

expected to draw attention to the molecular genetic events that play a role in the tumor progression and can give light on new approaches to early detection and treatment of ovarian cancer.

Conventional treatment of EOC

Early disease: FIGO stage I-II

Due to the lack of effective screening programs, ovarian cancer is diagnosed at an early stage only in about 25% of the cases. In most of these cases surgery is able to cure the disease, and the five-year survival rate for early-stage (stage I or II) ovarian cancer is around 90% [3]. Adjuvant chemotherapy for early stage ovarian cancer is still controversial but some studies have shown its benefit under confined conditions. According to the results of two studies from the International Collaborative Ovarian Neoplasm group and the EORTC, patients with IA or IB FIGO stage, non-clear-cell histology, well-differentiated (G1) tumors, and an "optimal" surgery (performed according to international guidelines, with pelvic and retroperitoneal assessment), appear not to benefit from chemotherapy [8]. Thus, it is commonly believed that, at least in these cases chemotherapy can be probably avoided and patients can be advised to undergo clinical and instrumental follow-up. In all the other (early stage) patients (adjuvant) chemotherapy is indicated [3].

Advanced disease: FIGO III-IV

The standard treatment for patients with advanced ovarian cancer is maximal surgical cytoreduction (total abdominal hysterectomy, bilateral salpingo-oophorectomy, pelvic and para-aortic lymphadenectomy and omentectomy) followed by systemic platinum-based chemotherapy and, actually, is reasonable to expect a 5-year survival for 10-30% of women diagnosed with ovarian cancer at stage III or IV [3]. The concept of primary debulking surgery is to diminish the residual tumor burden to a point at which adjuvant therapy will be optimally effective. The percentage of patients with advanced ovarian cancer who can optimally undergo cytoreductive surgery seems to range from 17%-87% [9], depending on the report reviewed. This percentage can largely depend on the experience of the surgeon.

Recently, an interesting randomized control trial on treatment of advanced ovarian cancer was conducted by Vergote et al. [10]. This phase III randomized study compared primary debulking surgery followed by chemotherapy with neoadjuvant chemotherapy followed by interval debulking surgery in patients with advanced ovarian cancer (Table 3). The median overall survival was 29 months in the primary-surgery group and 30 months in the neoadjuvant chemotherapy group and this difference was not statistically significant. Also, n difference was observed in median progression-free survival. These results are thoroughly discussed among the experts in this field; it is believed that upfront maximal cytoreduction is still the standard, although further research should focus on how to select patients that cannot receive optimal cytoreduction and that can benefit from a neoadjuvant strategy. When deciding debulking surgery, we should assess predictive factors with respect to recidual macroscopic disease after debulking surgery which is the strongest independent variable in predicting survival [10].

Table 3 Comparison of primary debulking surgery and neoadjuvant chemotherapy

	Primary debulking surgery	Neoadjuvant chemotherapy
Number of patients	336	334
Age: Median (range)	62 (25-86)	63 (33-81)
Stage	257 (76.5%)	253 (75.7%)
IIIc	77 (22.9%)	81 (24.3%)
IV	2 (0.6%)	0 (0%)
PFS	12 months	12 months
OS	29 months	30 months

Recurrent disease

Despite the activity of first-line chemotherapy, which gives response rates up to 80% in first line treatment, the majority of patients die of their recurrent disease [2]. Therefore, a large proportion of patients are candidates for second-line treatment. Platinum sensitivity, which is defined by a response to first-line platinum-based therapy, has been found to predict the response to subsequent retreatment with a platinum-containing regimen frequently used for salvage therapy.

In general, patients who progress or have stable disease during first-line treatment or who relapse within 1 month are considered to be 'platinum-refractory'. Patients who respond to primary treatment and relapse within 6 months are considered 'platinum-resistant', and patients who relapse more than 6 months after completion of initial therapy are characterized as 'platinum-sensitive' [11]. It is known that longer platinum free interval (PFI) increases the chances for a benefit by platinum re-challenge. This has been reported especially for PFI longer than 12 months. Patients who are relapsing 6-12 months following the end of their initial regimen may benefit less and are, usually classified as so-called 'partially sensitive' [12] (Table 4).

Several randomized trials have been performed in platinum-sensitive patients. The ICON-4/OVAR 2.2 study compared the combination chemotherapy (platinum plus paclitaxel) to single chemotherapy (platinum alone) in 802 patients with 'platinum-sensitive' relapsed ovarian

Table 4 Association of platinum sensitivity and PFI

Platinum sensitivity	resistant		sensitive	
	refractory	resistant	partially sensitive	sensitive
PFI	during/immediately after chemotherapy	< 6 months	6-12 months	> 12 months

cancer. Results demonstrated that both survival and progression free survival were significantly longer in combination therapy compared to platinum alone [13].

The optimal treatment of patients with partially platinum-sensitive recurrent ovarian cancer is not clearly defined. Trabectedin, a marine-derived antineoplastic agent initially isolated from the tunicate *Ecteinascidia turbinate*, has recently been introduced to this setting of patients. This agent is currently produced synthetically and its mechanism of anti-cancer action is based on DNA minor-groove binding [14].

Patients with platinum refractory and resistant are good candidates for novel investigational approaches and studies of drug resistance. Single-agent therapy is considered the standard treatment in these patients. Low response rates are recorded in these patients with the use of topotecan, docetaxel, oral stoposide, pegylated liposomal doxorubicin (PLD), gemcitabine, ifosfamide and hexamethylmelamine. The pegylated liposomal doxorubicin (PLD), a new formulation of doxorubicin, compared with the conventional, assumes favorable pharmacokinetic properties such as a lower plasma concentration peak, lower clearance, smaller distribution volume, longer half-life and higher AUC, resulting in a different and more convenient toxicity and efficacy profile [15]. The efficacy of PLD has been clearly documented in recurrent ovarian cancer giving the rationale for its use also in the first-line setting. The MITO-2 (Multicenter Italian Trials in Ovarian cancer) phase III was designed to compare the combinations of carboplatin plus paclitaxel to an experimental arm with carboplatin plus PLD in first-line treatment of ovarian cancer patients. Results have been presented at ASCO 2010 showing that carboplatin plus PLD is not superior to carboplatin plus paclitaxel in terms of PFS; the median PFS was 19 and 16.8 months in the former and the latter arms, respectively. However, given the observed confidence interval and the different toxicity profile it has been proposed that carboplatin plus PLD could be considered an alternative to standard therapy [16].

Several randomized trials have been performed in platinum-sensitive patients. A multicenter phase III study, recently published, the Calypso study [12], has compared efficacy and safety of PLD-carboplatin and carboplatin-paclitaxel in 976 relapsed platinum-sensitive ovarian cancer patients. The trial showed superiority of the experimental arm in terms of PFS (11.3 months versus 9.4; HR = 0.821, 95% CI 0.72-0.94; P = 0.005). The safety profile of PLD-carboplatin appears remarkably different from that of carboplatin plus paclitaxel. The PLD-carboplatin combination was associated with a higher incidence of anemia and thrombocytopenia (rarely requiring transfusions) and a higher incidence of stomatitis and cutaneous toxicity (that were rarely severe, 14% of G1-2). Notably, however, the PLD-carboplatin combination was associated with a very low incidence of hair loss and neurotoxicity compared between the 2 arms was found in terms of response rate [16]. One interesting observation of this trial was in PLD-carboplatin arm compared to carboplatin-paclitaxel there was the reduction in the rate of hypersensitive reaction (grade > 2: 5.6% versus 18.8%) Therapeutic Strategies in Epithelial Ovarian Cancer and this is important information since hypersensitive reactions are reported in the general practice in patients treated with carboplatin up to 25%.

Treatment of clear cell type of EOC

Although clear cell type is categorized in Type I (indolent) ovarian cancer, it is known to show relatively strong resistance to carboplatin and paclitaxel regimen and thus poor prognosis compared to serous adenocarcinoma (SAC), especially in advanced stages. Previously Sugiyama et al. investigated clinical characteristics of clear cell carcinoma (CCC) of the ovary and showed that patients with CCC were significantly more likely to have FIGO Stage I disease than were patients with SAC (48.5% versus 16.6%). However, a high recurrence rate was noted in those patients with Stage IC CCC (37%) and the survival rates for those stage IC CCC patients were lower than those for patients with SAC. Also, the 3-year and 5-year survival rates for Stage III CCC patients were significantly lower compared with Stage III SAC patients [17].

Enomoto et al. demonstrated that clear cell or mucinous carcinoma histologic type did not respond to the carboplatin-paclitaxel combination chemotherapy (response rates 18%, 13%, respectively compared to 81% for serous adenocarcinoma and 89% for endometrioid adenocarcinoma) [18]. Considering those previous reports, alternative chemotherapy regimens or novel treatment for clear cell and mucinous carcinoma should be investigated.

Takakura et al. performed phase II trial of paclitaxel-carboplatin therapy (TC arm) versus irinotecan plus cisplatin therapy (CPT-P arm) as first-line chemotherapy for clear cell adenocarcinoma of the ovary [19]. PFS

showed no significant difference between the 2 treatment groups. Because there were more patients with large residual disease in the CPT-P arm, they performed a subset analysis by removing those patients, and then compared the PFS with that of patients without residual disease less than 2 cm. The PFS tended to be longer in the CPT-P group, although the difference was not statistically significant. A phase III randomized trial of CPT-P arm versus TC arm undertaken by JGOG (Japanese Gynecologic Oncology Group) has closed and we are waiting for the results. According to a small retrospective in Japan, gemcitabine showed modest activity and is the most effective agent to clear cell adenocarcinoma of the ovary [20].

History of chemotherapy regimens for EOC

Over the years, experts and research groups have explored different combinations of antitumor drugs in order to improve the prognosis of ovarian cancer (Table 5). In 1976, the report by Witshaw and Kroner on the efficacy of cisplatin in ovarian cancer produced the modern era of combination chemotherapy (platinum-based combination therapy).

In the 1980s/early 1990 another turning point in the treatment of ovarian cancer was related to the discovery of paclitaxel, and active constituent of bark of the Pacific Yew tree, *Taxus brevifolia*. This agent acts by promoting microtubular assembly and stabilizes tubulin polymer formation and has a great deal of activity in epithelial ovarian cancer. Two randomized trials, the GOG 111 and the OV-10, comparing cisplatin/paclitaxel with cisplatin/cyclophosphamide, showed additional clinical benefit when cyclophosphamide was replaced by paclitaxel in the first-line setting [21-23].

Carboplatin, a cisplatin analogue is reported to have fewer marked side effects, especially such toxicities as nausea, renal toxicity, hearing loss, and neuromuscular toxicities than cisplatin. The carboplatin-paclitaxel combination is now considered an almost universal regimen in the management of epithelial ovarian cancer, and with a response rate of about 65%, PFS of 16-21 months and an OS of 32-57 months it is the standard arm in all the recent trials performed in this disease.

Table 5 The history of chemotherapy regimens for ovarian cancer

Study	Chemotherapy	regimen Reference
GOG22	Melphalan < CA	Cancer 51:783, 1983
GOG47	CA < CAP	Cancer 57:1725, 1986
GOG52	CAP = CP	JCO 7:457, 1989
GOG111	CP < TP	NEJM 334:1, 1996
OV10	CP < TP	JNCI 92:699, 2000
GOG158	TP = TC	ASCO 1999; #1373, 1374
SCOTROC	TC = DC	ASCO 2002; #804

In the last two decades, some studies have been performed in order to improve the efficacy of first-line chemotherapy such as by delivering drugs in epithelial ovarian cancer through the intraperitoneal (IP) route.

GOG 172 phase III trial revealed a prolonged survival in the arm of intraperitoneal (IP) therapy compared to the arm of intravenous (IV) therapy (65.6 and 49.7 months respectively; $P = 0.03$). Also PFS was better in the IP-therapy arm than in the IV-therapy group (23.8 versus 18.3 months, $P = 0.05$) [24]. However, a significantly higher rate of both hematologic and non-hematologic toxicities, including catheter related complications was observed in the arm of IP chemotherapy in this study. In most countries the intravenous route of administration of chemotherapy is still preferred.

Some studies have investigated the possibility to substitute paclitaxel with other drugs in order to improve the efficacy of treatment and to reduce toxicities, in particular alopecia and neurotoxicity (Table 6) [25].

The first attempt to develop this strategy was performed with docetaxel, a semisynthetic taxane with pharmacologic and pharmacokinetic advantages, compared to paclitaxel. This approach was sustained by emerging evidences suggesting superiority over anthracyclines and paclitaxel in metastatic breast cancer [26,27].

In ovarian cancer, docetaxel demonstrated activity [28], both in paclitaxel-resistant patients [29], and in primary ovarian cancer, in association with carboplatin [30]. To further investigate these promising findings, the SCOTROC-1 phase III study was performed. 1077 patients with ovarian cancer were randomly assigned to receive carboplatin IV (AUC 5) plus either docetaxel at 75 mg/m2 (1-h intravenous infusion) or paclitaxel at 175 mg/m2 (3-h intravenous infusion) [31]. Contrary to the previous results from several preclinical studies, which suggested that docetaxel might be more beneficial to paclitaxel, this phase III study did not demonstrate a survival advantage for carboplatin plus docetaxel over carboplatin plus paclitaxel treatment.

Carboplatin plus paclitaxel combination was associated with higher neurotxicity than carboplatin plus docetaxel therapy. Conversely, treatment with carboplatin plus docetaxel was associated with statistically more events of G3-4 neutropenia (94% versus 84%, $P<0.001$) and neutropenic complications than other treatment, requiring the frequent use of G-CSF support. Based on these data docetaxel with carboplatin has been considered a possible alternative to carboplatin-paclitaxel treatment in patients at very high risk of neurotoxicity, but has not replaced carboplatin-paclitaxel as standard treatment.

Table 6 Comparative investigations of the possibility to substitute paclitaxel with other drugs

Study	Treatment arms	FIGO stage	n	PFS (m)	OS(m)	p
SCOTROC-1		III-IV				0.71
	Carboplatin (AUC5)+Paclitaxel (175 mg/mq)		539	14.8	N.A	
	Carboplatin (AUC5)+Docetaxel (75 mg/mq)		538	15.0	N.A	
MITO-2		IC-IV				N.S.
	Carboplatin (AUC5) + Paclitaxel (175 mg/mq)		410	16.8	53.2	
	Carboplatin (AUC5) + Liposomal doxorubicin (30 mg/mq)		410	19.0	61.6	

N.A.: not accessed
N.S.: not significant

According to a recent review article [32], gemcitabine was the most common drug used in clinical trials. Gemcitabine-based combination therapy showed an average response rate of 27.2%, and was the most common therapy among the group of regimens with above average response rate and progression-free survival.

Novel treatment strategies of EOC

The larger expectation for improved prognosis in EOC is related to the use of the new biological agents. The deeper knowledge of ovarian cancer biology has led to the identification of multiple molecular targets, such as growth factor receptors, signal transduction pathways, cell cycle regulators, and angiogenic mechanisms. In this section, we overlook the major two molecular targeted agents applied to ovarian cancer treatment; anti-VEGF antibody bevacizumab and PARP inhibitor Olaparib.

Bevacizumab

One of the most investigated and promising molecular targeted drugs in ovarian cancer is bevacizumab, a monoclonal antibody directed against VEGF. VEGF expression is higher in ovarian cancer tumors than in normal ovarian tissue or benign ovarian tumors, and increasing VEGF expression in either cytosolic fractions derived from ovarian cancer tumors or serum VEGF levels in preoperative serum is considered to be associated with advanced stage and worse survival.

In order to inhibit the VEGF pathway, there are two primary strategies: (1) inhibition of the VEGF ligand with antibodies or soluble receptors and (2) inhibition of the VEGF receptor (VEGFR) with tyrosine kinase inhibitors (TKIs), or receptor antibodies. Of the VEGF targeting therapies, the most experience has been with a monoclonal antibody that binds the VEGF ligand, known as bevacizumab (Avastin). Bevacizumab is a 149-kDa recombinant humanized monoclonal IgG1 anti-VEGF antibody. It has been FDA-1 approved for the treatment of metastatic colorectal, breast, and non-small cell lung cancer and shows promise in the treatment of ovarian cancer. Several phase II studies have shown that bevacizumab is active in recurrent ovarian cancer [33,34].

Two phase III trials (GOG218, ICON 7) have recently evaluated the role of bevacizumab in first-line chemotherapy as an adjunct to carboplatin and paclitaxel. The GOG 218 is a multicenter, placebo-controlled trial with the primary end point to determine whether the addition of bevacizumab (15 mg/kg every 21 days) to standard chemotherapy is able to prolong PFS after primary cytoreductive surgery. Recently bevacizumab plus chemotherapy (carboplatin-paclitaxel) and bevacizumab maintenance was demonstrated to be able to prolong PFS of about 4 months (10.3 months versus 14.1 months) compared to carboplatin-paclitaxel alone [35]. Another multicenter trial is the ICON 7, an open label, two-arm trial, enrolling patients with high risk or advanced (stage I-IV) epithelial ovarian cancer to receive carboplatin plus paclitaxel or carboplatin-paclitaxel plus bevacizumab given concurrently and as maintenance up to 18 cycles. The bevacizumab used in this trial was half of that given in the GOG 218 study. This trial also showed that the addition of bevacizumab is able to prolong PFS compared to standard carboplatin-paclitaxel [36].

Another study, OCEANS trial, showed that addition of bevacizumab prolonged PFS in platinum-sensitive recurrent ovarian carcinoma cases [37].

PARP inhibitor, olaparib

The poly (ADP-ribose) polymerases (PARPs) are a large family of multifunctional enzymes [38]. PARP-1, the most abundant isoform, plays a key role in the repair of DNA single-strand breaks through the repair of base excisions. The inhibition of PARPs leads to the accumulation of DNA single-strand breaks, which causes DNA double-strand breaks at replication forks. These double-strand breaks are repaired in normal cells mainly by the error-free homologous recombination double-stranded DNA repair pathway, in which essential components are the tumor-suppressor proteins BRCA1 and BRCA2. In the absent of either BRCA1 or BRCA2, these lesions are not repaired, which results in cell cycle arrest and cell death, although there is an alternate pathway to non-homologous end-joining for DBS repair [39].

Women with inherited mutations in BRCA1 on chromosome 17q21 or BRCA2 on chromosome 13q31 are at significantly higher risk of developing breast and ovarian cancer than women in the control population. The lifetime risks of ovarian cancer are 54% for BRCA1 and 23% for BRCA2 mutation carriers [40]. Inherited mutations in those genes are found in 5-10% of all ovarian cancer patients. However, over 50% of high-grade serous or undifferentiated carcinomas (Type II ovarian cancer) showed loss of BRCA function, either by genetic or epigenetic events, which resulted in HR DNA repair defects [41].

The discovery of epigenetic mechanism of BRCA1/2 germinal mutation and the association of this mutation with ovarian cancer in 5-10% of the cases, led to the therapeutic concept of "synthetic lethality" [42]. In fact, in patients carriers BRCA mutation, PARP inhibition results in unrepaired DNA single-strand and double strand breaks and so cell death [43].

Fong et al. administered to fifty patients, the majority of which were platinum refractory, the PARP inhibitor olaparib with a favorable safety profile and a high response rate, in particular in patients with BRCA mutation. In patients with platinum-resistant and even platinum-refractory disease the response rate (of PARP inhibitor, olaparib) was of 41.7% and 15.4%, respectively [44]. Olaparib (AZD2281) was tested in BRCA-mutated patients with ovarian, primary peritoneal, and fallopian tube cancer. In the study, 20 patients (40%) responded to the therapy. Currently, randomized trials of olaparib and other PARP inhibitors in patients with ovarian cancer are underway.

Conclusion

Maximal surgical cytoreduction followed by systemic taxane and platinum-based chemotherapy is the standard treatment for patients with ovarian cancer. Molecular targeting therapy may improve the prognosis of them.

Abbreviations

CA: Cyclophosphamide + Adriamycin; CAP: Cyclophosphamide + Adriamycin + Cisplatin; CP: Cyclophosphamide+ Cisplatin; TP: Paclitaxel + Cisplatin; TC: Paclitaxel + Carboplatin; DC: Docetaxel + Carboplatin

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Authors' contributions

Dr. K wrote the manuscript, and Dr. E, Dr. U and Dr. N approved it. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Article

$p57^{Kip2}$ and $p27^{Kip1}$ Cooperate to Maintain Hematopoietic Stem Cell Quiescence through Interactions with Hsc70

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SUMMARY

Cell cycle regulators play critical roles in the balance between hematopoietic stem cell (HSC) dormancy and proliferation. In this study, we report that cell cycle entry proceeded normally in HSCs null for cyclin-dependent kinase (CDK) inhibitor p57 due to compensatory upregulation of p27. HSCs null for both p57 and p27, however, were more proliferative and had reduced capacity to engraft in transplantation. We found that heat shock cognate protein 70 (Hsc70) interacts with both p57 and p27 and that the subcellular localization of Hsc70 was critical to maintain HSC cell cycle kinetics. Combined deficiency of p57 and p27 in HSCs resulted in nuclear import of an Hsc70/cyclin D1 complex, concomitant with Rb phosphorylation, and elicited severe defects in maintaining HSC quiescence. Taken together, these data suggest that regulation of cytoplasmic localization of Hsc70/cyclin D1 complex by p57 and p27 is a key intracellular mechanism in controlling HSC dormancy.

INTRODUCTION

HSCs play an essential role in the maintenance of multiple lineages of blood cells through their dual ability to self-renew and to differentiate into progenitors of various lineages. By protecting HSCs from proliferative exhaustion, cell cycle quiescence is essential for the long-term engraftment potential and maintenance of stem cells. A balance of cell-extrinsic and -intrinsic regulators normally maintains HSCs in a state of relative dormancy. Our previous studies indicate that HSC quiescence is regulated by several extracellular factors, including Ang1/Tie2 (Arai et al., 2004), THPO/MPL (Yoshihara et al., 2007), and N-cadherin/β-catenin (Hosokawa et al., 2010). Because these pathways must converge on cell cycle regulation, it might be possible to drive HSC fate determination by directly manipulating the expression of intracellular cell cycle regulators. However, little is known about the intracellular mechanisms of cell cycle factors that regulate HSC quiescence and proliferation.

An early stage of the cell cycle is regulated by the action of the D-type cyclins that, together with their catalytic partner CDKs, function as intracellular sensors of extracellular signals. Their activities are carefully regulated by the INK4 family (p15, p16, p18, p19) and the Cip/Kip family (p21, p27, p57) of CDK inhibitors. Both positive and negative cell cycle regulators, including the D-type cyclins (Kozar et al., 2004), Cdk4/6 (Malumbres et al., 2004), and the INK4 family of CDK inhibitors (Oguro et al., 2006; Yuan et al., 2004), are critical for various aspects of HSC proliferation. In the Cip/Kip family gene products, p21 is critical for preventing HSC exhaustion by regulating quiescence (Cheng et al., 2000b), whereas p27 regulates the proliferation and pool size of hematopoietic progenitor cells (HPCs) rather than that of HSCs (Cheng et al., 2000a).

Among all CDK inhibitors, p57 is unique because of its essential role in development (Pateras et al., 2009). Recent studies have shown that p57 is highly expressed in the Thy1.1^{int}Flk2^{-}LSK (Passegué et al., 2005) and CD34^{-}MPL^{+}LSK (Yoshihara et al., 2007) quiescent populations of HSCs. Furthermore, TGF-β-induced cell cycle arrest in HPCs (Scandura et al., 2004) and HSCs (Yamazaki et al., 2009) is correlated with an increase in p57 expression. These observations suggest that HSC quiescence is potentially maintained by the modulation of p57 expression. However, the role of p57 in HSCs has not been determined, because of the neonatal lethality of $p57^{-/-}$ mice.

In this study, p57 was found to contribute to the maintenance of HSC quiescence, and p27 compensated for p57 function in HSCs with a p57 deletion. An association between p57, p27, and Hsc70 was found to maintain the cytoplasmic localization of the Hsc70/cyclin D1 complex and regulate the cell cycle entry of HSCs. These findings provide insights into the physiological function of p57 and p27 in HSCs and have implications for the roles of CDK inhibitors in the maintenance of tissue homeostasis.

RESULTS

Deletion of p57 in Embryonic HSCs Did Not Affect Cell Cycle Progression

Bone marrow (BM) lineage$^-$ Sca1$^+$ c-Kit$^+$ (LSK) cells can be divided into populations of long-term reconstituting HSCs (LT-HSCs; CD34$^-$/Flt3$^-$ or CD150$^+$/CD48$^-$), which are capable of extensive self-renewal, and short-term reconstituting HSCs (ST-HSCs; CD34$^+$/Flt3$^-$ or CD150$^-$/CD48$^-$), which self-renew for a limited time before giving rise to a population of non-self-renewing multipotent progenitors (MPPs; CD34$^+$/Flt3$^+$ or CD48$^+$) (Adolfsson et al., 2001; Kiel et al., 2005). To gain insights into G_0/G_1 cell cycle control in these populations, we analyzed the expression levels of G1 phase cyclins via qRT-PCR. Within the pool of LSK cells, LT-HSCs expressed the highest levels of cyclin D1 and D2 and the lowest levels of cyclin D3 (Figure 1A). However, no induction of cyclin E1 or E2 was observed in LT-HSCs, although their expression is correlated with cyclin D-dependent Rb-mediated transcription. Furthermore, both of the G_1 phase CDKs were expressed at low levels in LT-HSCs (Figure 1B). In contrast, the differentiation of LT-HSC into ST-HSC and then into MPP specifically correlated with decreased expression of p57 and increased expression of p21, whereas p27 expression did not change significantly (Figure 1C).

Immunocytochemistry showed that most Ki67$^-$LSK cells (quiescent HSCs) exhibited abundant expression of p57 in the cytoplasm, whereas most Ki67$^+$LSK cells (cycling HSCs or MPPs) showed only weak expression of p57. In contrast, no significant difference in the expression of p21 or p27 was detected between Ki67$^-$ and Ki67$^+$LSK cells (see Figure S1A available online). To understand how cell cycle entry is regulated in HSCs, freshly isolated CD34$^-$LSK cells were stimulated with SCF (stem cell factor). SCF stimulation caused the degradation of p57 and nuclear translocation of cyclin D1, which were followed by Rb phosphorylation (Figure S1B). Interestingly, CDK2, CDK4, and CDK6 were not detected in the cytoplasm of CD34$^-$LSK cells although their nuclear expression was induced after SCF stimulation (Figure S1C and data not shown). These results suggest that p57 in HSCs may regulate cell cycle progression by controlling the subcellular localization of cyclin D, which is independent of the expression of the CDKs.

To investigate whether the proliferation of hematopoietic cells was affected by p57 deficiency, cell cycle progression was examined in fetal liver (FL) LSK cells, common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) (Figure S2A). Hoechst staining showed that the absence of p57 did not affect the proliferation of FL LSK cells or progenitors (Figure S2B). In addition, no significant difference was detected in the total number of LSK cells between $p57^{+/+}$ and $p57^{-/-}$ embryos (Figure S2C).

Next, in vitro colony-forming assays were performed with freshly isolated FL Flt3$^-$ or Flt3$^+$LSK cells. Equal numbers of $p57^{+/+}$ or $p57^{-/-}$ cells were plated, and equivalent numbers of colonies were generated (Figure 2A). To determine whether p57 is involved in adult BM HSC reconstitution, the long-term repopulating capacity of $p57^{-/-}$ FL HSCs was examined in vivo. Flow cytometric analysis of the peripheral blood (PB) of the transplant recipients revealed that $p57^{+/+}$ and $p57^{-/-}$ FL LSKs were equally capable of supplying hematopoietic cells (Figure 2B). Serial BMTs also were performed to analyze the self-renewal capacity of $p57^{-/-}$ HSCs. Examination of donor-derived BM (Figure 2C) or LSK (Figure 2E) cells after the third transplantation revealed a substantial reduction in the reconstitution capacity of $p57^{-/-}$ HSCs compared to $p57^{+/+}$ HSCs. The proliferation of donor-derived LSK cells was then determined by Ki67 staining (Figure 2D). There was no difference in the frequency of G_0 cells between $p57^{+/+}$ and $p57^{-/-}$ LSK cells at either the first or the third BMT (Figure 2F). These data indicate that p57 may mediate the self-renewal capacity of FL donor-derived HSCs during serial BMT, but that the changes are not caused by alterations in the cell cycle.

Deficiency of p57 Leads to the Upregulation of p27 in HSCs

Although p57 deficiency did not affect the maintenance of quiescence in HSCs, molecular redundancy or compensatory mechanisms may exist that compensate for the loss of p57. The expression of CDK inhibitors was examined in LSK cells isolated from $p57^{+/+}$ or $p57^{-/-}$ donor-derived BM at 4 months post-BMT. The effects of THPO administration on gene expression in donor-derived LSK cells of recipient mice was also investigated (Figure 3A) because THPO/MPL signaling is involved in the maintenance of HSC quiescence and is associated with the regulation of p57 signaling pathways in HSCs (Yoshihara et al., 2007). Consistent with previous studies, upregulation of p57 occurred in wild-type (WT) donor-derived LSK cells after 3 days of daily THPO administration. Interestingly, a significant increase in p27 expression was detected in $p57^{-/-}$ LSK cells, and the increase became more distinct after THPO administration, whereas p21 expression was the same in all groups (Figure 3B). In addition, $p57^{-/-}$ LSK cells exhibited a greater level of p18 expression, which is correlated with the self-renewal of HSCs (Yuan et al., 2004). However, THPO administration did not increase the expression of p18 in $p57^{-/-}$ LSK cells, indicating a difference between the regulation of p18 and p27.

In the serial BMT, p18, but not p27, was upregulated in the reconstituted $p57^{-/-}$ LSK cells during the transplantation (Figure 3C). Furthermore, $p57^{-/-}$ donor-derived CD34$^-$LSK cells showed a high level of p18 expression in the nucleus (Figure 3D; Figure S3A), whereas, in contrast, p27 was expressed predominantly in the cytoplasm of these cells (Figure 3E; Figure S3B). In addition, the upregulation of p21 expression in donor-derived LSK cells was not observed when p57 was deleted (Figure S3C). Taken together, these data suggest that p27 may compensate for the loss of p57 function in the cytoplasm for the maintenance of quiescence in $p57^{-/-}$ HSCs.

Critical Role of p57 in the Maintenance of $p27^{-/-}$ BM LSK Cells

Given the upregulation of p27 in $p57^{-/-}$ FL donor-derived HSCs at post-BMT, a p57 knockdown (KD) strategy was utilized in $p27^{-/-}$ adult BM LSK cells to reduce the expression of p57 in the absence of p27 (Figure 4A). As shown in Figure S4A, the expression of p57 in $p27^{-/-}$ LSK cells was not affected. Three retroviral p57 shRNAs were introduced into MEFs, and the efficient reduction of p57 expression by sh-1 was verified (Figure S4B). LSK cells were isolated from $p27^{+/+}$

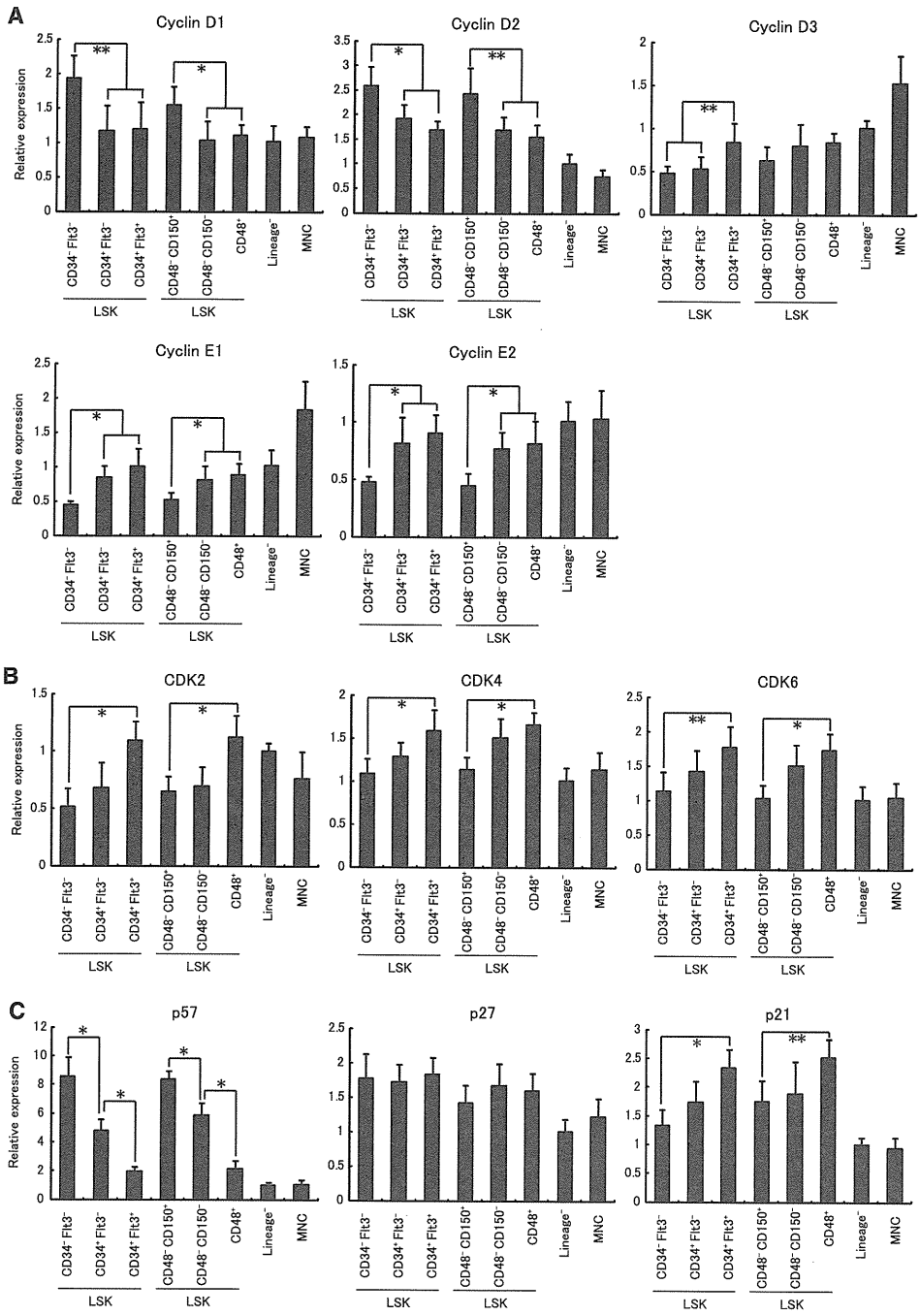

Figure 1. qRT-PCR Analysis of Cell Cycle-Related Genes in LT-HSC, ST-HSC, MMP, Lineage⁻, and MNC Fractions

LT-HSC, ST-HSC, and MMP fractions are from $CD34^-/Flt3^-$ or $CD48^-/CD150^+$; $CD34^+/Flt3^-$ or $CD48^-/CD150^-$; and $CD34^+/Flt3^+$ or $CD48^+$ populations, respectively.

(A) Expression levels of G_1 phase cyclins.

(B) Expression levels of G_1 phase CDKs.

(C) Expression levels of the p21, p27, and p57 CDK inhibitors.

Data represent mean values from three samples, all performed in duplicate. Error bars indicate the SD (*$p < 0.01$, **$p < 0.05$, $n = 3$).

or *p27*$^{-/-}$ BM and were transduced with either the scrambled control (CON) or the p57 sh-1 KD vector. At day 2 after viral infection, each group of LSK GFP^+ cells was sorted for functional assessment.

Long-term colony-initiating cell (LTC-IC) assays were first performed for GFP^+ cells on a layer of OP9 stromal cells. In this assay, the number of colony-forming cells after 6 weeks of culture reflects HSC function. After 6 weeks, the number of

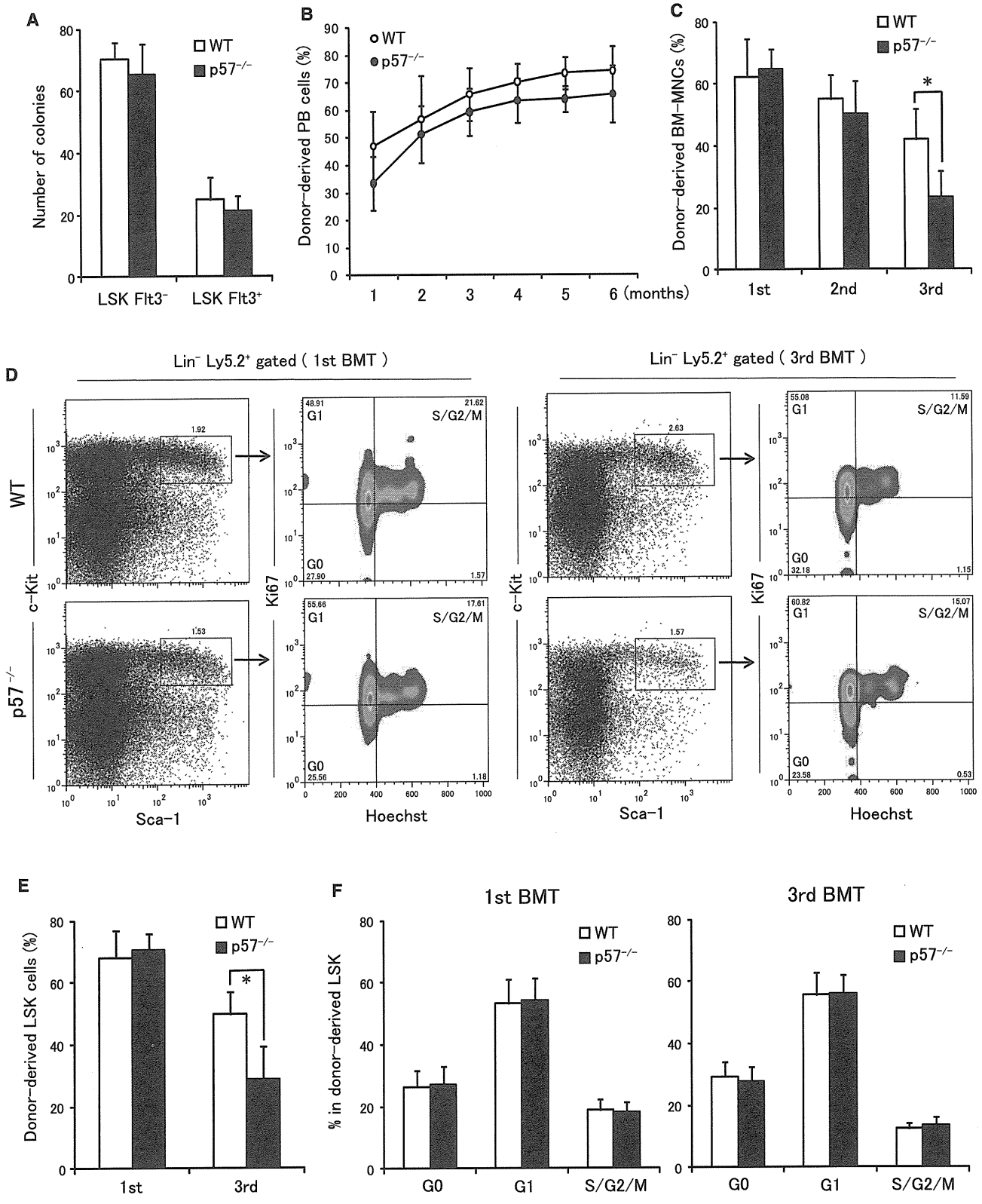

Figure 2. p57 Deficiency Affects HSC Repopulating Ability upon Serial BMT, but Is Not Caused by an Altered Cell Cycle

(A) Normal clonogenic capacity of *p57*$^{-/-}$ FL HSCs in vitro. Flt3^{-} or Flt3^{+} LSK cells were flow sorted from *p57*$^{+/+}$ or *p57*$^{-/-}$ E14.5 FLs and cultured in methylcellulose medium for 7 days. Data shown are the mean number of colonies (±SD) formed per 200 cells (n = 3).

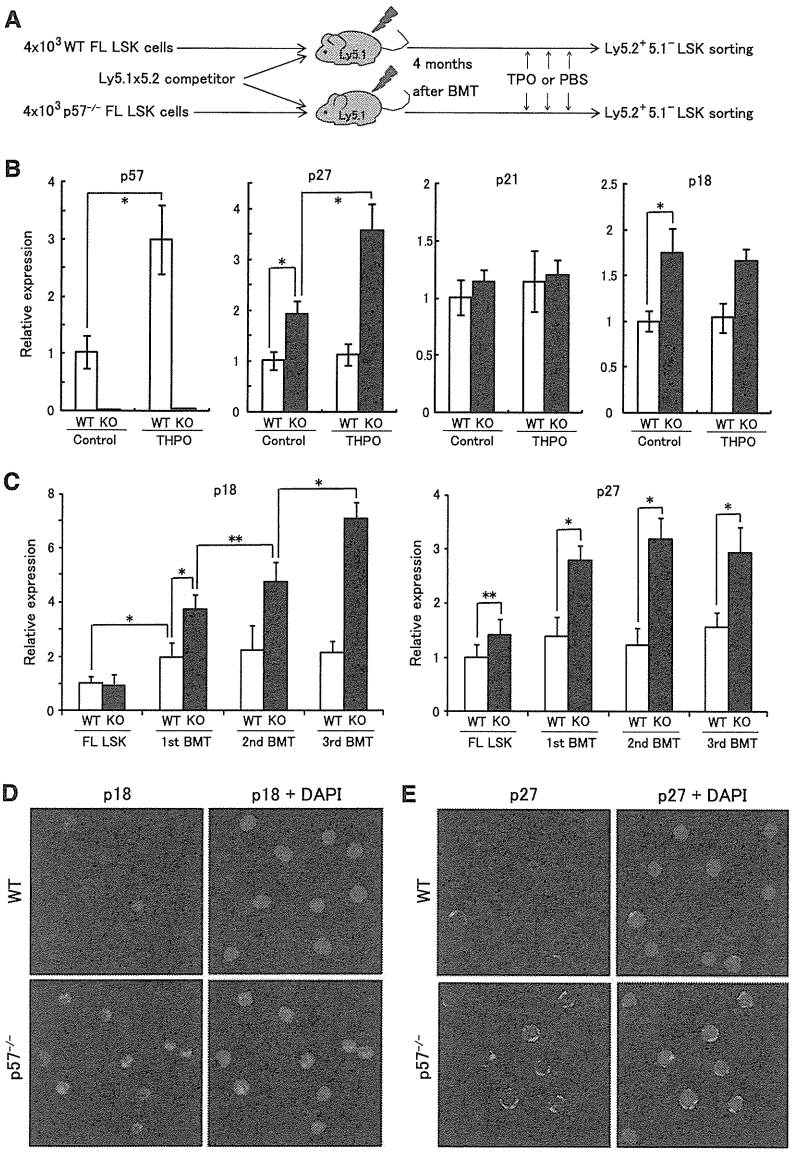

Figure 3. Expression of CDK Inhibitors in $p57^{+/+}$ or $p57^{-/-}$ FL Donor-Derived LSK Cells after Transplantation

(A) Scheme of the preparation of donor-derived LSK cells.

(B) Increased p27 and p18 expression in $p57^{-/-}$ LSK cells at 4 months posttransplantation. Donor-derived LSK cells were sorted after 3 days of daily THPO or PBS (control). The relative expression of CDK inhibitors (p21, p27, p57, and p18) was analyzed by qRT-PCR. Data shown are mean (±SD) values from two independent experiments (*$p < 0.01$, $n = 3$).

(C) Increased p18 expression in $p57^{-/-}$ LSK cells during serial BMT. FL LSK cells before transplantation or FL donor-derived LSK cells were sorted after each BMT. The relative expression of p18 and p27 was analyzed. Data shown are mean (±SD) values from two independent experiments (*$p < 0.01$, **$p < 0.05$, $n = 3$).

(D) Expression of p18 was upregulated in the nucleus of $p57^{-/-}$ donor-derived CD34⁻LSK cells at 4 months posttransplantation. Freshly isolated donor-derived cells were stained with anti-p18 (green) and DAPI (blue).

(E) Expression of p27 was upregulated in the cytoplasm of $p57^{-/-}$ donor-derived CD34⁻LSK cells at 4 months posttransplantation. Freshly isolated donor-derived cells were stained with anti-p27 (green) and DAPI (blue).

colonies derived from $p27^{-/-}p57^{KD}$ LSK cells was significantly less than that derived from $p27^{+/+}p57^{CON}$, $p27^{+/+}p57^{KD}$, or $p27^{-/-}p57^{CON}$ LSK cells, although there was no significant difference in the short-term cultures (less than 2 weeks) (Figure 4B). In addition, almost no large LTC-IC colonies (>2 mm in diameter) were generated from $p27^{-/-}p57^{KD}$ LSK cells (Figure S4C). Because these colonies represent the more primitive hematopoietic cell populations, these data suggest that p57 and p27 cooperate to maintain the immature HSC phenotype in vitro.

To evaluate HSC function in vivo, the repopulating capacity of $p27^{-/-}p57^{KD}$ LSK cells was next examined via competitive reconstitution assays. The $p27^{-/-}p57^{KD}$ cell recipients showed low donor-derived cell chimerism in the PB at 2 months after transplantation, and this proportion decreased thereafter. In contrast, the transplanted $p27^{+/+}p57^{CON}$, $p27^{+/+}p57^{KD}$, or $p27^{-/-}p57^{CON}$ cells showed a steady increase in chimerism from 2 to 4 months posttransplantation (Figure 4C). These results indicate that $p57^{KD}$ may cause an in vivo defect that impairs the function of LSK cells after BMT when p27 expression is deficient. To characterize the defect in $p27^{-/-}p57^{KD}$ LSK cells, the homing capacity and viability of transplanted cells were tested. 30 hr after BMT, homing capacity and apoptotic cell ratio of transplanted GFP⁺ cells in the BM were equivalent in each group (Figures S4D and S4E). Therefore,

(B) Normal long-term reconstitution capacity of $p57^{-/-}$ FL LSK cells. Irradiated recipient mice were transplanted with 4×10^3 LSK cells from $p57^{+/+}$ or $p57^{-/-}$ FLs plus 2×10^5 BM-MNCs. Data shown are the mean percentages (±SD) of donor-derived cells in the PB at the indicated times after transplantation ($n = 5$).

(C) Defective repopulation capacity of $p57^{-/-}$ BM MNCs during serial transplantation. 4×10^3 donor-derived LSK cells from the recipient mice were serially transplanted into recipient mice. The repopulating capacity of $p57^{+/+}$ or $p57^{-/-}$ donor cells was determined at 16 weeks posttransplant for each BMT. Data shown are the mean percentages (±SD) of donor-derived cells in BM MNCs (*$p < 0.01$, $n = 5$).

(D) Equivalent cell cycle status of posttransplant $p57^{+/+}$ and $p57^{-/-}$ LSK cells. Results shown are one analysis representative of two independent experiments.

(E) Defective repopulation of $p57^{-/-}$ LSK cells derived from BM cells in serial transplantation. BM cells from the recipient mice were analyzed for the frequency of donor-derived LSK cells at 16 weeks post-BMT (±SD, *$p < 0.01$, $n = 5$).

(F) Cell cycle status in the pool of donor-derived LSK cells. Cell cycle analyses of LSK populations by Ki67 and Hoechst staining in (D). The percentages of cells in G_0, G_1, or $S/G_2/M$ phase are indicated (±SD).