Keratinocyte G2/M Growth Arrest by 1,25-Dihydroxyvitamin D3 Is Caused by Cdc2 Phosphorylation Through Wee1 and Myt1 Regulation

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1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) has an antiproliferative effect on keratinocyte growth, and its derivatives are used for the treatment of psoriasis. It was reported previously that 1,25[OH]$_2$VD3 induced cell cycle arrest not only at the G0/G1 phase but also at the G2/M phase. However, the mechanism of 1,25[OH]$_2$VD3-induced G2/M phase arrest in keratinocytes has not been fully understood. The addition of 10^{-8} to 10^{-6} M 1,25[OH]$_2$VD3 to cultured normal human keratinocytes enhanced the expression of Myt1 mRNA preceding Wee1 mRNA; 10^{-6} M 1,25[OH]$_2$VD3 unregulated Myt1 mRNA from 6 h to 24 h and Wee1 mRNA from 12 to 48 h. Interestingly, the levels of phosphorylated Cdc2 were increased between 6 h and 48 h after 1,25[OH]$_2$VD3 treatment, although the expression levels of Cdc2 mRNA and its protein production were reduced. 1,25[OH]$_2$VD3 also decreased the expression of cyclin B1, which forms a complex with Cdc2. These data indicated that the increase of Myt1 and Wee1 induced the phosphorylation of Cdc2 leading to G2/M arrest. In conclusion, the induction of Cdc2 phosphorylation due to the increase of Wee1 and Myt1 as well as the reduction of Cdc2 and cyclin B1 are involved in 1,25[OH]$_2$VD3-induced G2/M arrest of keratinocytes.

Key words: 1,25-dihydroxyvitamin D3/Keratinocyte/Wee1/Myt1/Cdc2/cell cycle
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The compound 1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3), which is the hormonal form of vitamin D3, regulates cell growth in a variety of cells (Walters, 1992). The skin is one of the target organs for 1,25[OH]$_2$VD3, and the vitamin D receptor (VDR) is present in the cells of both the epidermis and the dermis (Stumpf *et al*, 1979, 1984; Pillai *et al*, 1988; Milde *et al*, 1991). The effects of 1,25[OH]$_2$VD3 on keratinocytes have been investigated extensively, and well characterized *in vitro* and *in vivo*. In the *in vitro* studies, 1,25[OH]$_2$VD3 demonstrated a potent inhibitory effect on proliferation, and promoted the differentiation of both murine and human keratinocytes (Hosomi *et al*, 1983; Smith *et al*, 1986; Matsumoto *et al*, 1990; Kobayashi *et al*, 1993; Gniadecki, 1996; Kobayashi *et al*, 1998; Segaert *et al*, 2000). The critical effects on the epidermis are also manifested *in vivo* (Holick *et al*, 1987; Dubertret *et al*, 1992; el-Azhary *et al*, 1993; Langner *et al*, 1993).

Several reports have demonstrated that 1,25[OH]$_2$VD3 suppresses keratinocyte growth through cell cycle regulation. The most distinguished effect of 1,25[OH]$_2$VD3 on cell cycle regulation is G1 block. G1 block is associated with changes of various kinds of cell cycle regulatory molecules: increased levels of p21 (Segaert *et al*, 1997; Zhuang and Burnstein, 1998; Moffatt *et al*, 2001), p27 (Segaert *et al*, 1997; Wang *et al*, 1997), and transforming growth factor-βGF-β (Segaert *et al*, 1997), decreased Cdk2 activity (Wang *et al*, 1997; Zhuang and Burnstein, 1998), reduced levels of cyclin E (Zhang *et al*, 1996), repressed E2F transactivation (Zhuang and Burnstein, 1998), and retinoblastoma hypophosphorylation (Kobayashi *et al*, 1993; Segaert *et al*, 1997). Interestingly, 1,25[OH]$_2$VD3 induces the accumulation of cells not only in the G1 compartment but also in the G2/M compartment. These features have been noted in HL60 cell cultures (Godyn *et al*, 1994; Zhang *et al*, 1996; Harrison *et al*, 1999), in breast cancer cell lines (Eisman *et al*, 1989a, b), and also in human keratinocytes (Kobayashi *et al*, 1993). In contrast to the numerous studies on the mechanism of G1 block in 1,25[OH]$_2$VD3-treated keratinocytes, the underlying mechanism of G2 block induced by 1,25[OH]$_2$VD3 in keratinocytes has not been reported.

Orderly progression through the cell cycle is mediated by the activation of a highly conserved family of protein kinases, namely, cyclin-dependent kinases (Cdks) (Pines, 1995; Morgan, 1997). Activation of a particular Cdk requires binding to a specific regulatory subunit, which is termed a cyclin. A pivotal regulatory step for G2/M transition in eucaryotes is the activation of the cell division cycle (Cdc)2/cyclin B complex (initially called the maturation or mitosis promoting factor, MPF) (Coleman and Dunphy, 1994; Morgan, 1997). Cdc2/cyclin B is maintained in an inactive

Abbreviations: ATP, adenosine triphosphate; Cdc, cell division cycle; Cdk, cyclin-dependent kinase; 1,25[OH]$_2$VD3, 1,25-dihydroxyvitamin D3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPA, ribonuclease protection assay; RXR, retinoid X receptor; Thr, threonine; Tyr, tyrosine; VDR, vitamin D receptor; VDRE, vitamin D response elements

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form during the S and G2 phases by inhibitory phosphorylation of the Cdc2 residues threonine 14 (Thr14) and tyrosine 15 (Tyr15), which are positioned within the adenosine triphosphate (ATP)-binding cleft (Gould and Nurse, 1989; Krek and Nigg, 1991; Jin *et al*, 1996). The inhibitory phosphorylation of Cdc2 is modulated by the Wee1 family protein kinases, i.e., human Wee1 and Myt1. The Wee1 gene product is a tyrosine-specific protein kinase, which locates in nucleus and phosphorylates Cdc2 exclusively at Tyr15 (McGowan and Russell, 1993, 1995). The Myt1 kinase, which is a homologue of the product of the *mik1* gene, is a dual-specificity protein kinase that phosphorylates Cdc2 at both the Thr14 and Tyr15 residues (Booher *et al*, 1997; Fattaey and Booher, 1997; Gould and Nurse, 1989; Krek and Nigg, 1991; Mueller *et al*, 1995). Myt1 kinase is a membrane-associated protein that localizes to the endoplasmic reticulum and Golgi complex (Liu *et al*, 1997); except for inhibitory phosphorylation of Cdc2, Myt1 seems to influence normal shuttling of the Cdc2/cyclin B complex into the nucleus (Liu *et al*, 1999). Phosphorylation of Tyr15 and Thr14 suppresses catalytic activity of Cdc2 by disrupting the orientation of the ATP molecule to the ATP-binding cleft of Cdc2 (Atherton-Fessler *et al*, 1993; De Bondt *et al*, 1993). Cdc25 family members, which are dual-specificity phosphatases, have been identified as positive regulators of Cdc2. They counteract Wee1/Myt1 activities (Russell and Nurse, 1986; Sebastian *et al*, 1993). Dephosphorylation of the Thr14 and Tyr15 residues of Cdc2 by Cdc25B (in the cytoplasm) and Cdc25C (in the nucleus) in the late G2 phase activates the cyclin B/Cdc2 complex directly, and the accumulated, active Cdc2/cyclin B1 then triggers the initiation of mitosis (Dunphy and Kumagai, 1991; Strausfeld *et al*, 1991; Coleman and Dunphy, 1994; Karlsson *et al*, 1999). Therefore, the active status of the Cdc2/cyclin B complex is controlled by several G2/M-specific cell cycle regulatory kinases and phosphatases that reversibly phosphorylate Cdc2.

To elucidate the mechanism of G2 block induced in human keratinocytes by 1,25[OH]$_2$VD3, we investigated the effects of 1,25[OH]$_2$VD3 on the regulatory molecules of G2/M transit. We demonstrated here that 1,25[OH]$_2$VD3 increased Wee1 and Myt1, and the phosphorylated Cdc2. This provides a potential mechanism for the accumulation of VD3-treated keratinocytes in the G2/M compartment.

Results

cDNA microarray analysis of the effect of 1,25[OH]$_2$VD3 on Wee1, cyclin B1, Cdc25B, and Cdc25C in human keratinocytes We initially studied the expression of cell cycle-related genes in 1,25[OH]$_2$VD3-treated keratinocytes using the cDNA microarray technique. Six hours of stimulation with 1,25[OH]$_2$VD3 modified the expression patterns of four genes that are thought to be important regulators of G2/M transition (Fig 1). The expression of the Wee1 mRNA, whose protein product phosphorylates Cdc2 at Tyr15 and suppresses Cdc2 activity, was induced after 1,25[OH]$_2$VD3 treatment. In contrast, the expression levels of cyclin B1, Cdc25B, and Cdc25C were suppressed to various degrees by 1,25[OH]$_2$VD3. These data suggest the possibility that 1,25[OH]$_2$VD3 induces G2/M cell cycle arrest in keratinocytes by affecting Cdc2/cyclin B1.

Effect of 1,25[OH]$_2$VD3 on Wee1 and Myt1 in human keratinocytes The cDNA microarray analysis showed that 1,25[OH]$_2$VD3 upregulated the expression of the Wee1 gene. To ascertain the effect of 1,25[OH]$_2$VD3 on Wee1, we examined the level of Wee1 mRNA, and Wee1 protein in 1,25[OH]$_2$VD3-treated human keratinocytes. Wee1 mRNA expression was upregulated gradually in a time-dependent manner from 12 h up to 48 h by 10^{-6} M of 1,25[OH]$_2$VD3 (Fig 2*A*). The optimum induction of Wee1 mRNA was 6-fold at 48 h compared with the control (vehicle). Wee1 protein began to increase at 12 h and reached the maximum, 3-fold at 48 h (Fig 2*B*). This time course of Wee1 protein induction is consistent with that of Wee1 mRNA. Furthermore, 1,25[OH]$_2$VD3 increased Wee1 mRNA expression in a concentration-dependent manner, 1.6-fold at 10^{-8} M, 2.4-fold increase at 10^{-7}M, and 4.0-fold 10^{-6} M (Fig 2*C*). Wee1 protein was increased almost similarly by the addition of 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} (Fig 2*D*). These data demonstrate that 1,25[OH]$_2$VD3 increases both Wee1 mRNA and Wee1 protein similarly in human keratinocytes.

Since Myt1 is a Wee1 family kinase that has been shown to phosphorylate Cdc2 both at Tyr15 and Thr14 (Mueller *et al*, 1995; Booher *et al*, 1997; Fattaey and Booher, 1997), we examined the effect of 1,25[OH]$_2$VD3 on Myt1 expression. 1,25[OH]$_2$VD3 enhanced the expression of Myt1 mRNA (Fig 3*A*). But the time course of Myt1 mRNA induction by 1,25[OH]$_2$VD3 was distinguished from that of Wee1 mRNA. Myt1 mRNA increased markedly from 6 to 12 h, and decreased at 24 h. Then, Myt1 mRNA expression returned to the baseline level at 36 h. Myt1 protein increased and decreased in the same time course as Myt1 mRNA (Fig 3*B*). 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M increased Myt1 mRNA, 2.3-, 3-, and 1.5-fold, respectively, compared with the control (vehicle) (Fig 3*C*). The optimum induction of Myt1 protein was also observed at 10^{-7} M of 1,25[OH]$_2$VD3 (Fig 3*D*). Taken together, 1,25[OH]$_2$VD3 induces Myt1 kinase preceding Wee1 kinase in human keratinocytes. Myt1 is localized in the cytoplasm and Wee1 is localized in the nucleus. This difference of localization may affect the time lag of Myt1 and Wee1 induction by 1,25[OH]$_2$VD3 in human keratinocytes.

Effect of 1,25[OH]$_2$VD3 on Cdc2 and phosphorylation of Cdc2 in human keratinocytes Next, we assessed the

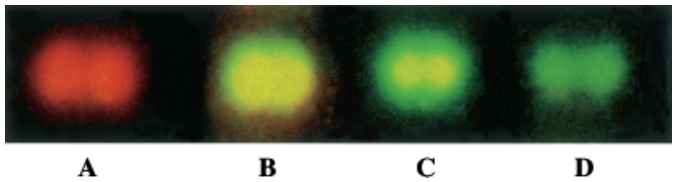

Figure 1
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) regulates the expression of regulators of G2/M transit. Normal human keratinocytes were cultured for 6 h in MCDB medium that contained 1,25[OH]$_2$VD3 (10^{-6} M) or vehicle, and RNA samples from these cells were analyzed using the cDNA microarray. Red, green, and yellow signify increased, decreased, and unchanged gene expression, respectively, in response to 1,25[OH]$_2$VD3. *A*, Wee1; *B*, cyclin B1; *C*, Cdc25B; *D*, Cdc25C.

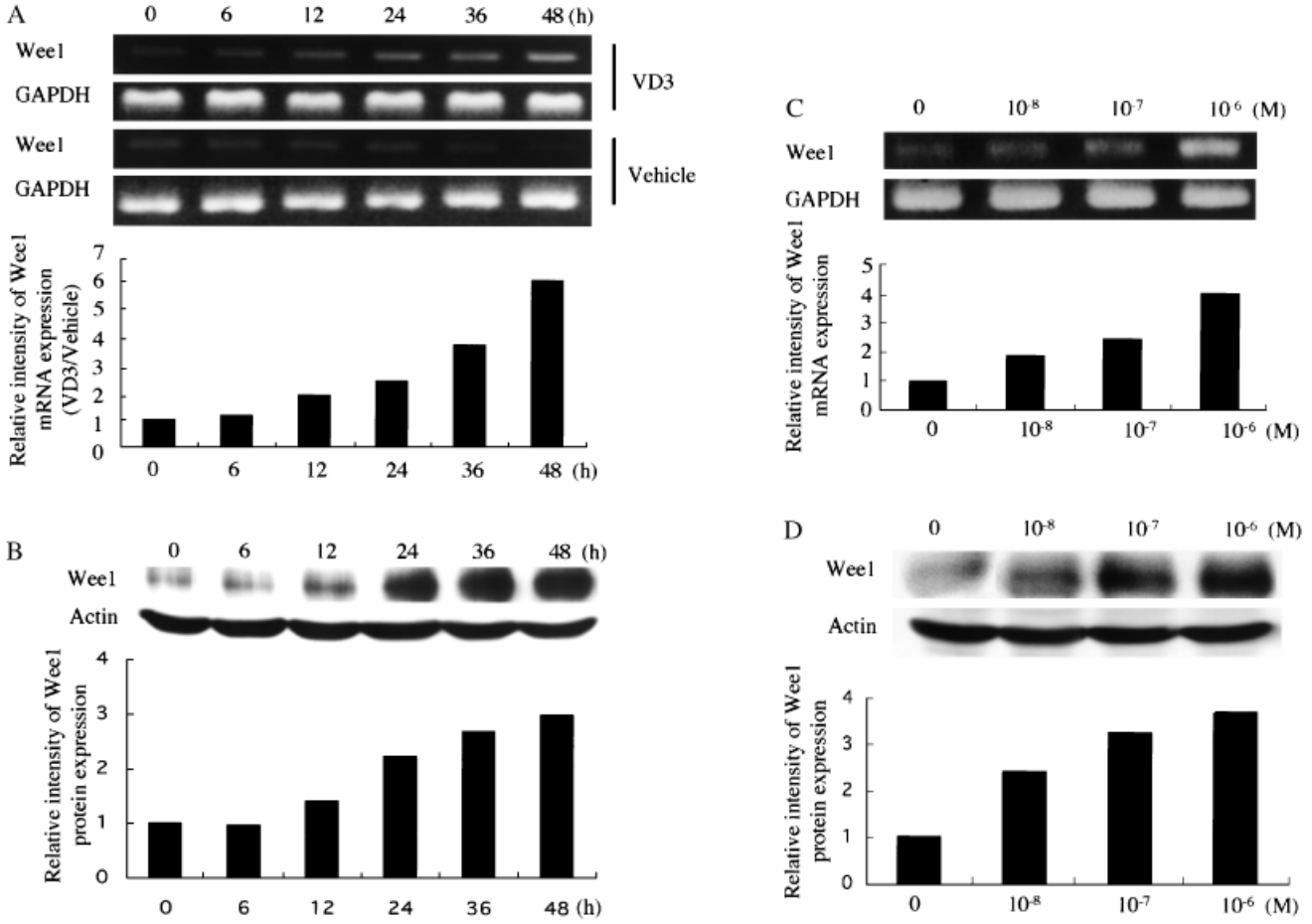

Figure 2
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) increases the expression of Wee1 mRNA and protein. (*A*) Keratinocytes were incubated for the indicated time periods in MCDB medium that contained either 1,25[OH]$_2$VD3 (10^{-6} M) or the vehicle, and total RNA samples were collected. RT-PCR was performed using specific primers for human Wee1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Wee1 mRNA expression was firstly estimated using GAPDH as the internal reference, and then the relative levels of Wee1 mRNA for each time point in 1,25[OH]$_2$VD3-treated cells was estimated by using that of vehicle-treated cells at a corresponding time point. Lastly, the relative Wee1 mRNA expression was normalized against that at 0 h as 1 U, and plotted in the graph. (*B*) Keratinocytes were cultured and stimulated as described in (*A*), and protein was extracted at the indicated time. Western blotting was performed, and the levels of Wee1 protein expression were estimated using actin as the internal reference and normalized against the value of 0 h as 1 U. (*C*) Keratinocytes were cultured in different concentrations of 1,25[OH]$_2$VD3 or vehicle for 24 h. Wee1 mRNA expression was estimated using GAPDH as the internal reference, and normalized against the relative value for the vehicle-treated sample (0 M) as 1 U. (*D*) Keratinocytes were cultured and stimulated as described in (*C*), and protein was extracted after 24 h of stimulation. Western blotting was performed and the levels of Wee1 protein expression were estimated using actin as the internal reference and normalized against the value for the vehicle-treated sample (0 M) as 1 U.

effect of 1,25[OH]$_2$VD3 on Cdc2 and phosphorylated Cdc2, since Wee1 and Myt1 participate in the inhibitory phosphorylation of Cdc2 (McGowan and Russell, 1993; McGowan and Russell, 1995; Mueller *et al*, 1995; Booher *et al*, 1997; Fattaey and Booher, 1997).

The addition of 10^{-6} M 1,25[OH]$_2$VD3 suppressed the expression of the Cdc2 mRNA in a time-dependent manner (Fig 4*A*). Cdc2 mRNA began to decrease at 12 h, and was barely detectable at 36 h. Western blotting demonstrated that 10^{-6} M of 1,25[OH]$_2$VD3 reduced Cdc2 protein at 12 h, and was barely detectable at 36 and 48 h like Cdc2 mRNA expression (Fig 4*B*).

Interestingly, phosphorylated Cdc2 was upregulated by 1,25[OH]$_2$VD3, although the overall level of Cdc2 protein expression was decreased (Fig 4*B*). The level of phosphorylated Cdc2 increased from 6 h and reached a plateau at 24–48 h. Therefore, the relative ratio of phosphorylated Cdc2/total Cdc2 increased in a time-dependent manner, about 10-fold at 48 h. Cdc2 protein was markedly reduced in a concentration-dependent manner by addition of 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M (Fig 4*C*). In contrast, phosphorylated Cdc2 was increased almost equally by 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M (Fig 4*C*).

Taken together, these data indicate that treatment with 1,25[OH]$_2$VD3 suppresses Cdc2 production and inhibits its activity in human keratinocytes. This seems likely to result in G2/M growth arrest of 1,25[OH]$_2$VD3-treated keratinocytes eventually.

Effect of 1,25[OH]$_2$VD3 on cyclin B1 in human keratinocytes The cyclin B1 subunit is essential for Cdc2 activity, and the level of cyclin B1 affects the activity of Cdc2 and progression from the G2 to the M phase. So, we examined the effect of 1,25[OH]$_2$VD3 on cyclin B1 mRNA and protein. The addition of 10^{-6} M 1,25[OH]$_2$VD3 induced the decrease of both cyclin B1 mRNA and cyclin B1 protein in a time-

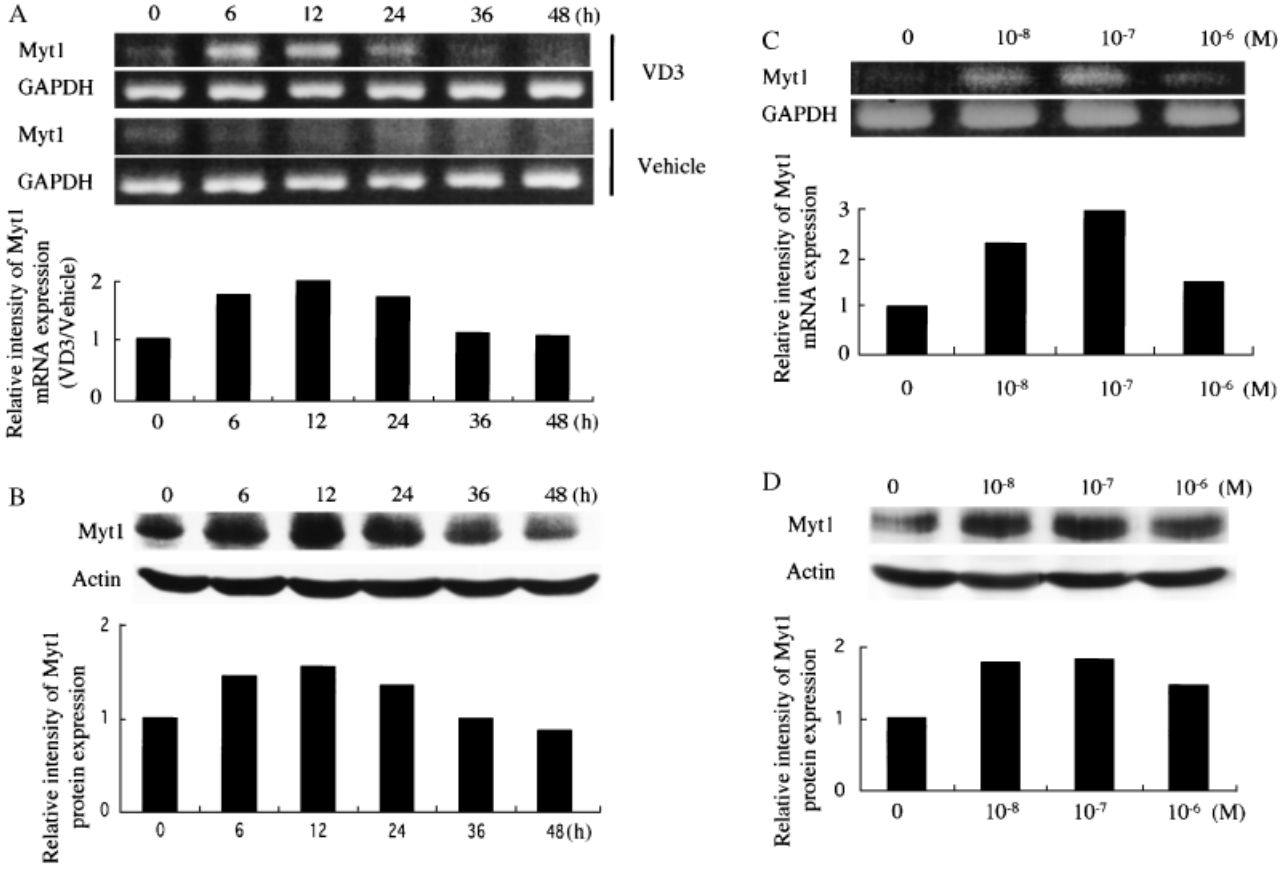

Figure 3
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) increases the expression of Myt1 mRNA and Myt1 protein. (*A*) RT-PCR was performed using specific primers for human Myt1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the relative intensity of Myt1 mRNA expression was estimated as described in Fig 2*A*. (*B*) Western blotting was performed as described in Fig 2*B*, and the levels of Myt1 protein expression were estimated and normalized as described in Fig 2*B*. (*C*) Keratinocytes were cultured in different concentrations of 1,25[OH]$_2$VD3 or vehicle for 12 h. The Myt1 mRNA expression was estimated and normalized as described in Fig 2*C*. (*D*) Keratinocytes were cultured and stimulated as described in (*C*), and proteins were extracted after 12 h of stimulation. Western blotting was performed, and the levels of Myt1 protein expression were estimated and normalized as described in Fig 2*D*.

dependent manner (Figs 5*A*, *B*). In addition, cyclin B1 mRNA and protein were reduced in a concentration-dependent manner by addition of 1,25[OH]$_2$VD3 at 10^{-8}, 10^{-7}, and 10^{-6} M(Figs 5*C*, *D*), although cyclin B1 mRNA decreased more remarkably than cyclin B1 protein. This suppression of cyclin B1 may also contribute to G2/M growth arrest of 1,25[OH]$_2$VD3-treated keratinocytes.

Discussion

We used a cDNA microarray to conduct a systematic analysis of 1,25[OH]$_2$VD3-dependent cell cycle regulation, and found that 1,25[OH]$_2$VD3 strongly induced the Wee1 family in keratinocytes. Accompanying Wee1 and Myt1 induction, 1,25[OH]$_2$VD3 induced inhibitory phosphorylation of Cdc2. This paper reports that Wee1 and Myt1 are target molecules of 1,25[OH]$_2$VD3, and that Wee1 and Myt1 might be key molecules in 1,25[OH]$_2$VD3-induced G2/M arrest in keratinocytes. 1,25[OH]$_2$VD3 is reported to induce G2/M arrest in other cells (Godyn *et al*, 1994; Harrison *et al*, 1999). Wee1 and Myt1 might be involved in the 1,25[OH]$_2$VD3-induced G2/M arrest of these cells.

Wee1 inhibits mitosis (McGowan and Russell, 1995). Despite extensive studies of the functions of the Wee1 family, there are few reports on their mechanisms. Interestingly, we found a time lag between Myt1 (6–12 h post-stimulation) and Wee1 (12–48 h post-stimulation) induction, which resulted in continuous phosphorylation of Cdc2 (6–48 h post-stimulation). Wee1 phosphorylates Cdc2 in the nucleus, whereas Myt1 phosphorylates Cdc2 in the cytoplasm. The significance of this localization and the mechanism producing the time lag between Myt1 and Wee1 induction is unclear. We speculate that Myt1 kinase phosphorylates and inhibits Cdc2 activation and nuclear translocation of the Cdc2/cyclin B complex during the early period of 1,25[OH]$_2$VD3 treatment. Moreover, Cdc2 phosphorylation is retained as high in the late period of 1,25[OH]$_2$VD3 treatment, whereas the level of Wee1 increases steadily. Therefore, 1,25[OH]$_2$VD3 might keep Cdc2 inactive with the sequential increase in the two kinases: Myt1 and Wee1 (Fig 6).

1,25[OH]$_2$VD3 is a physiologically active ligand for VDR. VDR forms stable receptor complexes preferentially as heterodimers with the retinoid X receptor (RXR). The VDR-RXR dimer binds vitamin D response elements (VDRE), which are located in the promoters of 1,25[OH]$_2$VD3-

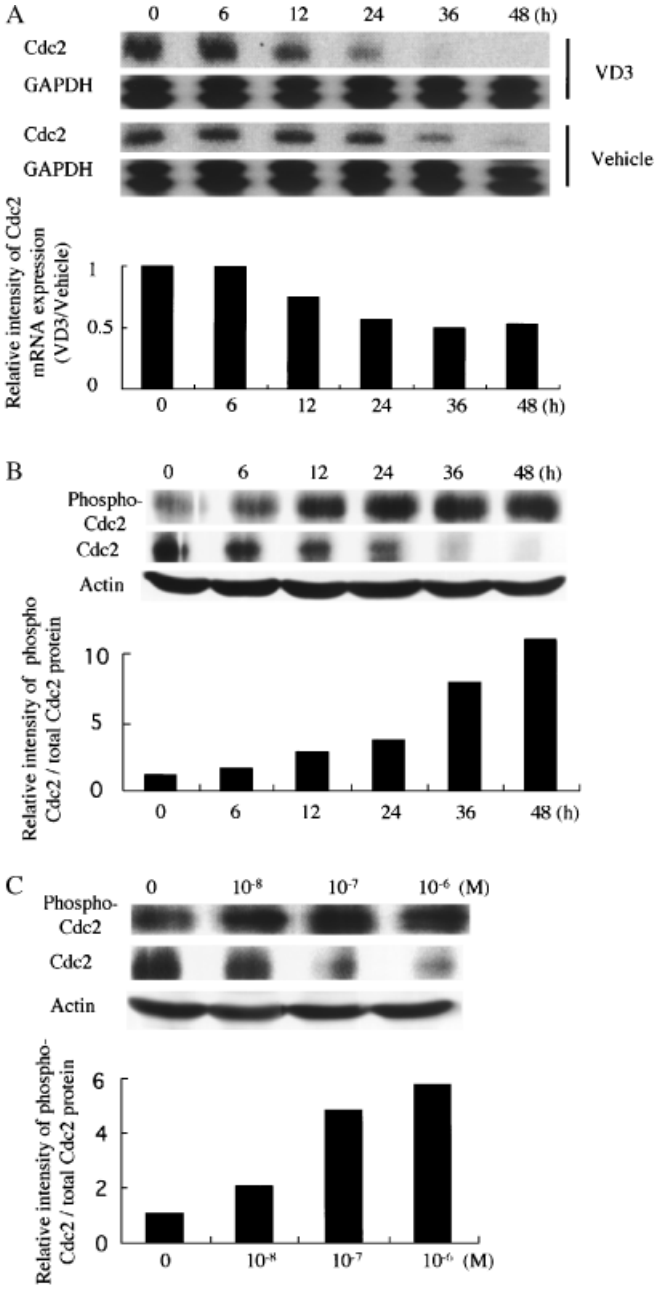

Figure 4
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3) downregulates the expression of Cdc2 mRNA and Cdc2 protein, and increases the level of phospho-Cdc2 protein. (*A*) Total RNA samples were collected as described in Fig 2*A*. Cdc2 mRNA was detected by ribonuclease protection assay, and the relative levels of Cdc2 mRNA expression were estimated as described in Fig 2*A* and plotted on the graph. (*B*) Western blotting was performed as described Fig 2*B*. The levels of Cdc2 and phospho-Cdc2 protein expression were, respectively, estimated against actin, the relative level of phospho-Cdc2 at each point was estimated using total Cdc2 expression as the reference, and then normalized against the relative value at 0 h as 1 U. (*C*) Keratinocytes were cultured and stimulated as described Fig 2*C*, and protein was extracted after 24 h of stimulation. Western blotting was performed, and Cdc2 and phospho-Cdc2 protein level at each point was estimated using actin as an internal reference. The relative levels of phospho-Cdc2 against total Cdc2 protein at each time point were normalized against that relative value at 0 M as 1 U.

regulated genes. The Wee1 promoter does not contain a confirmed VDRE, so the VDR-VDRE pathway might not regulate Wee1 directly. A recent study found one activator protein 1 (AP-1)-binding motif in the Wee1 promoter region, and c-Fos transactivates the Wee1 kinase gene directly (Kawasaki *et al*, 2001). In addition, prolonged c-Fos expression elicits abnormally increased expression of the Wee1 gene, which induces and maintains inactive phosphorylation of Cdc2 kinase. Another study reported that 1,25(OH)$_2$VD3 stimulates AP-1 DNA-binding activity in keratinocytes (Johansen *et al*, 2003), and we confirmed that 1,25(OH)$_2$VD3 increased c-Fos mRNA expression and AP-1 transcription beginning 6 h after stimulation in a time-dependent manner (unpublished data). The increased c-Fos and activated AP-1 transcription seem to be dependent on the binding of 1,25[OH]$_2$VD3 to its surface membrane receptor, annexin II (Johansen *et al*, 2003). Combined with these data, this AP-1 activation induced by 1,25(OH)$_2$VD3 might contribute to 1,25(OH)$_2$VD3-dependent Wee1 expression and inactive phosphorylation of Cdc2 kinase, and this signal pathway might be independent of VDR.

Previous reports have indicated that Cdc2 phosphorylation is not the only mechanism that regulates G2 arrest, but that Cdc2 and cyclin B1 levels are also related to the G2/M transition (Kao *et al*, 1997; McVean *et al*, 2002). We found that 1,25[OH]$_2$VD3 decreased Cdc2 and cyclin B1 expression in human keratinocytes. Combined with the fact that 1,25[OH]$_2$VD3 increases inhibitory phosphorylation of Cdc2, 1,25[OH]$_2$VD3 suppresses Cdc2 activation efficiently, since almost all the Cdc2 is inhibited by phosphorylation by 1,25[OH]$_2$VD3, as shown in Figs 4*B*, *C*. Cdc2 expression appears to be regulated at the mRNA level, and to begin 12 h after treatment. We postulate that 1,25[OH]$_2$VD3 suppresses Cdc2 transcription. The basal promoter region of human Cdc2 contains the E2F, E-box, and Sp1 motifs. Of these, the E2F motif is important for activating the Cdc2 promoter (Shimizu *et al*, 1995). In the pRb/E2F pathway, pRb is phosphorylated (inactive) and hypophosphorylated (active) during the cell cycle, and hypophosphorylated pRb appears to repress gene transcription via its interaction with E2F (Harbour and Dean, 2000). 1,25(OH)$_2$VD3 induces hypophosphorylation of pRb in keratinocytes within 6 h of treatment (Kobayashi *et al*, 1993). Therefore, 1,25(OH)$_2$VD3 might suppress Cdc2 transcription via pRb activation and consequent E2F silencing in keratinocytes. In fact, 1,25(OH)$_2$VD3 blocks the transcription of E2F-regulated genes, such as cyclins A and E, via the G1 CDK-pRb-E2F pathway in MCF-7 cells (Jensen *et al*, 2001). Treatment of keratinocytes with TGF-β1 also results in the formation of a DNA-binding complex between pRb and E2F, which contributes to suppressing the E2F-regulated Cdc2 gene and inhibiting cell cycle progression (Herzinger *et al*, 1995). Cyclin B1 and Cdc25 are also E2F-target genes. This suggests an interesting link in 1,25(OH)$_2$VD3-treated keratinocytes between the G1 CDk-pRb-E2F pathway and the mitotic kinase Cdc2. Since the CDk inhibitor p21 is a 1,25(OH)$_2$VD3-regulated gene and 1,25(OH)$_2$VD3 increases p21 expression in a VDRE-dependent manner (Liu *et al*, 1996), 1,25(OH)$_2$VD3 probably decreases Cdc2 expression via a VDR-dependent pathway. c-Myc deregulation by 1,25(OH)$_2$VD3 in keratinocytes (Matsumoto *et al*, 1990)

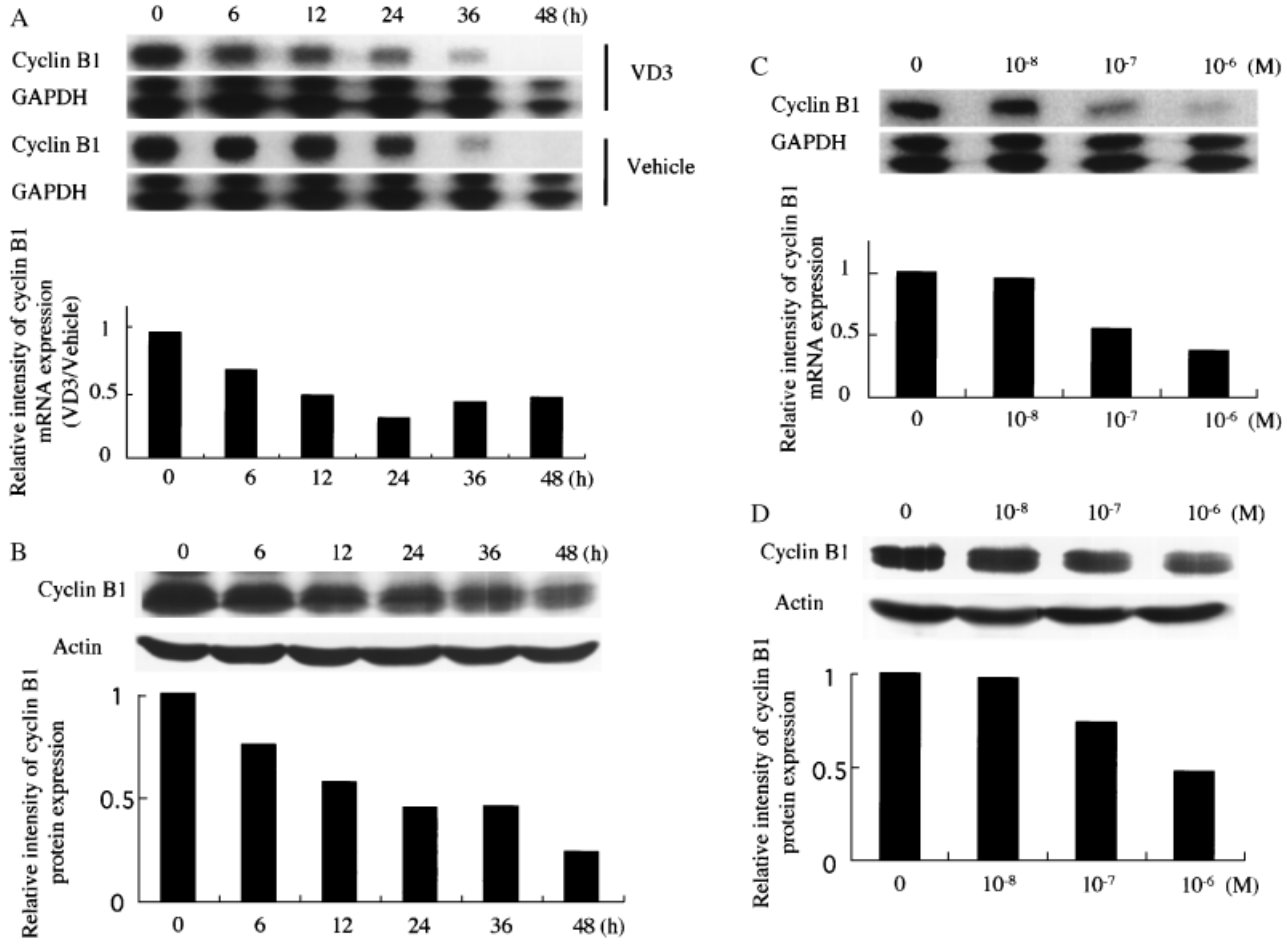

Figure 5
1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3 VD3) downregulates the expression of cyclin B1 mRNA and protein. (*A*) Ribonuclease protection assay was performed as described in Fig 4*A*. The relative levels of cyclin B1 mRNA expression were estimated and normalized as described in Fig 2*A*. (*B*) Western blotting was performed as described in Fig 2*B*, and the levels of cyclin B1 protein expression were estimated and normalized as described in Fig 2*B*. (*C*) Keratinocytes were cultured and stimulated as described in Fig 2*C*. Cyclin B1 mRNA expression was estimated and normalized as described in Fig 2*C*. (*D*) Keratinocytes were cultured and stimulated as described in Fig 2*B*, and protein was extracted after 24 h of stimulation. Western blotting was performed, and the levels of cyclin B1 protein expression were estimated and normalized as described in Fig 2*D*.

could also contribute partially to the decreased Cdc2 and cyclin B1 expression. c-Myc activates the transcription of Cdc2 and cyclin B1 by occupying the E-box (North *et al*, 1999; Menssen and Hermeking, 2002). Activation of JNK and p38 reduces cyclin B1 mRNA expression (Garner *et al*, 2002) and 1,25(OH)$_2$VD3 increases the activities of JNK and SAPK kinase in normal keratinocytes (our unpublished data). The reductions in Cdc2 and cyclin B1 expression might contribute to the G2 arrest of human keratinocytes via two pathways: (1) a reduction in the amount of Cdc2/cyclin B1 complex and (2) a decrease in cyclin B1-dependent Cdc2 activation.

In this report, we presented data obtained from samples treated with a dose of 1,25(OH)$_2$VD3 (10^{-6} M), which is slightly higher than the dose of vitamin D3 ointment that is used to treat psoriasis (25 $\sim$ 50 μg per g or about 10^{-7} M). Note that 10^{-7} M 1,25(OH)$_2$VD3 elicited a remarkable effect in our work in concentration-dependent experiments. We also studied the temporal effect of 10^{-7} M 1,25(OH)$_2$VD3 and found that its influence was apparent, but moderate, compared with that of 10^{-6} M (data not shown). We present the data for 10^{-6} M 1,25(OH)$_2$VD3 because Kobayashi *et al*, (1993) reported that G2/M accumulation was obvious in 10^{-6} M 1,25(OH)$_2$VD3-treated keratinocytes and was induced weakly by 10^{-7} M 1,25(OH)$_2$VD3. Therefore, these changes might occur under physiological conditions and might contribute to the improvement of psoriatic skin treated with vitamin D3 analogue ointments.

The 1,25(OH)$_2$VD3 signaling pathways, via either VDR or non-genomic signaling via a possible 1,25(OH)$_2$VD3 membrane receptor, are poorly understood. Our results suggest that 1,25[OH]$_2$VD3 induces G2/M arrest in normal human keratinocytes by increasing the levels of Wee1 and Myt1, which results in the inhibitory phosphorylation of Cdc2 (Fig 6). Furthermore, the decreased levels of Cdc2 and cyclin B1 contribute to G2/M arrest. This is the first report of the interaction of 1,25[OH]$_2$VD3, Wee1, and Myt1, and this paper presents new molecular mechanisms for 1,25[OH]$_2$VD3-induced biological function.

Materials and Methods

Cell culture Normal human keratinocytes were cultured with MCDB153 medium supplemented with insulin (5 μg per mL),

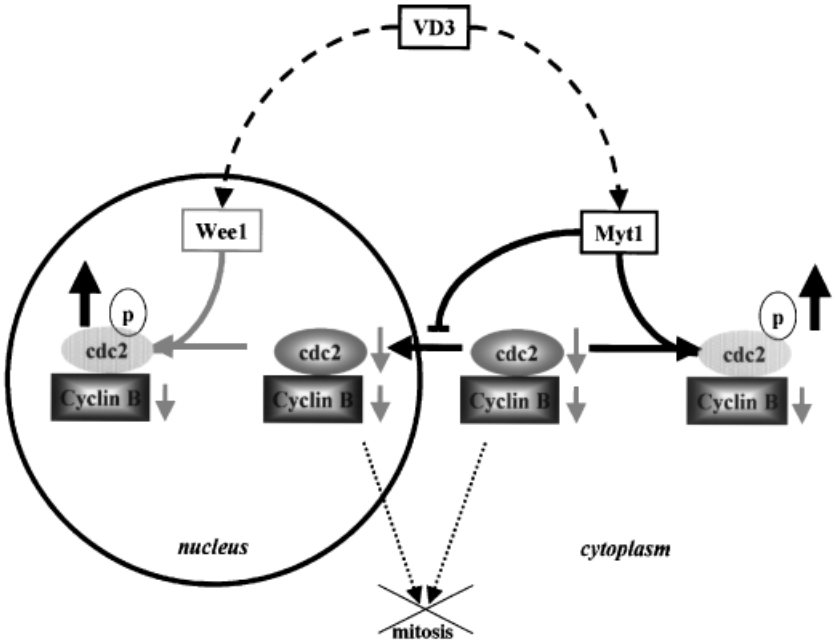

Figure 6
Schema of 1,25-dihydroxyvitamin D3 (1,25[OH]$_2$VD3)-induced G2/M arrest of keratinocytes. This summarizes the possible mechanisms of 1,25[OH]$_2$VD3-induced G2/M arrest of keratinocytes. 1,25[OH]$_2$VD3 induces Wee1 and Myt1 in keratinocytes, and these kinases phosphorylate Cdc2 and inactivate the Cdc2 protein in keratinocytes, resulting in G2/M arrest. The suppressed Cdc2 and cyclinB expression by 1,25[OH]$_2$VD3 may also contribute to G2/M blockade.

hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μg per mL), and Ca^{2+} (0.03 mM) as previously described (Yamasaki *et al*, 2003a). Second or third passage cells were used in all of the experiments. All the procedures that involved human subjects received prior approval from the Ethical Committee of Ehime University School of Medicine, and all subjects provided written informed consent.

Reagents 1,25[OH]$_2$VD3 was a generous gift from Teijin Pharmaceutical Co. Ltd (Tokyo, Japan). The rabbit anti-human-Wee1, goat anti-human-Myt1, and goat anti-human-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Mouse anti-human-cyclin B1 was obtained from PharMingen (San Diego, California). The rabbit anti-human-Cdc2 and rabbit anti-human-phospho-Cdc2 (Tyr15) antibodies were obtained from Cell Signaling Technology (Beverly, Massachusetts).

1,25[OH$_2$VD3 treatment of human keratinocytes Subconfluent human keratinocyte cultures were incubated with various concentrations (10^{-8}, 10^{-7}, and 10^{-6} M) of 1,25[OH]$_2$VD3, or vehicle (ethanol) alone. After 24 h, the cells were harvested and total RNA and protein were extracted. For the time-course experiment, we stimulated cells with 10^{-6} M 1,25[OH]$_2$VD3 and extracted total RNA and protein at 0, 6, 12, 24, 36, and 48 h.

cDNA microarray analysis The cDNA array analysis was performed with the Atlas cDNA expression array system, which includes human cell cycle-related genes (Clontech Laboratories Inc., Palo Alto, California), according to the manufacturer's instructions. Briefly, keratinocytes were treated with 10^{-6} M 1,25[OH]$_2$VD3 or vehicle for 6 h, and total RNA was isolated with the Atlas Pure System (Clontech). One microgram of total RNA was labeled with the cDNA Synthesis Primer Mix and [α-^{32}P]dATP. Each radioactively labeled probe mix was then hybridized with a separate array membrane. After a high-stringency wash, the hybridization pattern was analyzed by autoradiography. The relative mRNA expression levels of stimulated keratinocytes were assessed by comparison with those of the control cells. Red indicated a signal that was enhanced by 1,25[OH]$_2$VD3, and green indicated a signal that was suppressed by 1,25[OH]$_2$VD3. Yellow indicated unchanged expression of mRNA.

RT-PCR analysis Total RNA from cultured human keratinocytes was prepared using Isogen (Nippon Gene, Toyama, Japan), and treated with 50 U per mL of DNase I (Clontech) at 37°C for 30 min to remove any genomic DNA contamination. The following specific primers were used for the PCR: human Wee1, 5′-GGACAGTGTCGTCGTAGAAAG-3′ and 5′-GGCAGCATTTGGGATTGAGGT-3′; human Myt1, 5′-AAGCTGGGTGACTTCGGACT-3′ and 5′-ACAGAACGCAGCTCGGAAGA-3′; and human GAPDH (glyceraldehyde 3-phosphate dehydrogenase), 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. The RT-PCR was performed using RT-PCR High Plus (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was added to a 50 μL reaction mixture that contained 10 μL of 5 × reaction buffer, 6 μL of 2.5 mM dNTPs, 5 μL of 25 mM $Mn(OAc)_2$, 19 μL of RNase-free H_2O, 2 μL of 10 U per μL RNase inhibitor, 2 μL of 2.5 U per μL of rTth DNA polymerase, and 2 μL of 10 pmol per μL of each primer. The cDNA was reverse transcribed from total RNA for 30 min at 60°C and heated to 94°C for 2 min. Amplification was performed using the DNA Thermal Cycler (Astec, Fukuoka, Japan) for 23–25 cycles. A cycle profile consisted of 1 min at 94°C for denaturation, and 1.5 min at 53°C–60°C for annealing and primer extension. A 5 μL sample of the reaction mixture was electrophoresed on a 2.0% agarose gel that contained ethidium bromide. The PCR products were also sequenced to confirm proper amplification. We performed at least three independent studies and confirmed similar results. One representative experiment is shown in the figures. The intensity of each band was quantified using the NIH *Image* software. The relative levels of Wee1 and Myt1 mRNA expression were estimated using GAPDH mRNA expression of each time point as the internal reference, and normalized against the respective relative level of the control (the point at 0 h was considered as a control for the time course, whereas the point at 0 M of 1,25[OH]$_2$VD3 was considered as a control for the concentration-dependent experiments) as 1 U.

Ribonuclease protection assay (RPA) Single-stranded antisense riboprobes were prepared by *in vitro* transcription of human cDNA fragments using the RiboQuant In Vitro Transcription Kit (Pharmingen) in the presence of [α-^{32}P]UTP. The hCC-1 and hCYC-1 probe sets (Pharmingen) were used as the templates for *in vitro* transcription. Samples of total RNA (10 μg each) were hybridized with the ^{32}P-labeled riboprobe and digested with RNase using the RiboQuant RPA Kit (Pharmingen) according to the manufacturer's

instructions. The hybridization products were separated on a gel and exposed to a film as previously described (Yamasaki *et al*, 2003b). We performed at least three independent studies and confirmed similar results. One representative experiment is shown in figures. Band density was analyzed using the NIH *Image* program. GAPDH bands are doublet, which may be due to that the end of GAPDH mRNA is highly susceptible to the RNase digestion even though it is double stranded. So, the sum of the two bands' intensity was considered as GAPDH mRNA expression. The relative levels of Cdc2 and cyclin B1 mRNA were estimated using GAPDH as the internal reference, and normalized against the respective relative values of the control signal as 1 U. The relative values of Cdc2 and cyclin B1 mRNA were plotted on graphs.

Western blot analysis The cells were harvested by scraping in extraction buffer that contained 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), and protease inhibitors. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes. The analysis was performed using the Vistra ECF kit (Amersham Biosciences K.K., Tokyo, Japan) and FluoroImager (Molecular Dynamics Inc., Sunnyvale, California) as previously described (Yamasaki *et al*, 2003b). We performed at least three independent studies and confirmed similar results. One representative experiment is shown in figures. The intensity of each band was quantified with ImageQuant (Molecular Dynamics Inc.), the relative protein expressions were estimated using actin as the internal reference, and normalized to the control as 1 U. The relative values of proteins are plotted on the graphs.

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