OPN mRNA, but the level expressed by iDCs was exceeded by differentiation stage OC8 (Fig. 1B). To characterize the OPN-producing cells further, we performed immunofluorescence staining on samples of each cell population (monocytes, iDCs, OC4, OC8, and OC12). Monocytes and iDC were attached to slide glass by cytospin. OC4, OC8, and OC12 were cultured on glass coverslips. In accordance with the increase in OPN mRNA expression found over the course of culture, production of OPN increased with differentiation: monocytes did not produce OPN, iDCs started to produce relatively small amounts, and OC12 produced the highest levels of OPN (Fig. 1C). The concentration of both the full-length and the cleaved forms of OPN in the cell supernatant steadily increased during the course of OC-like MGC formation (Fig. 1D).

EXPRESSION OF OPN RECEPTORS DURING THE COURSE OF OC-LIKE MGC GENERATION FROM iDCs

Next, we investigated the expression of receptors for OPN, specifically αvβ3 integrin, CD44v6, and α9β1 integrin, during the course of OC-like MGC formation. The mRNA levels of αvβ3 integrin, CD44v6, and α9β1 integrin increased and peaked at OC8 or OC12 (Fig. 2A), following a similar pattern to OPN production. CD44v6 mRNA was highly expressed by monocytes; however, its expression by iDCs was low. To characterize OPN receptor-expressing cells further we examined αvβ3-, CD44v6-, and α9β1-expressing OC12 for TRAP and OPN expression using immunofluorescence staining. Furthermore, to know whether iDCs remain at the end of culture, OC12, and whether the remaining iDCs are involved in the formation of OC-like

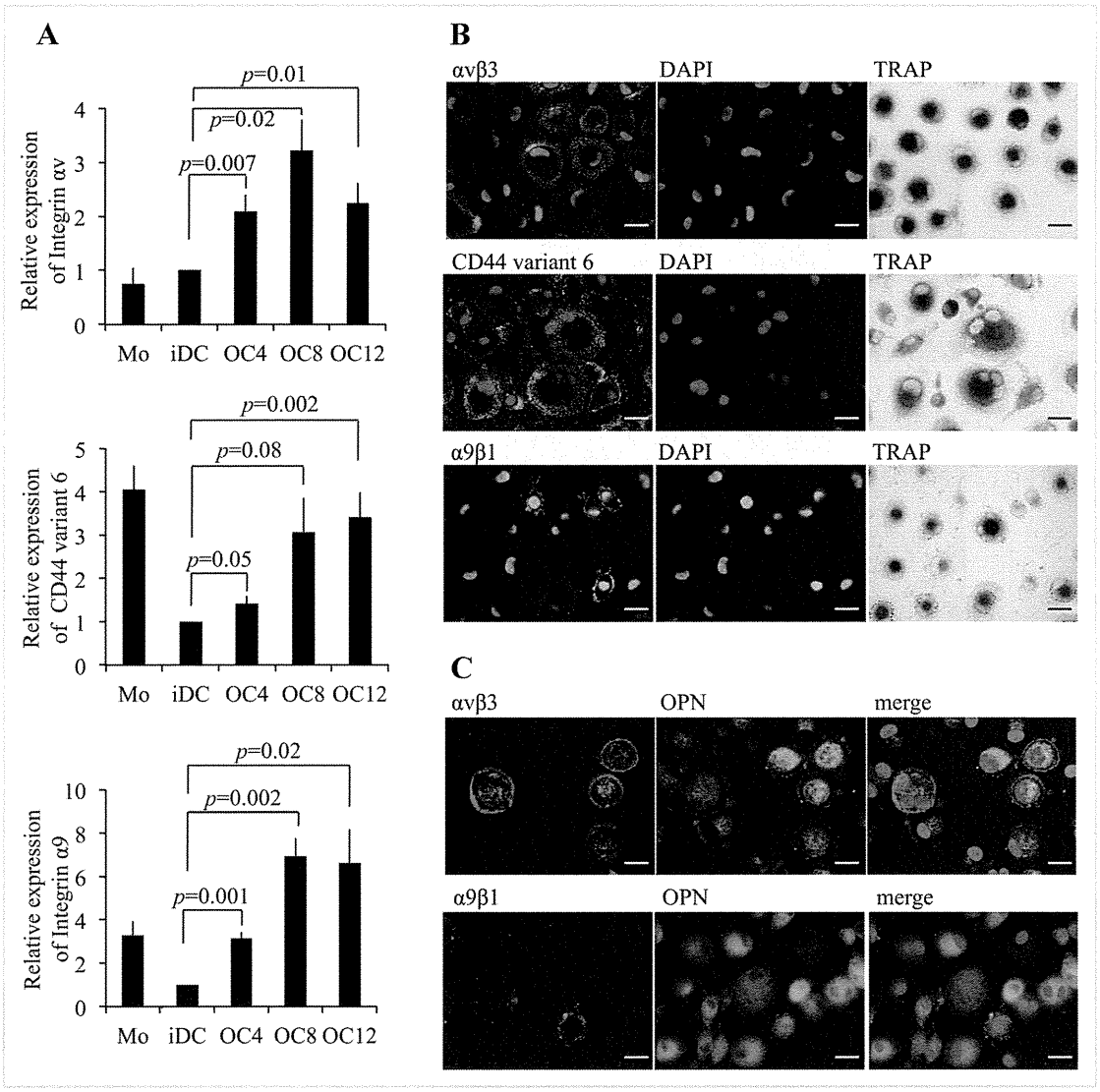

Fig. 2. OPN receptor expression during the course of OC-like MGC formation from iDCs and characterization of αvβ3-, CD44v6-, and α9β1-expressing cells. A: Expression of integrin αv mRNA (receptor for full-length OPN), CD44v6 mRNA (receptor for full-length OPN), and integrin α9 mRNA (receptor for cleaved OPN) measured by relative quantitative RT-PCR (data shown are the mean of experiments with cells from five donors). Error bars represent mean ± SEM. B: Detection of αvβ3, CD44v6 (receptors for full-length OPN), and α9β1 (receptor for cleaved OPN) by immunofluorescence staining (green) of OC12. Nuclei were stained with DAPI (blue). TRAP activity was assessed on the same glass coverslips. Bars: 20 μm. Data shown are representative of ten experiments. C: Detection of OPN (green) and its receptor (red) by double immunofluorescence staining. Nuclei were stained with DAPI (blue). Bars: 20 μm. Data shown are representative of 10 experiments. DAPI, 4′,6-diamidino-2-phenylindole; TRAP, tartrate-resistant acid phosphatase.

MGCs, we stained iDC marker, CD1a. Cells expressing αvβ3 were both TRAP- and OPN-positive but CD1a-negative, and some were multinucleated but with no more than four nuclei (Figs. 2B,C and 3A), suggesting that they were not fully mature OC-like MGCs. CD44v6-expressing cells were also TRAP-positive, and most were typical MGCs (Fig. 2B), suggesting that they were mature OC-like MGCs. On the other hand, α9β1-expressing cells were both OPN- and CD1a-positive but TRAP-negative with a single nucleus, and some were spindle shaped with processes characteristic of iDCs (Figs. 2B,C and 3A). Using fluorescence intensity scoring, we showed that cells expressing α9β1 existed before the start of OC differentiation and some iDCs expressed α9β1 integrin weakly but, while its expression became stronger during the course of OC-like MGC formation (Fig. 3B, Supplementary Fig. S1). The number of α9β1- and CD1a-positive cells did not increase (Fig. 3C,D). These data indicate that some remaining iDCs express α9β1 integrin strongly instead of the increased number of α9β1-positive cells. This suggests that α9β1- and CD1a-positive cells might have some role in OC-like MGCs formation.

OC-LIKE MGC FORMATION IS SUPPRESSED BY DOWN-REGULATION OF OPN

To study the role of OPN in OC-like MGC formation, we down-regulated the expression of OPN by transfection with OPN siRNA at the initial monocyte stage, or at the iDC stage (Fig. 4A). First, we generated iDCs from monocytes that had been transfected with either OPN siRNA or control siRNA. There was no difference in efficacy of differentiation of monocytes into iDCs between monocytes transfected with OPN siRNA or control siRNA, as judged by the expression of CD1a (Supplementary Fig. S2). Next, we prepared two types of iDCs, one derived from monocytes that had been treated with OPN

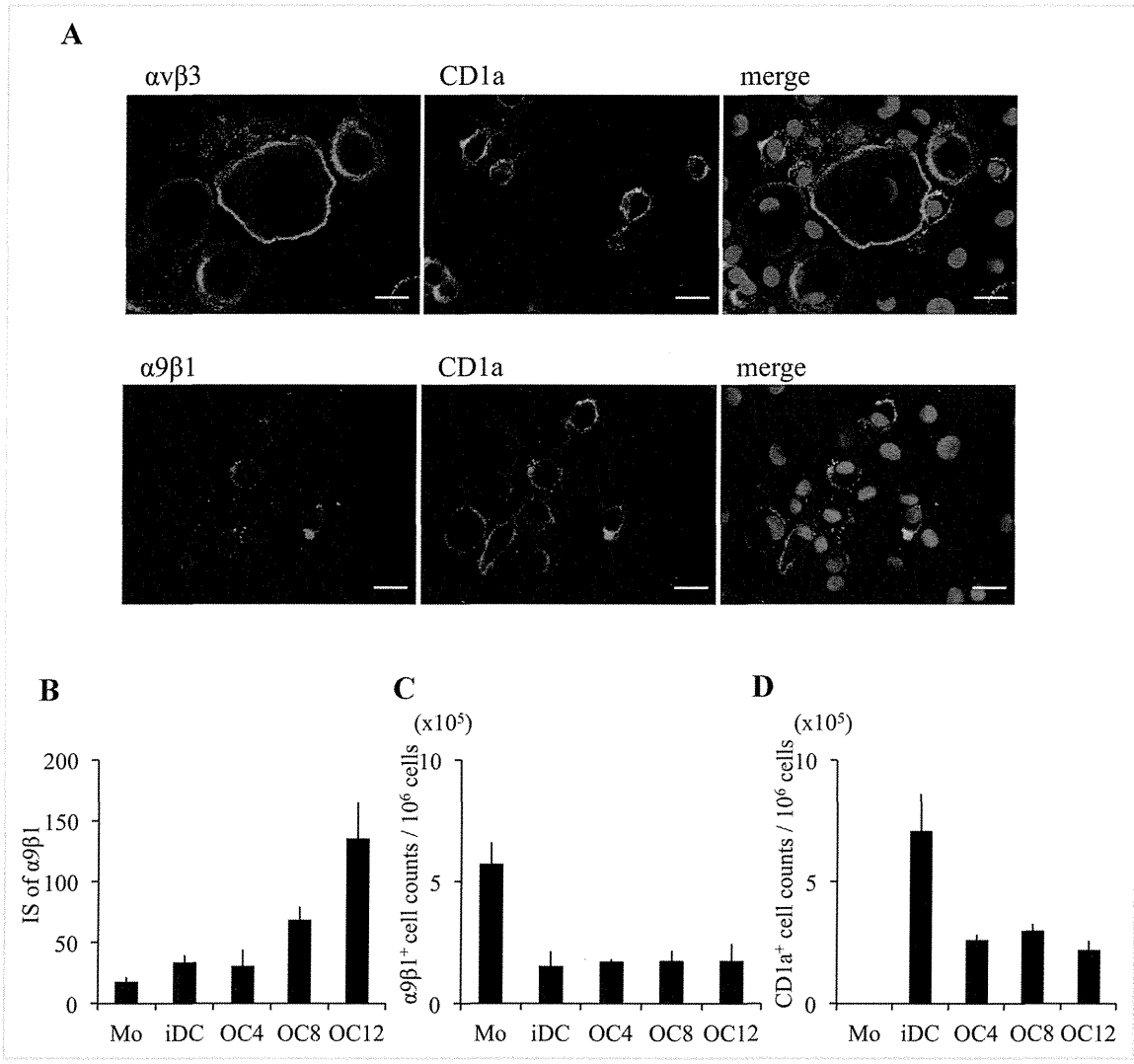

Fig. 3. The relationship between OPN receptors and CD1a. A: Detection of CD1a (green) and OPN receptors (red) by double immunofluorescence staining, Nuclei were stained with DAPI (blue). Bars: 20 μm. Data shown are representative of 10 experiments. B: Immunofluorescence data providing the IS of α9β1 expressed. The mean staining intensity was calculated as follows: IS, mean of brightness of selected cells' red channel score (in arbitrary units, AU) using Adobe Photoshop Elements, version 11. Data shown are the mean of experiments with cells from three donors. Error bars represent mean ± SEM. C: Number of α9β1-positive cells per 1×10^6 cells (data shown are the mean of experiments with cells from three donors). D: Number of CD1a-positive cells per 1×10^6 cells (data shown are the mean of experiments with cells from three donors). IS, intensity score.

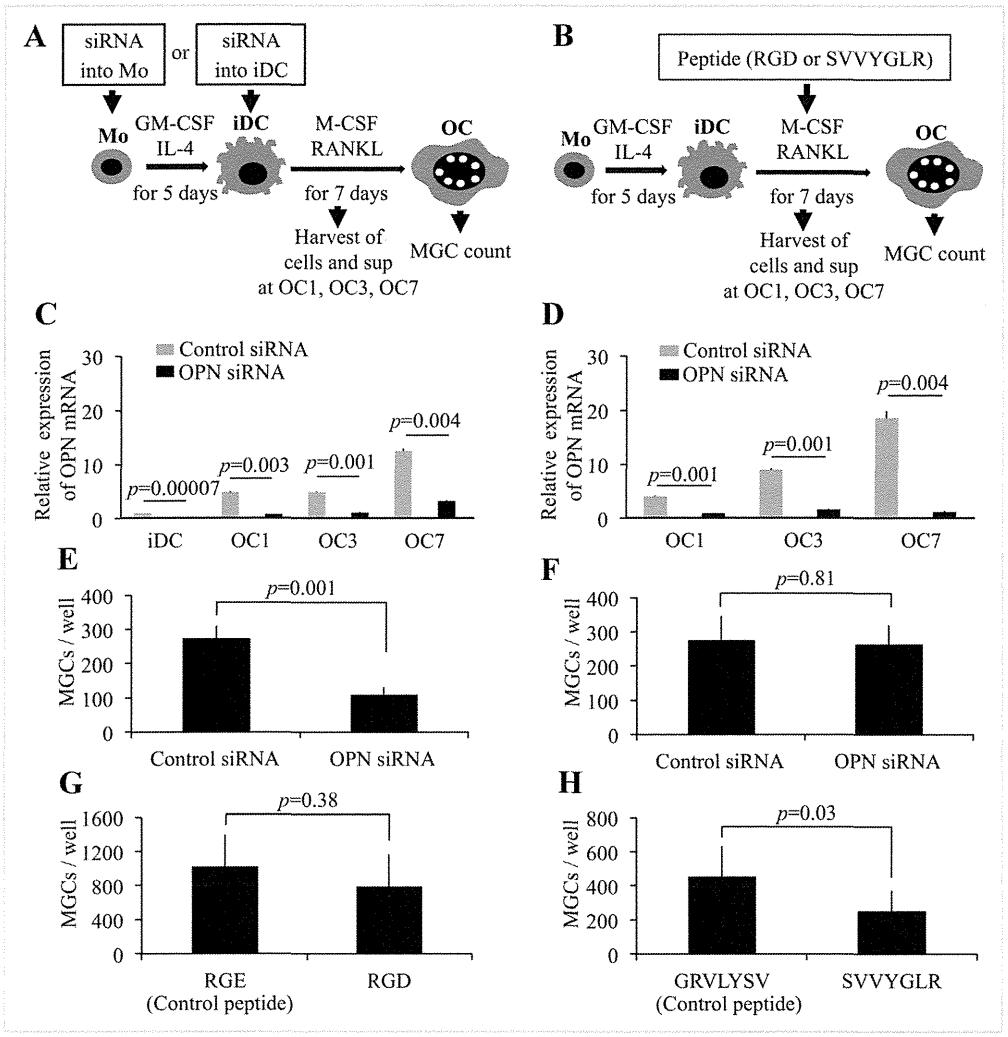

Fig. 4. The effect on MGC formation from iDC of down-regulating OPN with siRNA and inhibiting OPN binding. A: To study the role of OPN on OC-like MGC generation, OPN expression was down-regulated by transfection of OPN siRNA into monocytes or iDCs derived from non-treated monocytes. B: To identify the type of OPN receptor involved in MGC generation, two different synthetic peptides, RGD or SVVYGLR, were included in the culture medium during the differentiation of iDCs into OC-like MGCs. C, D: OPN mRNA levels measured by relative quantitative RT-PCR in OC-like MGC generated from (C) siRNA-transfected monocytes (data shown are the mean of experiments with cells from ten donors) or (D) siRNA-transfected iDCs (data shown are the mean of experiments with cells from ten donors). E, F: Number of MGCs differentiated from (E) iDCs generated from siRNA-transfected monocytes (data shown are the mean of experiments with cells from ten donors) or (F) siRNA-transfected iDCs (data shown are the mean of experiments with cells from ten donors). The number of MGCs was counted at OC7. G: Number of MGCs differentiated from iDCs in the presence of 10 μg/ml RGE (control) or RGD peptides (data shown are the mean of experiments with cells from five donors). H: Number of MGCs differentiated from iDCs in the presence of 1 μg/ml GRVLYSV (control) or SVVYGLR peptides (data shown are the mean of experiments with cells from six donors). MGCs were counted at OC7. Error bars represent mean ± SEM. OC1, OC-like MGCs on day 1; OC3, OC-like MGCs on day 3; OC7, OC-like MGCs on day 7; *sup, supernatants.*

siRNA (Fig. 4C,E), and the other derived from non-treated monocytes, which were treated with OPN siRNA at the iDC stage (Fig. 4D,F). Subsequently both types of iDCs were cultured for a further 7 days. Cells and supernatants were recovered at day 1, 3, and 7 and thus these cells were referred to as OC1, OC3, and OC7, respectively. The numbers of OC-like MGCs were counted on day 7 (OC7). With the exception of OC1, the concentration of full-length and cleaved forms of OPN in the supernatant of cells differentiated from both types of iDCs was reduced in OPN siRNA-transfected cells compared to cells transfected with control siRNA (Supplementary Fig. S3). Nevertheless, OPN mRNA expression was efficiently reduced by OPN siRNA treatment (Fig. 4C,D). By the end of culture on day 7, there was a significant reduction in MGC formation by iDCs derived from monocytes treated with OPN siRNA (Fig. 4E), while OPN siRNA-treated iDCs derived from non-treated monocytes were able to generate MGCs (Fig. 4F). These data suggest that OPN plays a role in the early phase of OC and/or MGC differentiation, specifically during the differentiation of monocytes to iDCs.

MGC FORMATION IS REDUCED BY SVVYGLR PEPTIDES

To investigate the type of OPN receptor involved in MGC formation, we used two different synthetic peptides, corresponding to internal

sequences of OPN, namely RGD, which binds RGD-recognizing integrins including αvβ3 and α5β1, and SVVYGLR, which is recognized by α4β1 and α9β1. These peptides were added to the culture medium during the differentiation of iDCs into OC-like MGCs (Fig. 4B). No obvious effect on MGC formation by RGD peptide compared to the RGE control peptide was observed (Fig. 4G). However, MGC formation was significantly reduced by SVVYGLR peptide as compared to the GRVLYSV control peptide (Fig. 4H), suggesting that α4β1 and/or α9β1 integrin receptors play a pivotal role in MGC formation.

OPN DID NOT AFFECT VIABILITY AND APOPTOSIS IN OC-LIKE MGC FORMATION

OPN is known to confer resistance to apoptosis [Tuck et al., 2007; Yamaguchi et al., 2013]. In inflammatory bone diseases, osteolytic lesions can be treated with bisphosphonates [Morimoto et al., 2011], which induce OC apoptosis [Abe et al., 2012]. Based on these reports, we hypothesized that OPN promote the survival and inhibit the apoptosis of OC precursor cells or OCs, consequently OC-like MGC formation is increased. We evaluated whether OPN affects cell viability and apoptosis in the course of OC-like MGC formation, using OPN siRNA-transfected or control siRNA-transfected monocyte-derived iDC. We determined cell viability and apoptosis by flow cytometry for floating cell and trypan blue stain and TUNEL stain for tightly adhering cells. Additionally, we performed Caspase-3 activity assay. Caspase-3 is an active cell-death protease involved in the execution phase of apoptosis, where cells undergo morphological changes such as DNA fragmentation, chromatin condensation, and apoptotic body formation [Porter and Janicke, 1999]. The number of viable cell and apoptotic cell, and caspase-3 activity were not affected by down-regulation of OPN (Fig. 5A–C).

OXIDATIVE STRESS IS NOT INVOLVED IN OPN PRODUCTION DURING THE COURSE OF OC-LIKE MGC FORMATION

In view of previous reports demonstrating that reactive oxygen species (ROS) may play a significant role as second messengers for the expression of osteopontin in mice [Umekawa et al., 2009; Lyle et al., 2012], it is an interesting issue whether ROS is linked to OPN production in human primary cells. To answer this question, we examined intracellular ROS activity and OPN production with or without ROS inhibitor of diphenyleneiodonium chloride (DPI) or *N*-acetyl-L-cysteine (NAC) in our culture system. DPI is a competitive inhibitor of flavin-containing cofactors and a very potent inhibitor of NADPH oxidase [Hancock and Jones, 1987]. NAC, in contrast, acts as a scavenger of ROS regardless of the source of production [Aruoma et al., 1989]. ROS was already generated at the differentiation into iDCs from monocytes, and came up to the highest levels at OC7 (Fig. 6A). As previous research has indicated [Del Prete et al., 2008], iDC differentiation from monocyte was suppressed when monocytes were pretreated with ROS inhibitor (Supplementary Fig. S4). We next treated iDCs with a non-cytotoxic concentration of DPI (100 nM) or NAC (20 mM), and evaluated the effect of ROS inhibitors on OPN production. Although ROS activity was significantly suppressed at OC7 (Fig. 6B), OPN production was not decreased (Fig. 6C,D). Because OPN is also known to reduce intracellular ROS during hypoxia/reperfusion to protect cells from oxidative injury [Denhardt et al., 1995], we examined intracellular ROS activity with or without down-regulation of OPN during the course of OC-like MGC formation. The down-regulation of OPN did not affect intracellular ROS activity (Supplementary Fig. S5).

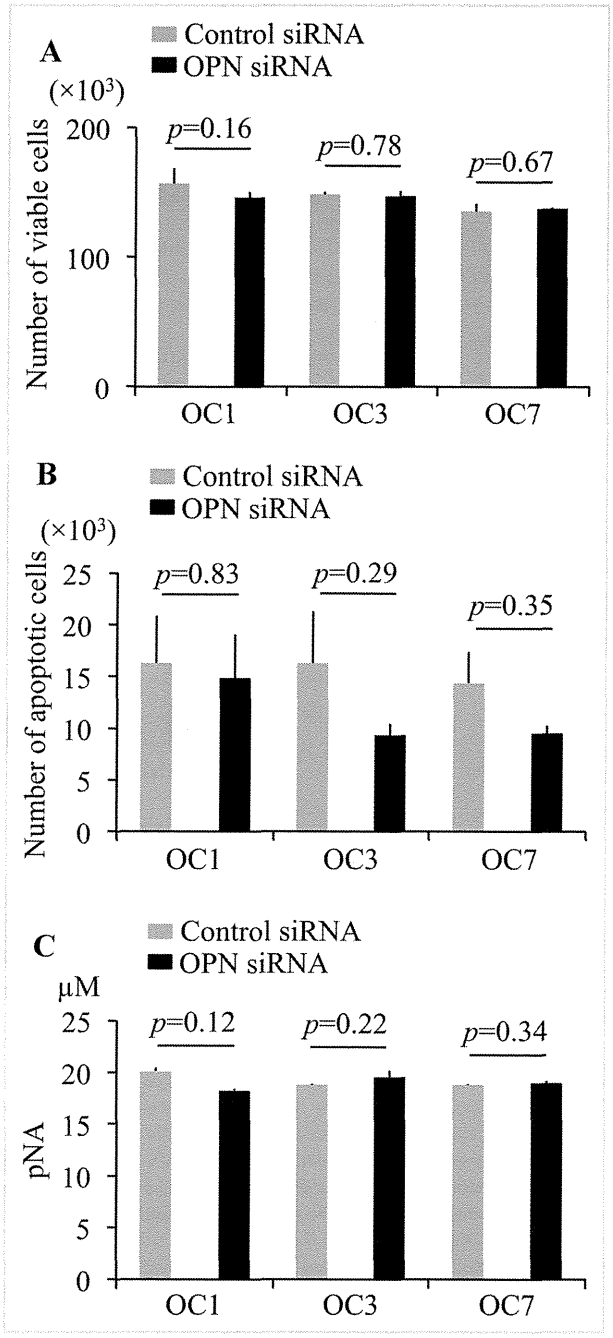

Fig. 5. Viability and apoptosis in the course of OC-like MGC differentiation from iDC of down-regulating OPN with siRNA. A: Number of viable cells. B: Number of apoptotic cells. C: Quantification of caspase-3 activity. We detected pNA as cleavage product by caspase-3. Data shown are the mean of experiments with cells from four donors. Error bars represent mean ± SEM. pNA, p-nitroaniline.

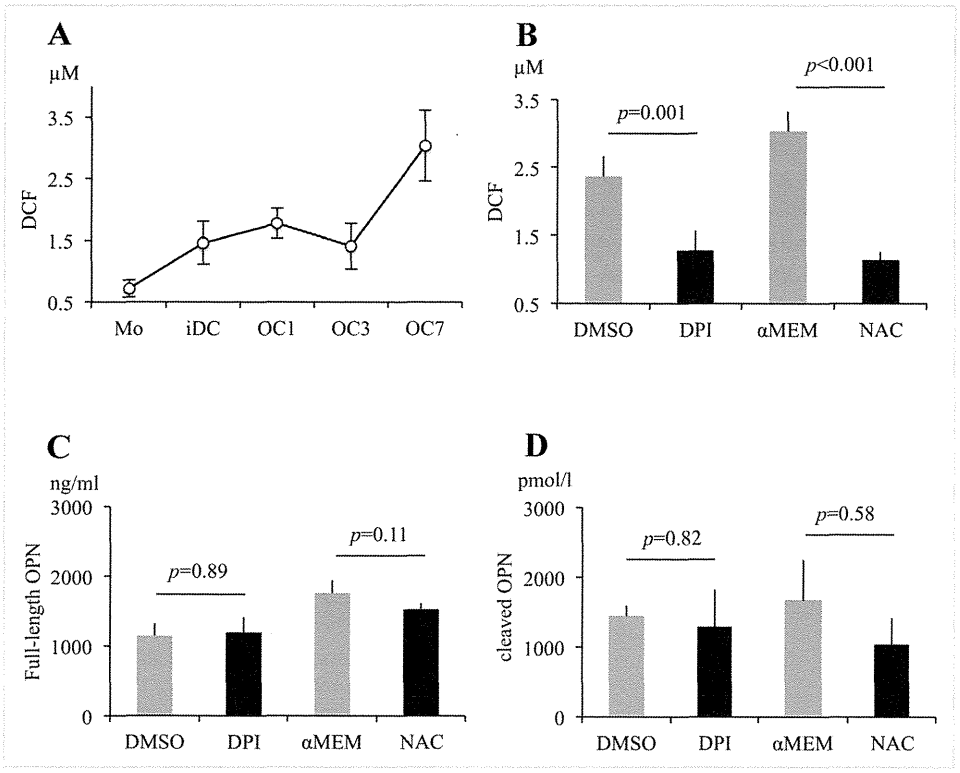

Fig. 6. The relation between ROS activity and OPN production. A: ROS activity during the course of MGC formation. We detected DCF as oxidized product by intracellular ROS. B: The effect of ROS inhibitor at OC7. C: Full-length OPN in the supernatants at OC7 differentiated from DPI- or NAC-treated iDC. D: Cleaved OPN in the supernatants at OC7 differentiated from DPI- or NAC-treated iDC. Data shown are the mean of experiments with cells from four donors. Error bars represent mean ± SEM. DCF, 2′, 7′-dichlorodihydrofluorescein; α-MEM, α-minimum essential medium; DMSO, Dimethyl sulfoxide; DPI, diphenyleneiodonium chloride; NAC, N-acetyl-L-cysteine.

DISCUSSION

In this study, we found that during the course of OC-like MGC formation from iDC, a large amount of OPN (both mRNA and protein) was produced, the cultured cells expressed OPN receptors, and inhibiting OPN expression suppressed OC-like MGC formation. These results indicate that OPN plays an important role in OC-like MGC formation from iDCs.

All cells expressed OPN during the course of OC-like MGC formation from iDCs in vitro. On the other hand, OPN receptors that recognize full-length OPN were expressed on TRAP-positive and CD1a-negative multinucleated cells, while the α9β1 integrin receptor, which recognizes the cleaved form of OPN, was expressed on TRAP-negative and CD1a-positive mononuclear cells. This indicates that full-length OPN stimulates cells that have differentiated into OC-like MGCs, while the cleaved form of OPN stimulates cells that have retained the character of iDCs. MGC formation from iDCs transfected with OPN siRNA was not suppressed, but that from iDCs generated from OPN-transfected monocytes was significantly suppressed. The differentiation of monocytes into iDCs was not itself influenced by OPN suppression. This indicates that OPN is crucial for OC-like MGC formation during the early phase, although OPN levels in OC1 cell culture supernatants were not significantly different between OPN siRNA- and control siRNA-transfected cells. There is a possibility that a critical OPN level for iDC fusion and OC-like MGC formation exists in the early phase of culture. MGC formation was not suppressed by the RGD peptide, which interferes with the interaction of full-length OPN with its receptor, but was significantly suppressed by SVVYGLR peptide, which interferes with the interaction of the cleaved form of OPN with its receptor. These findings suggest that cleaved OPN has a key role in stimulating iDC to differentiate into OC-like MGCs in an autocrine manner.

OPN has been known as a multi-functional secreted phosphoglycoprotein, which is involved not only in bone resorption by OCs but also in the immune defense system and autoimmune disease. Recently, an increasing number of reports describe the association between OPN and the inflammatory bone disease of RA and LCH. In mouse models of collagen-induced arthritis, OPN deficiency prevents development of the disease [Yumoto et al., 2002] and anti-OPN antibody, which blocks the interaction of OPN with its integrins, significantly inhibits disease development [Yamamoto et al., 2007]. Bronchoalveolar lavage cells from patients with pulmonary LCH spontaneously produce abundant amounts of OPN and OPN overexpression in rat lungs induces lesions similar to pulmonary LCH, with marked alveolar and interstitial accumulation of Langerhans cells [Prasse et al., 2009]. Furthermore, OPN is highly overexpressed in T cells and LCH cells of the LCH lesion [Allen et al., 2010].

T cells and antigen-presenting cells, such as DCs and macrophages, secrete OPN causing autocrine or paracrine stimulation that results in the secretion of other pro-inflammatory cytokines. This pro-

inflammatory action is more strongly induced by cleaved than full-length OPN [Uede, 2011]. For example, it was reported that the production by vascular smooth-muscle cells of free radicals related to oxidative stress was greater in response to cleaved OPN than in response to full-length OPN [Lai et al., 2006]. The adhesive ability of the cleaved OPN is also enhanced in comparison to that of full-length OPN [Gao et al., 2004]. Cleaved OPN and its receptors (the $\alpha4\beta1$ and $\alpha9\beta1$ integrins) are involved in the neutrophil infiltration and hepatic injury in inflammatory liver diseases [Diao et al., 2004]. Additionally, the cleaved form of OPN plays a critical role in RA [Morimoto et al., 2010; Uede, 2011], while a role for the cleaved form of OPN in LCH has not been revealed. In this paper, we demonstrate the role of cleaved OPN in the formation of OC-like MGCs from iDCs. Cleaved OPN could therefore plausibly play a role in the pathogenesis of both RA and LCH in which OCs are intimately involved [Redlich et al., 2002; da Costa et al., 2005].

OPN did not affect viability and apoptosis in OC-like MGC formation, suggesting OPN directly acts as a signal mediator for OC-like MGC formation. ROS activity increased during OC-like MGC formation, however, we could not discover any relation between ROS activity and OPN production.

ACKNOWLEDGMENTS

This study was supported by a grant for Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare, Japan and Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This study was carried out under the Joint Research Program of the Institute for Genetic Medicine, Hokkaido University, and with funds from the JKA Foundation raised through its promotion of KEIRIN RACE. We thank Dr. Hiroko Hayakawa, of JMU Core Center of Research Apparatus, for help with flow cytometry.

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