Pituitary tumor transforming gene 1 enhances proliferation and suppresses early differentiation of keratinocytes

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Running title: Suppression of keratinocyte differentiation by PTTG1

Abbreviations: PTTG1, pituitary tumor transforming gene 1.

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Abstract

The epidermis is a self-renewing tissue homeostasis of which is dependent upon the tight balance between proliferation and differentiation based on appropriate regulation of the cell cycle. The cell cycle regulation is dependent on the interactions among a number of cell cycle regulatory molecules, including the pituitary tumor transforming gene 1 (PTTG1), also known as securin, a regulator of sister chromatid separation and transition from metaphase to anaphase. The present study was performed to clarify the little-known functions of PTTG1 in the epidermis by use of keratinocytes cultured under 2D or 3D conditions. Forced overexpression of PTTG1 caused upregulation of cyclin B1, CDK1, and c-Myc, resulting in enhanced proliferation and suppression of early differentiation without apparent alterations in terminal differentiation, and the exogenous PTTG1 was downregulated in association with cell cycle exit. In contrast, depletion of PTTG1 caused their downregulation and constrained proliferation with retention of differentiation capacity. These findings suggested that PTTG1 can alter the proliferation status by modulating the expression levels of the other cell cycle regulatory proteins, and excess PTTG1 primarily affects early differentiation of keratinocytes under the stability regulation associated with cell cycle exit.

Introduction

Proper regulation of cell proliferation and differentiation is critical for the maintenance of self-renewing tissues, such as the epidermis, a stratified epithelium consisting of keratinocytes. Within the epidermis, proliferation is principally confined to the basal layer and differentiation occurs as keratinocytes migrate through the suprabasal layers after growth arrest, namely, cell cycle exit (Fuchs and Horsley, 2008; Gandarillas *et al.*, 2000). When the balance leans toward proliferation and differentiation is suppressed, epidermal proliferative disorders occur, such as squamous cell carcinoma and psoriasis. This relationship relies on the interactions among the numerous cell cycle regulatory molecules, such as cyclins, cyclin-dependent kinases (CDK), CDK inhibitors, and transcription factors such as p53 (Di Cunto *et al.*, 1998; Muller, 1995), which are known to alter keratinocyte proliferation and differentiation under several experimental settings (Di Cunto *et al.*, 1998; Hauser *et al.*, 2004; Truong *et al.*, 2006; Watt *et al.*, 2008; Wong *et al.*, 2010).

Pituitary tumor transforming gene 1 (PTTG1), initially identified as a protooncogene (Pei and Melmed, 1997) and later characterized as vertebrate securin (Zou *et al.*, 1999), is expressed abundantly in multiple cancer cell types and its transforming activity has been demonstrated experimentally (Panguluri *et al.*, 2008; Tong and Eigler, 2009).

PTTG1 possesses transcriptional activity in its C-terminal region and has several validated transcriptional targets, such as c-myc and basic fibroblast growth factor (bFGF). As a cell cycle regulatory protein, PTTG1 plays a role in the regulation of sister chromatid separation and transition from metaphase to anaphase, it functions as securin, and shows interactions with other cell cycle regulatory molecules, such as CDK1 (Ramos-Morales *et al.*, 2000), CDK inhibitor $p21^{WAF1/Cip1}$ (Chesnokova *et al.*, 2007), and p53 (Yu *et al.*, 2000a). In normal tissue, PTTG1 expression is restricted and highly expressed in the testis in a stage-specific manner during spermatogenesis, suggesting that PTTG1 may play a role in male germ cell differentiation (Ramos-Morales, 2000; Pei, 1998). These observations indicate that PTTG1 has important functions in proliferation and differentiation. However, its physiological function in the epidermis remains to be elucidated.

The present study was performed to investigate the role of PTTG1 in keratinocyte proliferation and differentiation using HaCaT cells, normal human keratinocytes (NHK), and neonatal murine keratinocytes (NMK) cultured under 2D or 3D conditions. PTTG1 overexpression enhanced proliferation and upregulated the levels of cyclin B1, CDK1, and c-Myc expression at both protein and mRNA levels. Excess PTTG1 protein was downregulated in association with cell cycle exit and suppressed early differentiation

without significant alterations in terminal differentiation. These findings suggested that PTTG1 may maintain the proliferative phenotype in keratinocytes by delaying cell cycle exit.

Results

PTTG1 expression in normal keratinocytes is under cell cycle-dependent regulation

The *in vivo* expression of PTTG1 in epidermal keratinocytes was confirmed by immunostaining of normal human skin with an antibody against PTTG1 protein. PTTG1 was shown to be expressed in the basal and peribasal keratinocytes, and was localized in the nucleus and cytoplasm (Fig. 1A). Some PTTG1-positive keratinocytes were not located in the basal layer but in the peribasal layer, indicating that some peribasal keratinocytes still possessed proliferative capacity and had not completed cell cycle exit, as reported previously (Bata-Csorgo *et al.*, 1993; Dover and Watt, 1987; Penneys *et al.*, 1970; Pinkus and Hunter, 1966; Regnier *et al.*, 1986; Zanet *et al.*, 2010). Next, NMK were cultured in low- or high-calcium-containing medium. NMK cultured under high-calcium conditions undergo a phenomenon known as calcium switch, which involves cell cycle exit followed by terminal differentiation (Rothnagel *et al.*, 1993).

The results of immunoblotting and quantitative RT-PCR indicated significantly elevated PTTG1 expression in undifferentiated keratinocytes cultured under low-calcium conditions (0.05 mM calcium) at both the mRNA and protein levels in comparison to those cultured under high-calcium conditions (0.12 mM calcium) (Fig. 1B and C). Therefore, these findings indicated that PTTG1 is expressed at high levels during the cell cycle, and is downregulated after cell cycle exit.

PTTG1 alters the thickness of epithelia formed by human keratinocytes cultured under 3D conditions

HaCaT cells and NHK were cultured under 3D conditions as described in *Materials and methods* to allow experimental investigation of the effects of PTTG1 expression in epidermal keratinocytes. In this experimental model, the thickness of epithelium-like structures formed by stratified keratinocytes can demonstrate the proliferation capacity. They were transduced with a retroviral vector encoding rat PTTG1 and nerve growth factor receptor (NGFR) to establish cells with constitutive PTTG1 expression. The vector containing only the NGFR sequence was used as a control. Exogenous PTTG1 expression was monitored at the protein level (Fig. 6-A). HaCaT cells and NHK were depleted of endogenous PTTG1 using a lentiviral vector encoding a short-hairpin RNA

(shRNA) against PTTG1, and a vector encoding scrambled shRNA sequence was used as a negative control. Sufficient knockdown was confirmed at the protein level (Fig. 6-A). Constitutive PTTG1 expression resulted in the formation of thicker epidermoid epithelia than the control, whereas PTTG1 depletion resulted in the formation of thinner epithelia than the negative control (Fig. 2). These results suggested that PTTG1 altered the thickness of the epidermoid epithelia formed by human keratinocytes because of its crucial influence on keratinocyte proliferation.

Overexpression of PTTG1 suppressed early differentiation of human keratinocytes cultured under 3D conditions

Keratinocytes cultured under 3D conditions can mimic their differentiation pattern under physiological conditions (Bell *et al.*, 1981). To analyze the effects of PTTG1 on the differentiation, immunostaining of 3D cultured HaCaT cells (A) and NHK (B) was performed using antibodies against early differentiation markers of keratinocytes, K1 and K10, along with K14 and PTTG1. In addition, NHK were stained with an antibody against the terminal differentiation marker loricrin. Constitutive PTTG1 overexpression in HaCaT cells caused decreases in thickness of K1- and K10-positive layers and increases in thicknesses of undifferentiated layers (Fig. 3-A), whereas NHK showed an

increase in the undifferentiated layers although substantial changes in the stained layers correlated with the total thickness change (Fig. 3-B). There was no apparent change in staining pattern for loricrin (Fig. 3-B). On the other hand, PTTG1 depletion had no significant effect on the staining patterns of any of the differentiation markers despite the changes in the total layer thickness in both HaCaT cells and NHK (Fig. 3). These results suggested that excess PTTG1 suppresses early differentiation specifically, but the absence of PTTG1 had no significant effect on the differentiation of human keratinocytes.

Overexpression of PTTG1 suppressed early keratinocyte differentiation under 2D conditions

The results of the study using HaCaT cells and NHK suggested that PTTG1 may suppress early keratinocyte differentiation. To demonstrate its role in keratinocyte differentiation under another experimental setting, an expression plasmid encoding the rat PTTG1 was transfected into NMK. The cells were induced to undergo differentiation by calcium switch, allowing us to observe their two independent biological conditions separately: during the cell cycle or after cell cycle exit. Differentiating keratinocytes cultured under high-calcium conditions showed marked decreases in K1 and K10

expression at both the mRNA and protein levels (Fig. 4). These observations suggested that PTTG1 also suppressed early differentiation of normal keratinocytes under 2D conditions.

PTTG1 altered keratinocyte proliferation patterns under both 2D and 3D conditions

Proliferation and differentiation processes are generally independent of each other. To verify whether the suppressed keratinocyte differentiation described above was due to enhanced proliferation, we examined the effects of the change in PTTG1 expression level on keratinocyte proliferation pattern. First, we examined the proliferative capacity by counting the proliferation rate of HaCaT cells. PTTG1 overexpression resulted in increased proliferation rate, whereas depletion of PTTG1 resulted in a decrease in proliferation rate (Fig. 5-A). Next, immunostaining using an antibody against Ki67, a classic proliferation marker, was performed to confirm the proliferative status under 3D conditions. HaCaT cells and NHK overexpressing PTTG1 showed increased positivity for Ki67 (Fig. 5-B and C), whereas PTTG1-depleted keratinocytes showed decreased positivity (Fig. 5B and C). These results suggested that PTTG1 plays a critical role in maintaining proliferative capacity in HaCaT cells and NHK.

PTTG1-induced hyperproliferation is due to the upregulation of Cyclin B1, CDK1, and c-Myc oncoprotein

As enhanced proliferation is related to faster progression of the cell cycle, we assayed the expression of endogenous cell cycle regulatory proteins by immunoblotting. HaCaT cells, NHK, and NMK overexpressing PTTG1 showed upregulation of cyclin B1, CDK1, and c-Myc (Fig. 6-A and -B). Cyclin B1, which is expressed in G2/M phase of the cell cycle, forms a complex also known as mitosis-promoting factor (MPF) with the master mitotic kinase CDK1 and promotes cell cycle progression. These upregulated cell cycle regulatory proteins may contribute to the more rapid cell cycle progression, resulting in enhancement of proliferation and delayed cell cycle exit, suppressing early differentiation. Moreover, the results of quantitative RT-PCR suggested that this upregulation was at the mRNA level (Fig. 6-C). In contrast, immunoblotting of PTTG1-depleted HaCaT cells and NHK revealed downregulation of these cell cycle regulatory proteins (Fig. 6). In addition, NHK overexpressing PTTG1 caused significant upregulation of Cyclin A, which is expressed in S/G2 phase of the cell cycle, and in contrast, PTTG1 depletion caused its downregulation (Fig. 6-A). However, there was no such change in the expression level of Cyclin D1, which is highly expressed in G1

phase of the cell cycle (Fig. 6-A). These observations suggested that PTTG1 may modulate the expression levels of these endogenous cell cycle regulatory proteins that are abundant in G2/M phase of the cell cycle. Notably, NMK overexpressing PTTG1 cultured under high-calcium conditions showed markedly decreased expression of PTTG1 protein along with Cyclin B1 and CDK1, despite its constitutive expression as confirmed by the mRNA level (Fig. 6-C), indicating the stability regulation of the exogenous PTTG1 protein in association with cell cycle exit along with these upregulated proteins.

Discussion

In the epidermis, PTTG1 expression is confined to the proliferative layers, and undifferentiated NMK specifically showed PTTG1 mRNA and protein expression. These results indicated the cell cycle-dependent regulation of PTTG1 expression, as reported previously (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996; Ramos-Morales *et al.*, 2000; Yu *et al.*, 2000b; Zou *et al.*, 1999). In addition, we showed that constitutive PTTG1 overexpression caused upregulation of cyclin B1 and CDK1, which are known to form a complex also referred to as MPF. In contrast, PTTG1 depletion resulted in their downregulation, affecting cell cycle progression.

In the eukaryotic cell cycle, the tightly regulated expression and degradation of cell cycle regulatory proteins are indispensable, and degradation of these molecules is dependent on the ubiquitin – proteasome pathway (Ciechanover, 1998). In the G2/M phase of the cell cycle, securin (PTTG1) and cyclin B1 accumulate at the metaphase stage of mitosis followed by their rapid degradation, which promotes exit from mitosis (Clute and Pines, 1999; Hagting *et al.*, 2002; Herbert *et al.*, 2003; Peters, 2002; Zur and Brandeis, 2001). During this process, anaphase-promoting complex (APC), a ubiquitin ligase, mediates proteolysis by recognizing polyubiquitinization targets that will be subjected to degradation *via* the proteasome, leading to the periodic expression of these cell cycle regulatory proteins (King *et al.*, 1995). A growing body of evidence suggests that PTTG1 expedites M phase progression *via* stabilization of cyclin B with its inhibitory effect on the APC activity, as demonstrated by experiments using murine oocytes (Marangos and Carroll, 2008) or budding yeast (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). Thus, the results of the present study may illustrate the universal feature of PTTG1 protein regarding M phase regulation beyond the cell division patterns or biological classification.

We also showed significant changes in the expression level of Cyclin A, which is predominantly expressed in S/G2 phase of the cell cycle with no such change in Cyclin

D1, a G1 phase cyclin, using NHK. These observations indicated that with regard to the effect on cell cycle progression, PTTG1 protein may have a greater influence on G2/M phase where it is abundantly expressed under physiological conditions (Ramos-Morales *et al.*, 2000). A study using securin knockout cells, which showed elongation of the metaphase to anaphase interval in M phase without significant changes in other phases of the cell cycle (Jallepalli *et al.*, 2001), may support our findings. In addition, we showed that exogenous PTTG1 protein was downregulated in calcium-induced differentiating NMK despite its constitutive overexpression, suggesting the existence of a stability regulation mechanism similar to the ubiquitin – proteasome pathway known to play a role in M phase regulation. Briefly, excess PTTG1 protein appears to enhance proliferation *via* upregulation of G2/M cell cycle accelerators, but once the keratinocytes exit the cell cycle and undergo differentiation, it may have little effect on later differentiation processes because of its own downregulation, which may be consistent with the staining pattern of loricrin without significant alterations in NHK overexpressing PTTG1 cultured under 3D conditions. This implies an important cell cycle regulatory machinery in keratinocyte differentiation. As demonstrated by the experiments involving culture under 3D conditions, absence of PTTG1 protein appeared not to disturb the differentiation capacity of keratinocytes. This is consistent with the

phenotype of PTTG knockout mice, which were viable and fertile but showed hypoplasia in multiple organs including the thymus, testis, and spleen, in which the levels of PTTG1 expression levels are high (Wang *et al.*, 2001). In keratinocytes, PTTG1 can alter the proliferation status by modulating the expression levels of other cell cycle regulatory proteins, especially those expressed in G2/M phase of the cell cycle, but may have little effect on terminal differentiation itself.

In conclusion, the results of the present study suggested that PTTG1 alters the proliferation status by modulating the expression levels of other cell cycle regulatory proteins, such as CyclinB1, CDK1, and c-Myc. This aberrant cell cycle regulation due to the overexpression of PTTG1 suggested that it may play important roles in hyperproliferative disorders in the epidermis, such as psoriasis or squamous cell carcinoma. In addition, the stable regulation of excess PTTG1 protein in association with cell cycle exit implies the existence of an important mechanism of cell cycle regulation involved in keratinocyte differentiation.

Materials and methods

Plasmids

The plasmid pBKCMV-PTTG1 encoding the full-length PTTG1 (NM_022391) was a kind gift from Dr. Songguang Ren (Cedars-Sinai Medical Center, Los Angeles, CA). The construct was confirmed by enzymatic digestion and sequenced to verify the correct reading frame before use.

Cell culture

HaCaT cells were maintained in MCDB153 growth medium (Sigma-Aldrich, St. Louis, MO) supplemented with 0.1 mM calcium chloride, 10 ng/mL EGF (Sigma-Aldrich), 1% penicillin–streptomycin solution (Sigma-Aldrich), and decalcified fetal bovine serum (Gibco-BRL, Paisley, UK) as described previously (Kato *et al.*, 1995). Normal human keratinocytes (NHK) (KJB-100; DS Pharma, Osaka, Japan) were cultured in serum-free growth medium (KJB-200; DS Pharma) in accordance with the manufacturer's instructions. Keratinocytes within fourth passage were used for the experiments. Primary mouse keratinocyte cultures were prepared as described previously (Rothnagel *et al.*, 1993). Three-dimensional (3D) cell culture was performed as described previously (Taguchi *et al.*, 2011). Briefly, human dermal fibroblasts (Kurabo, Osaka, Japan) were cultured in type I collagen gel (Cellmatrix type IA; Nitta Gelatin Inc., Osaka, Japan), in 12-well plates at a density of 3.0×10^5/well (HaCaT) or 5.0×10^5/well

(NHK). After solidification of the gels, HaCaT cells and NHK were seeded onto each well at densities of 1.3×10^5/well and 5.0×10^5/well, respectively. After incubation for 72 h, the double-layered cultures were exposed to the air-liquid interface, underneath which the inverted cell strainers contained 3D medium consisting of a 1:1 mixture of MCBD153 medium (HaCaT) or KJB-200 medium (NHK) with Dulbecco's Modified Eagle's Medium (Wako, Osaka, Japan) supplemented with 10% decalcified FBS sand 1% penicillin–streptomycin solution (Falcon, Franklin Lakes, NJ) in 6-well plates. The cultures in triplicate wells were analyzed on the indicated dates. The thicknesses of the epithelia formed were measured as described previously (Taguchi *et al.*, 2011).

DNA transfection

Keratinocytes from newborn mice were transfected using TransIT®-Keratinocyte Transfection Reagent (Mirus Bio, Madison, WI). To examine mRNA or protein expression in undifferentiated keratinocytes, cells were cultured in low-calcium-containing medium for 48 h after transfection and then harvested. Expression in differentiated keratinocytes was assessed by cultivation for 24 h in low-calcium medium (0.05 mM calcium) without EGF, followed by 48 h in high-calcium medium (0.12 mM calcium).

Retroviral production and transduction

cDNA encoding PTTG1 was digested from the plasmid and subcloned into the corresponding sites of the retroviral vector GCDNsap containing nerve growth factor receptor (NGFR) and transduced into the packaging cell line 293gpg along with the control vector encoding NGFR, as described previously (Fujisawa *et al.*, 2009). The virus titers were approximately 1.0×10^5 infectious units/mL on Jurkat cells.

The retroviral vectors were transduced into HaCaT cells or NHK cultured in 6-well plates and then subcultured into several dishes 10 cm in diameter. Flow cytometry was performed to detect successful transduction using a FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ) and LNGFR-APC antibody (Miltenyi Biotec, Bergisch Gladbach). The efficacy of transduction was 80% – 90%.

Immunoblotting

Cells at 80% confluency in 10-cm culture dishes were solubilized using Sample Buffer Solution (Wako). The whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, Hercules, CA), and the proteins were transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were incubated overnight with primary antibodies against PTTG1 (10 μg/mL; ab-3305;

ABcam, Cambridge, UK), keratin 10 (0.2 μg/mL; PRB-159P; Covance, Emeryville, CA), keratin 1 (0.2 μg/mL; PRB-165P; Covance), keratin 5 (0.2 μg/mL; PRB-160P; Covance), cyclin A (10 μg/mL RB-1548; Thermo Fisher Scientific, Newark, DE), cyclin B1 (0.5 μg/mL 05-373SP; Millipore, Billerica, MA), cyclin D1 (2 μg/mL; sc-718; Santa Cruz Biotechnology, Santa Cruz, CA), c-Myc (2 μg/mL; sc-40; Santa Cruz Biotechnology), β-actin (0.2 μg/mL; BioVision, Mountain View, CA), and CDK1 (1 μg/mL; Millipore), followed by incubation for 60 min with HRP-labeled secondary antibodies against rabbit IgG (0.04 μg/mL; Santa Cruz) and mouse IgG (0.4 μg/mL; Santa Cruz). Antibody binding was visualized and enhanced with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and Hyperfilm™ MP (GE Healthcare). The intensities of the bands were quantified by densitometry, and the expression levels were normalized relative to β-actin.

Immunohistochemical staining

Sections of 3D cultures were treated by autoclaving in 10 mM citrate buffer (pH 6.0) for antigen retrieval and incubated overnight with primary antibodies against keratin 14 (0.1 μg/mL; MS-115; Thermo Fisher Scientific), keratin 1 (0.5 μg/mL; PRB-149P; Covance), keratin 10 (100×; MON 3025; Sanbio B.V., Uden, The Netherlands), loricrin

(0.5 μg/mL; PRB-145P; Covance), and Ki67 (0.05 μg/mL; ab833; ABcam), followed by visualization using an EnVision™ kit according to the standard protocol (Dako, Glostrup, Denmark) and counterstaining with hematoxylin.

Quantitative real-time PCR analysis

Total RNA was extracted from 10-cm culture dishes at 80% – 90% confluency using ISOGEN® (Nippon Gene, Tokyo, Japan) and cDNA was synthesized using a Prime Script® RT reagent kit (Takara Bio, Shiga, Japan). An ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for SYBR® Green (Takara Bio)-based quantitative analysis. The quantity of mRNA was normalized relative to that of GAPDH. Quantification was performed in triplicate. The primers used were as follows:

Keratin 10: (forward: 5'-CTGACAATGCCAACGTGCTG-3'; reverse: 5'-GCAGGGTCACCTCATTCTCGTA-3')

PTTG1 (mouse): (forward: 5'-TTTGGCATCTAAGGATGGGTTGA-3'; reverse: 5'-AGCATTGAACACTTTGCCGACTC-3')

PTTG1 (rat) (forward: 5'-GGCTTGCCTAAAGCCACCAG-3'; reverse: 5'-GTAGGCATCATCAGGAGCAGGAG-3')

Cyclin B1 (forward: 5'-TGAGCCTGACCTGAACCTG-3'; reverse: 5'-GTTTCCATCGGGCTTGGAGA-3')

CDK1 (forward: 5'-ACTGGCTGATTTCGGCCTTG -3'; reverse: 5'-CAGCAACACTTCTGGAGATCGGTA-3')

c-Myc (forward: 5'-CCTAGTGCTGCATGAGGAG -3'; reverse: 5'-GGATGGAGATGAGCCCGAC -3')

GAPDH (forward: 5'-AAATGGTGAAGGTCGGTGTGAAC-3'; reverse 5'-CAACAATCTCCACTTTGCACTG-3')

Depletion of PTTG1 *via* shRNA

Lentiviral particles encoding shRNA targeting PTTG1 mRNA were purchased from Santa Cruz Biotechnology (sc-37491-V). Lentiviral particles encoding a scrambled shRNA sequence (sc-108080) were also purchased from the same source for use as a control. HaCaT cells and NHK were transduced with these lentiviral particles in accordance with the manufacturer's instructions, and the knockdown efficacy was assessed at the protein level by immunoblotting.

Measurement of HaCaT cell proliferation rate

HaCaT cells of each strain were seeded into 6-well plates at a density of 5×10^4 cells/well in the growth medium described above. The next day, the medium was replaced with fresh medium, and the numbers of cells in triplicate wells were counted by trypsinization every 24 h, from 48 h to 120 h after seeding.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Comparisons among groups were performed with the paired Student's *t* test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Conflict of Interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1

PTTG1 expression in normal keratinocytes is under cell cycle-dependent regulation

(a) Immunostaining of normal human skin with an antibody to PTTG1 protein. PTTG1 was expressed in basal and peribasal keratinocytes, and localized in the cytoplasm and nucleus. Bar = 100 μ m (b) Total RNAs extracted from neonatal murine keratinocytes (NMK) cultured in low- or high-calcium-containing medium were subjected to quantitative real-time PCR. Relative expression of PTTG1 (left) and K10 (right) mRNA in NMK under low- and high-calcium conditions. PTTG1 mRNA expression level was 6.6-fold higher under low-calcium conditions than under high-calcium conditions. The results indicated cell cycle-dependent transcriptional regulation of PTTG1. The expression levels were normalized relative to GAPDH mRNA expression. All values represent means ± SEM from three independent experiments. $^{**}P < 0.01$. (c) Immunoblotting of whole-cell extracts from NMK cultured under low- or high-calcium conditions. PTTG1 protein expression became almost undetectable as NMK underwent differentiation, also indicating cell cycle-dependent regulation.

Figure 2

PTTG1 altered the thickness of epithelia formed by human keratinocytes cultured under 3D conditions

(a) (b) HaCaT cells were transduced with a retroviral vector encoding rat PTTG1 and nerve growth factor receptor (NGFR) and endogenous PTTG1 was depleted from HaCaT cells using a lentiviral vector encoding shRNA against PTTG1. PTTG1 expression and sufficient knockdown were monitored by immunoblotting (see Fig. 6−a). Constitutive PTTG1 expression resulted in formation of thicker epidermoid epithelia than the control, whereas absence of endogenous PTTG1 in HaCaT cells resulted in formation of thinner epithelia: epidermoid epithelia formed 10 days after exposure to the air-liquid interface (hematoxylin-eosin staining) Bar = 20 μ m (a); temporal thickness measurement of the epithelia (b).

(c) (d) NHK were transduced with the PTTG- or sh-RNA-encoding retroviral vectors and cultured under 3D conditions as were HaCaT cells. Successful PTTG1 overexpression or endogenous PTTG1 knockdown was monitored by immunoblotting (see Fig. 6-b). PTTG1 expression resulted in formation of thicker epidermoid epithelia than the control, whereas absence of endogenous PTTG1 NHK resulted in formation of thinner epithelia: epidermoid epithelia formed 4 days after exposure to the air-liquid

interface (hematoxylin-eosin staining) Bar = 20 μ m (a); thickness measurement of the epithelia (b).

All values represent means ± SEM from three independent experiments $^{**}P < 0.01$ *vs.* control.

Figure 3

Overexpression of PTTG1 suppressed early differentiation of human keratinocytes cultured under 3D conditions

Representative figure of HaCaT cells and NHK cultured under 3D conditions stained with antibodies against K1 and K10 (keratinocyte early differentiation markers), along with K14 and PTTG1. NHK was also stained with an antibody against loricrin (a terminal keratinocyte differentiation marker).

(a) Constitutive PTTG1 overexpression in HaCaT cells caused decreases in thickness of K1- and K10-positive layers and increases in thicknesses of undifferentiated layers. (b) Constitutive PTTG1 overexpression in NHK was associated with an increase in the undifferentiated layers although substantial changes in the stained layers were correlated with the total thickness. There was no apparent change in loricrin staining pattern. Note the bottom-heavy staining pattern of PTTG1 despite its constitutive

overexpression.

(a) (b) PTTG1 depletion had no significant effect on staining patterns of any of the differentiation markers despite the changes in total layer thickness. Bars = (a) 20 μ m; (b) 50 μ m.

Figure 4

Overexpression of PTTG1 suppressed early keratinocyte differentiation under 2D conditions

NMK were transfected with an expression plasmid encoding PTTG1 and induced to differentiate by calcium switch.

(a) Representative results of immunoblotting of whole-cell lysate from differentiating NMK cultured under high-calcium conditions. NMK constitutively overexpressing PTTG1 showed decreases in K1 and K10 compared with NMK transfected with pCMV empty vector. The band intensities quantified by densitometry were significantly different. The expression levels were normalized relative to β-actin.

(b) Total RNAs extracted from NMK were subjected to quantitative real-time PCR. Relative expression levels of K1, K10, and PTTG1 mRNA. NMK constitutively expressing PTTG1 showed decreases in K1 and K10 compared with NMK transfected with pCMV empty vector. The expression levels were normalized relative to GAPDH

mRNA expression.

(a) (b) The exogenous PTTG1 protein was downregulated under high-calcium conditions despite its constitutive expression as confirmed by detection of its mRNA.

All data are means ± SEM form three independent experiments. $^{**}P < 0.01$ *vs.* control; n.s. not significant.

Figure 5

PTTG1 altered keratinocyte proliferation patterns under both 2D and 3D conditions

(a) Proliferation rate was assayed by cell counting. HaCaT cells constitutively expressing PTTG1 showed increased proliferation rate compared with controls, whereas PTTG1-depleted HaCaT cells showed a decreased proliferation rate.

(b) (c) HaCaT cells (b) and NHK (c) cultured under 3D conditions were stained with an antibody against Ki67. Constitutive expression of PTTG1 resulted in an increase in number of Ki67-positive cells, whereas depletion of PTTG1 resulted in a decrease. All values represent means ± SEM from three independent experiments. $^{**}P < 0.01$ *vs.* control. Bars = (a) 20 μ m; (b) 50 μ m

Figure 6

PTTG1-induced keratinocyte hyperproliferation is due to the upregulation of Cyclin B1, CDK1 and c-Myc oncoprotein

(a), (b) Representative immunoblotting of whole-cell lysates from HaCaT cells, NHK, and NMK. Cyclin B1, CDK1, and c-Myc were upregulated due to constitutive expression of PTTG1, whereas all of these molecules were downregulated due to depletion of PTTG1. The band intensities quantified by densitometry showed significant differences (graphs). Expression levels of Cyclin A (S/G2 phase cyclin) and Cyclin D1 (G1 phase cyclin) in NHK were analyzed by immunoblotting. Compared with Cyclin D1, Cyclin A expression level changed significantly according to PTTG1 expression level, showing similarity to PTTG1, Cyclin B1, and CDK1. The expression levels were normalized relative to β-actin.

(c) Total RNAs extracted from NMK were subjected to quantitative real-time PCR. Relative expression levels of PTTG1, Cyclin B1, CDK1, and c-Myc mRNA. NMK constitutively expressing PTTG1 showed upregulation of these molecules. The expression levels were normalized relative to GAPDH mRNA expression. PTTG1 protein was downregulated along with Cyclin B1 and CDK1 despite constitutive mRNA expression.

All data are means ± SEM form three independent experiments. **$P < 0.01$ *vs.* control; n.s. not significant.

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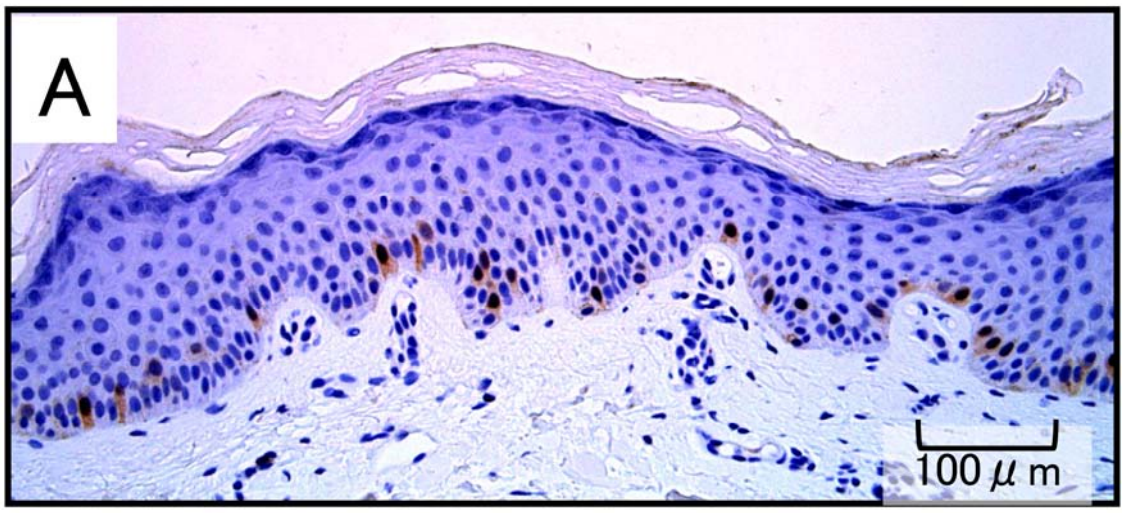
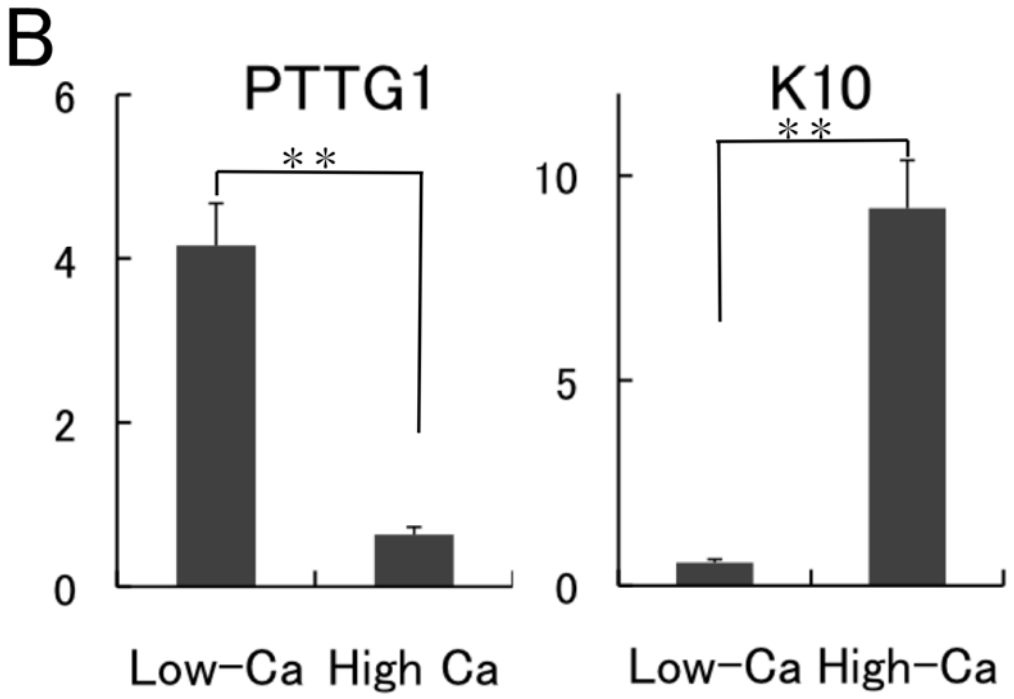
fig.1


A
100 μ m


B
PTTG1
K10
**
**
6
4
2
0
10
5
0
Low-Ca High Ca
Low-Ca High-Ca

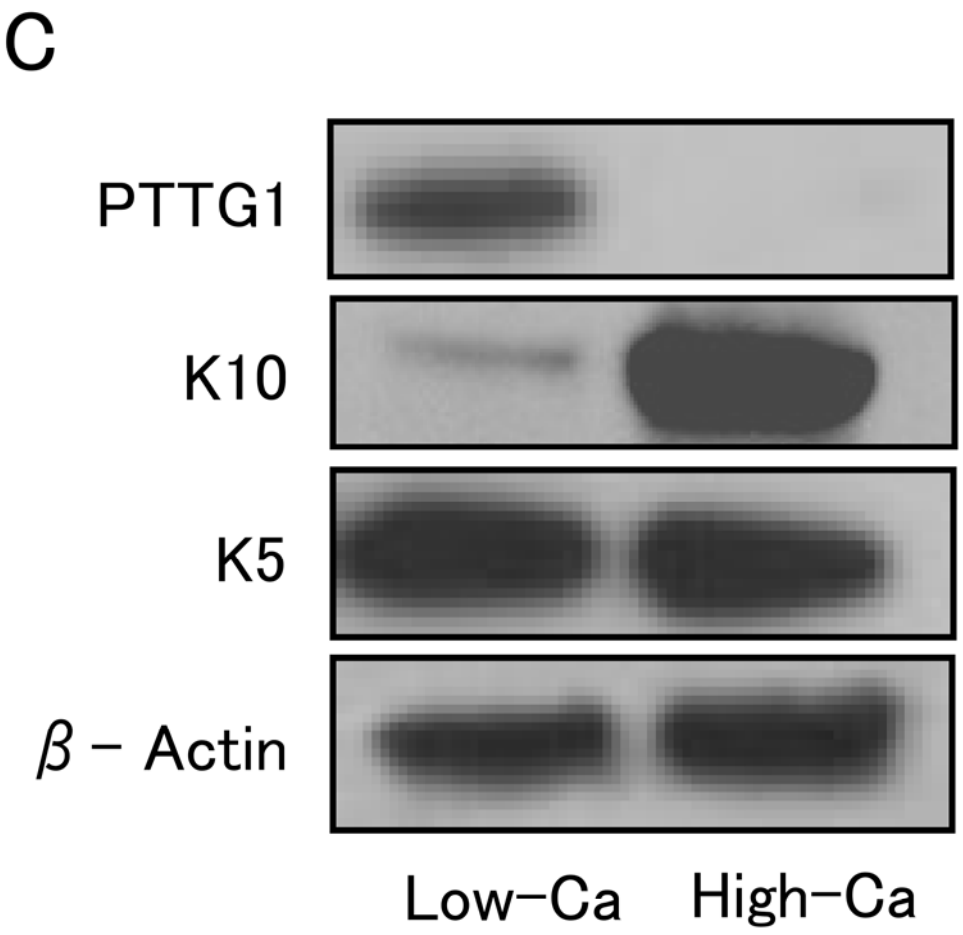

C
PTTG1
K10
K5
β - Actin
Low-Ca
High-Ca

fig.2

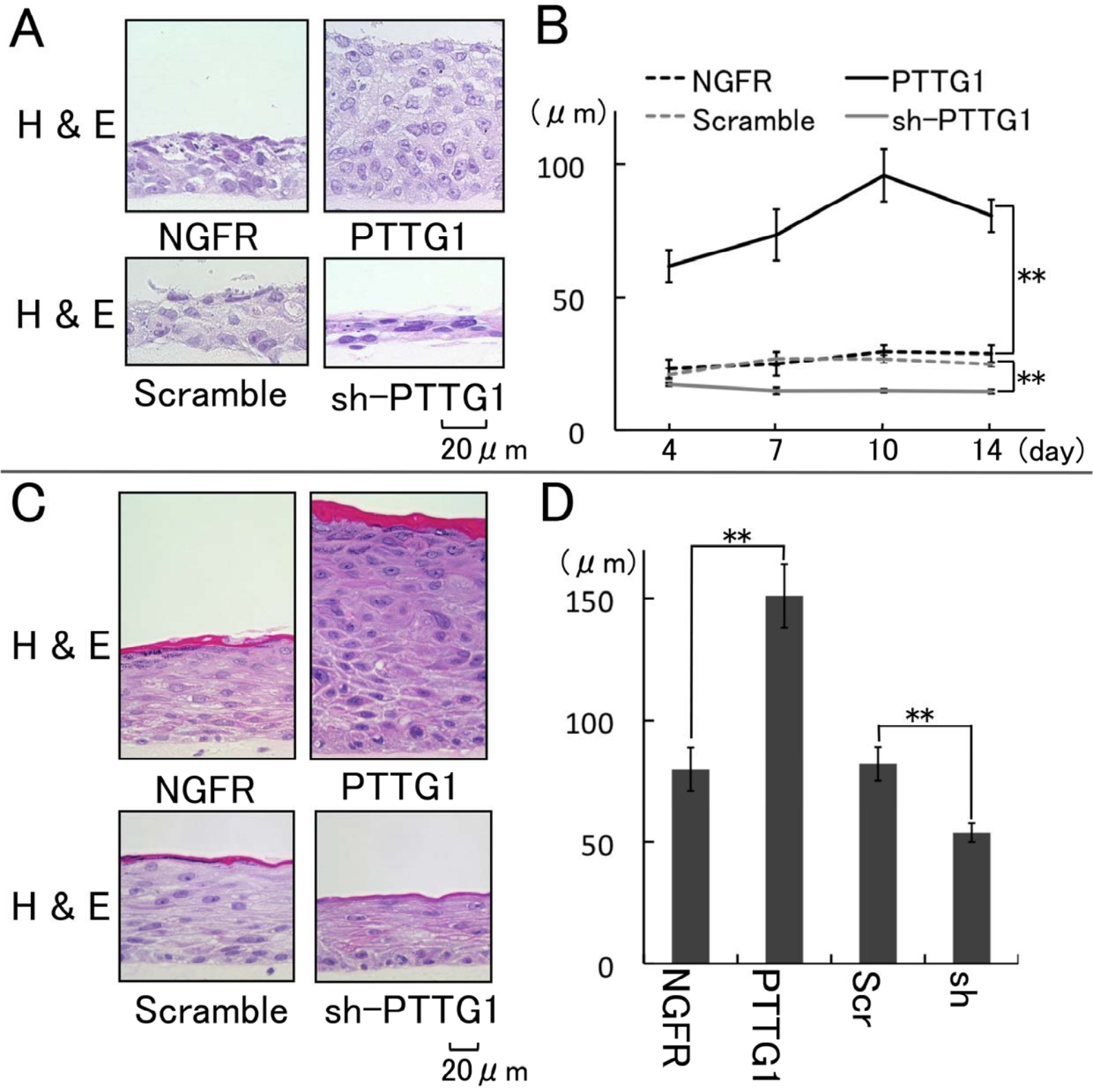
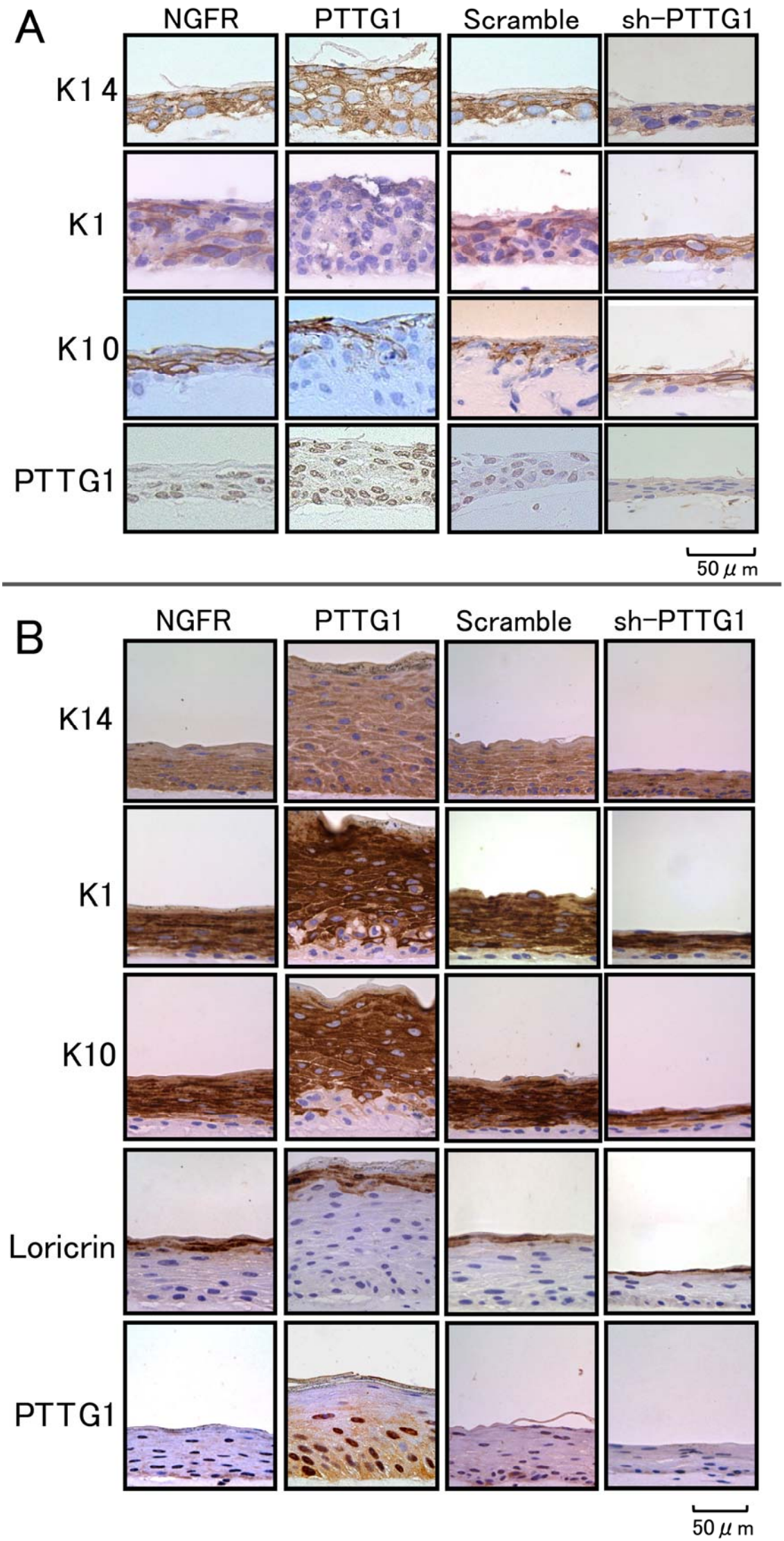

A
H & E
NGFR
PTTG1
H & E
Scramble
sh-PTTG1
20 μ m
B
NGFR
PTTG1
Scramble
sh-PTTG1
(μ m)
100
50
0
4
7
10
14 (day)
**
**
C
H & E
NGFR
PTTG1
H & E
Scramble
sh-PTTG1
20 μ m
D
(μ m)
150
100
50
0
**
**
NGFR
PTTG1
Scr
sh

fig.3


A
NGFR
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Scramble
sh-PTTG1
K14
K1
K10
PTTG1
50 μm
B
NGFR
PTTG1
Scramble
sh-PTTG1
K14
K1
K10
Loricrin
PTTG1
50 μm

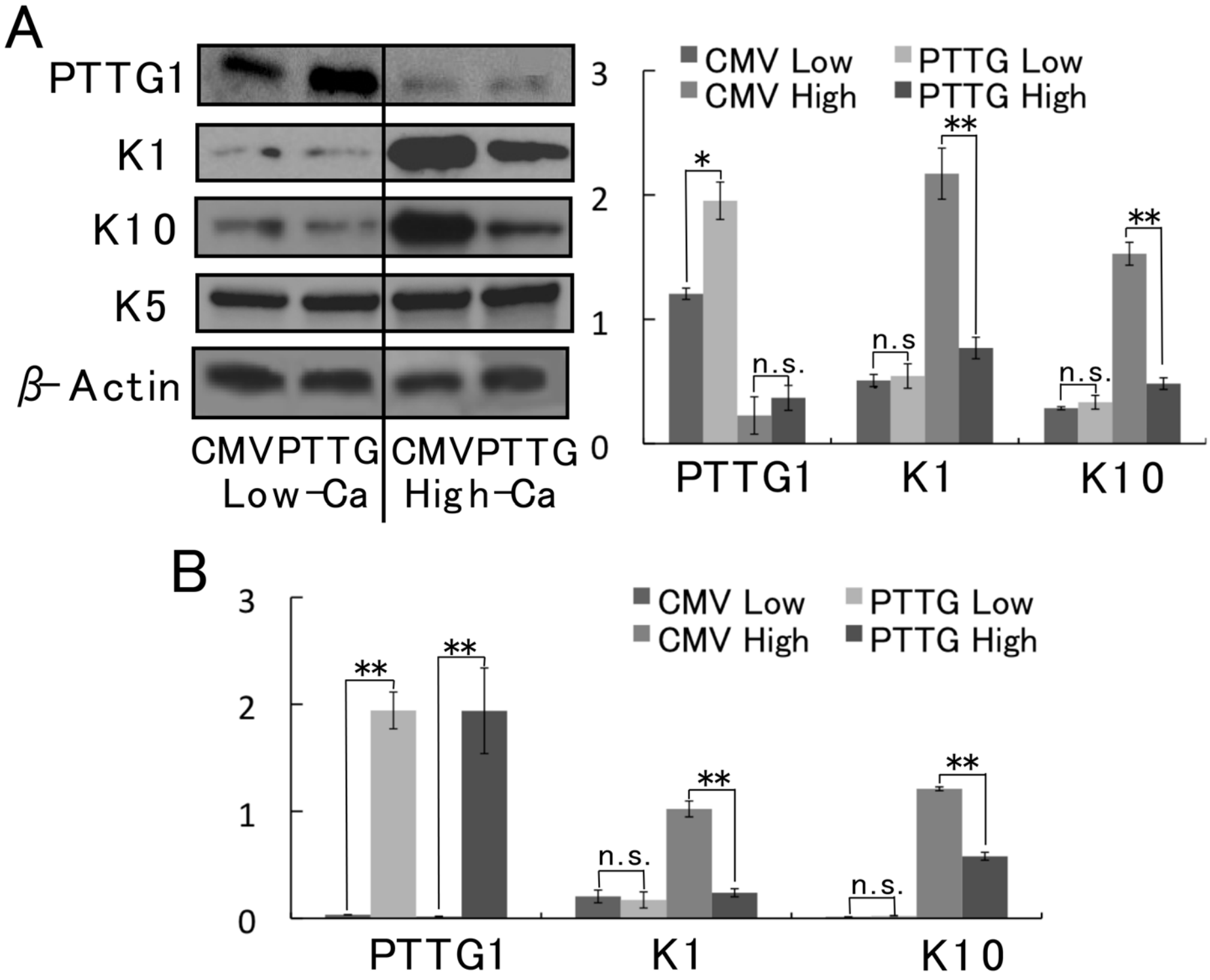
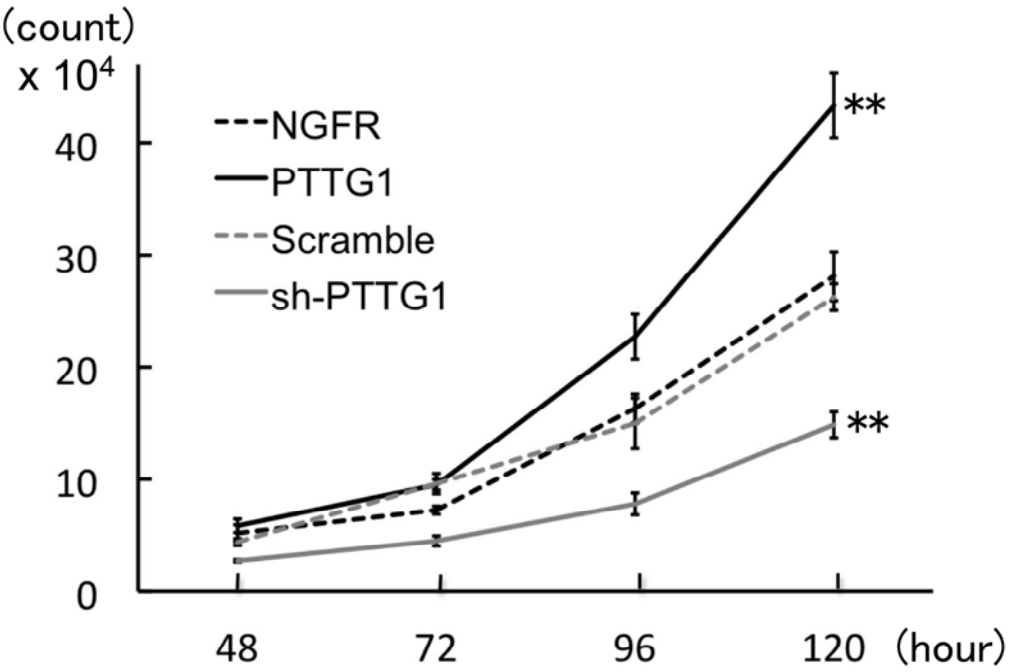
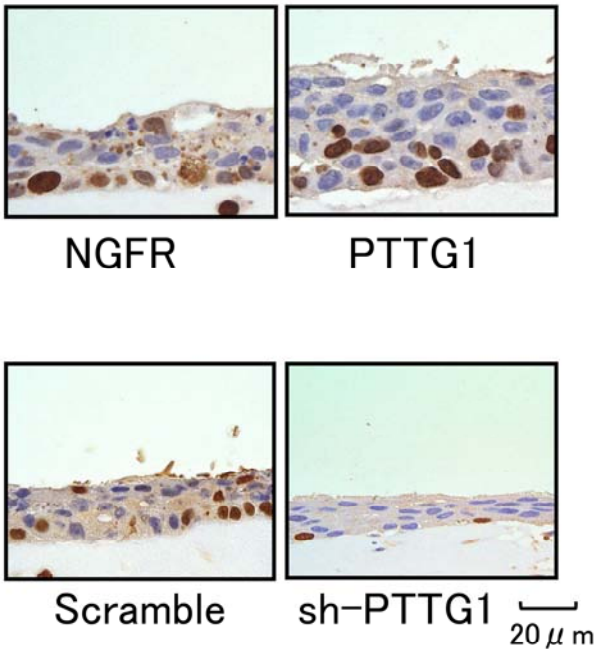

A
PTTG1
K1
K10
K5
β-Actin
CMVPTTG
Low-Ca
CMVPTTG
High-Ca
3
2
1
0
CMV Low
CMV High
PTTG Low
PTTG High
*
n.s.
**
n.s
n.s.
**
PTTG1
K1
K10
B
3
2
1
0
CMV Low
CMV High
PTTG Low
PTTG High
**
**
n.s.
**
n.s.
**
PTTG1
K1
K10

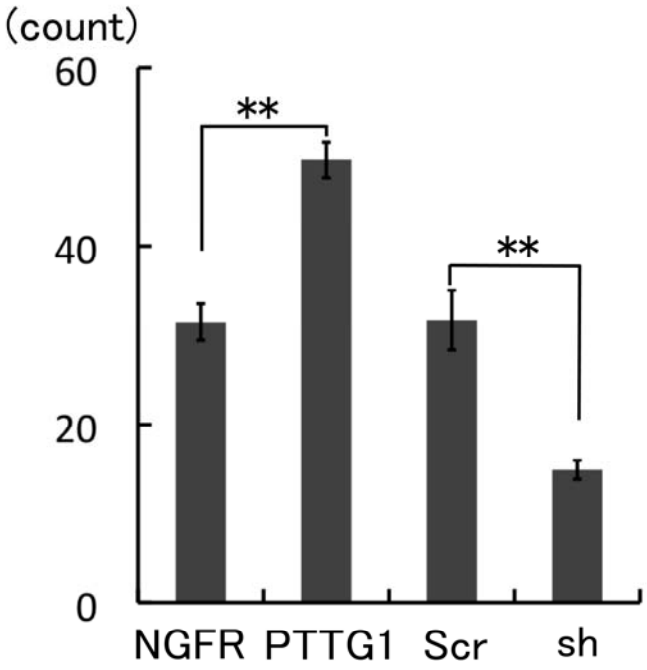
fig.5

A

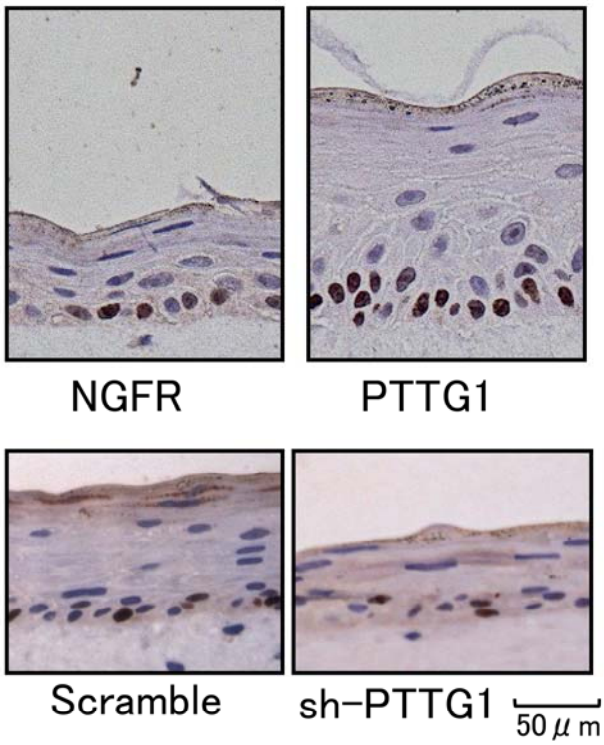

(count)
x 10^4
40
30
20
10
0
NGFR
PTTG1
Scramble
sh-PTTG1
**
**
48
72
96
120
(hour)

B


NGFR
PTTG1
Scramble
sh-PTTG1
20 μm


(count)
60
40
20
0
**
**
NGFR
PTTG1
Scr
sh

C


NGFR
PTTG1
Scramble
sh-PTTG1
50 μm

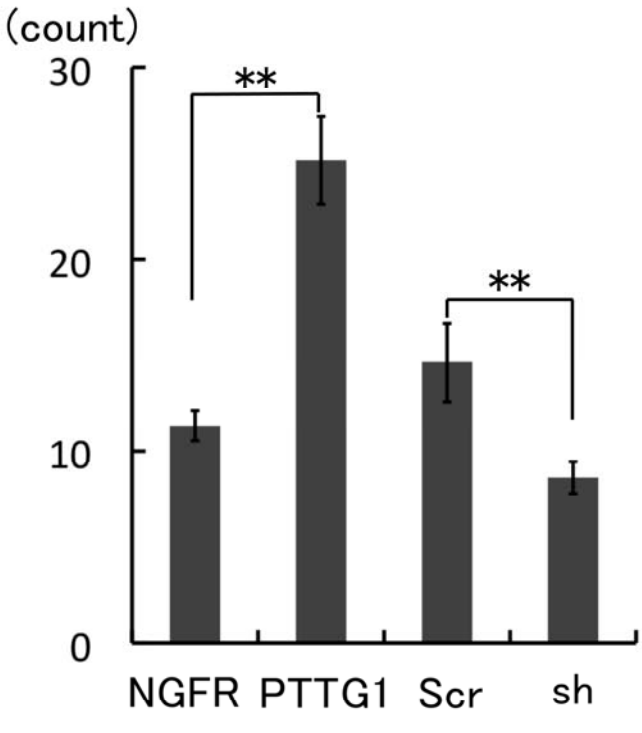
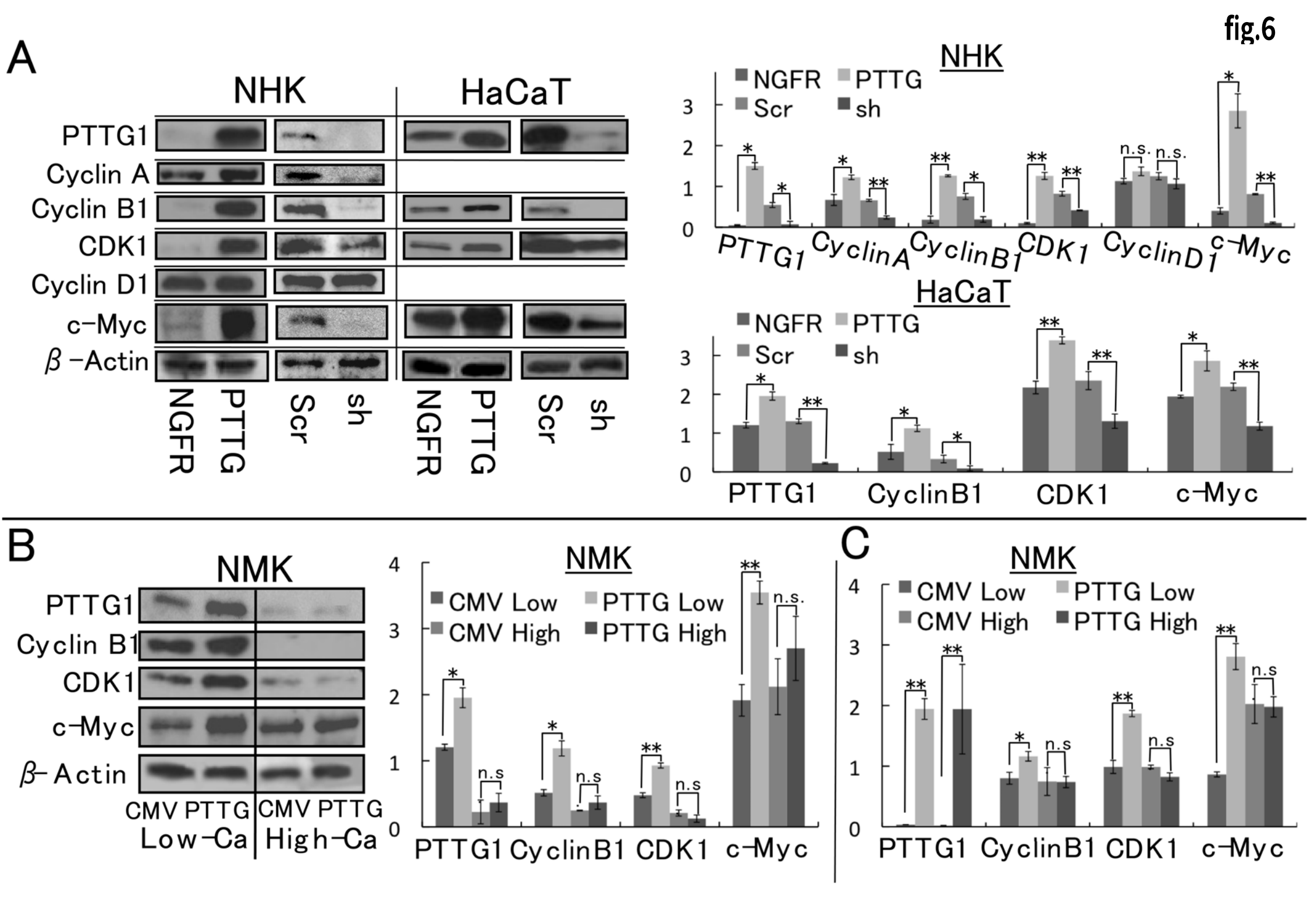

(count)
30
20
10
0
**
**
NGFR
PTTG1
Scr
sh

fig.6


A
NHK
HaCaT
PTTG1
Cyclin A
Cyclin B1
CDK1
Cyclin D1
c-Myc
β-Actin
NGFR
PTTG
Scr
sh
NHK
NGFR
PTTG
Scr
sh
PTTG1
CyclinA
CyclinB1
CDK1
CyclinD1
c-Myc
n.s.
HaCaT
PTTG1
CyclinB1
CDK1
c-Myc
B
NMK
PTTG1
Cyclin B1
CDK1
c-Myc
β-Actin
CMV PTTG
Low-Ca
CMV PTTG
High-Ca
NMK
CMV Low
PTTG Low
CMV High
PTTG High
n.s
PTTG1
CyclinB1
CDK1
c-Myc
C
NMK
CMV Low
PTTG Low
CMV High
PTTG High
n.s
PTTG1
CyclinB1
CDK1
c-Myc