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PTH-1R responses to PTHrP and regulation by vitamin D in keratinocytes and adjacent fibroblasts☆

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Abstract

Vitamin D and PTHrP are essential for the differentiation of keratinocytes and epidermal development. The action of PTHrP on skin is mediated via its PTH-1R receptors present in both epidermal and dermal cells. This suggests that PTHrP may have a paracrine/autocrine role, and its receptors may act in association or in negative cooperativity. We compared the intracellular signaling pathways in response to PTHrP (1–34) and to various PTHrP peptides, the N-terminal (1–34), Mid region (67–89), and C-terminal (107–139) fragments, and the possible modulation of PTHrP and its receptor mRNA expressions by vitamin D. Adjacent dermal fibroblasts as freshly isolated keratinocytes expressed both PTHrP and PTH-1R mRNAs, and responded to the various PTHrP fragments. bPTH and PTHrP(1–34) increased both cellular cAMP and $[Ca^{2+}]i$ in keratinocytes and fibroblasts. In contrast, PTHrP (107–139) increased $[Ca^{2+}]i$ but not cAMP in the two cell types. PTHrP (67–89) had no effect in keratinocytes, and only increased $[Ca^{2+}]i$ in fibroblasts. Vitamin D deficiency in weaned rats increased the expression of PTHrP mRNA in keratinocytes, and decreased it in fibroblasts and kidneys. Vitamin D deficiency increased PTH-1R mRNA expression in keratinocytes and kidneys, but not in fibroblasts. Although keratinocytes and skin fibroblasts are target cells for PTHrP and express PTH-1R, the two adjacent cell types differ as regards their intracellular signaling in response to PTHrP peptides. Moreover vitamin D regulates PTHrP and PTH-1R in a cell-specific manner.
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Keywords: PTHrP; PTH/PTHrP receptor; Keratinocytes; Fibroblasts; PTHrP peptides

1. Introduction

PTHrP is highly expressed in normal skin, and regulates the differentiation of epidermal cells [1,2]. PTHrP may function as a paracrine factor in the epidermis since dermal fibroblasts bear type I PTH/PTHrP receptors (PTH-1R). However, type I PTH/PTHrP receptors have recently been identified in keratinocytes [3]. Bringing together these findings raises questions on the function of PTH-1R in both cell types. Binding of PTHrP (1–34) to PTH-1R may activate two signaling pathways: cAMP/adenylyl cyclase/protein kinase A and/or cytosolic free calcium/inositol trisphosphate/protein kinase C [4]. Adenylyl cyclase-linked PTH-1R route has only been localized in an immortalized human keratinocyte cell line (RHEK) [5], but PTHrP increased membrane-associated protein kinase C (PKC) activity without affecting adenylyl cyclase activity in cultured differentiation-competent mouse skin keratinocytes [6]. Underlying dermal fibroblasts exhibited a cAMP response only to PTHrP (1–34). Finally, it is not known whether only the N-terminal PTHrP (1–34) or other PTHrP fragments play a physiological role in the epidermis. We therefore evaluated the two cell signal transduction responses to PTHrP (1–34) in freshly isolated newborn keratinocytes and in adjacent dermal fibroblasts, and extended our study to the other PTHrP peptides, namely the human Mid-region (67–89), and C-terminal (107–139) PTHrP peptides.

Vitamin D may play a paracrine role during the differentiation of keratinocytes [7,8], and regulates the production of PTHrP [9]. The vitamin D receptor (VDR) is present in both keratinocytes and fibroblasts. Vitamin D negatively regulates PTHrP expression in keratinocytes and cancer cells [9–12]. In contrast, PTH-1R is differentially regulated by vitamin D in classical target tissues of PTHrP as kidneys [13], in ROS cells, and in bone and cartilage cells that are adjacent [14]. We therefore studied the modulation of PTHrP and PTH-1R mRNA in keratinocytes and fibroblasts isolated from vitamin D-deficient rats. Our data demonstrate that PTHrP and its receptors are expressed in both cell types. PTH-1R seems

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structurally similar in both cell types. However, PTH-1R acts differently in fibroblasts because of different biological responses to the various fragments of PTHrP. Moreover, PTHrP and PTH-1R mRNA are regulated in a different way by vitamin D in keratinocytes and fibroblasts.

2. Materials and methods

2.1. Animals

Weaned 21-day-old male Wistar rats were divided in two groups. Group1 (D^+D^+, $n = 5$) served as controls, and was fed a diet containing 0.75% calcium and 2000 IU/kg vitamin D. The rats in Group 2 (D^-D^-, $n = 5$) were raised in the dark, and given a vitamin D-deficient diet containing 0.4% calcium. After 8 weeks, both groups were anesthesized by i.p. phenobarbital injection before death. Blood sample was taken for biochemical analysis. Skins were used for keratinocytes and fibroblasts preparation. Kidneys were rapidly excised and frozen in liquid nitrogen.

2.2. Keratinocytes and fibroblasts preparation

Total keratinocytes were isolated from skin samples of 1-day-old, vitamin D-deficient or normal rats, by a modification of the method of Yuspa and Harris [15]. Fibroblasts outgrew from skin biopsies, and became confluent after 3 weeks in culture. Kidneys of the same animals were used as a tissue control for the expression of PTHrP and PTH-1R.

2.3. Parathyroid hormone-related protein and its receptor mRNA assay

RNA was extracted from the cells, and used for RT-polymerase chain reaction (PCR). We used primers to amplify 611 bp corresponding to exons 3 and 4 of the rat PTHrP cDNA, primers to amplify 910 bp of PTH-1R cDNA, and rat G3PDH cDNA primers as an internal control [6]. Controls included water instead of cDNA in the PCR samples, omitting the reverse transcriptase during the synthesis of cDNA. The conditions for reverse transcriptase and PCR of PTHrP and PTH-1R were as previously described [3]. RT-PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and hybridized after Southern blotting with specific synthetic oligomer probes.

2.4. Cyclic AMP assay

Freshly isolated keratinocytes were cultured in 6-well plates for 8 days in KGM medium (low calcium medium 0.1 mM), while fibroblasts were cultured in DMEM medium (normal calcium, 1.8 mM). The medium was removed, and the cells were incubated for 5–10 min in medium containing 1% bovine serum albumin, 0.2 mM isobutyl methylxanthine (a phosphodiesterase inhibitor), and various doses of the PTHrP fragment or its solvent (10 mM acetic acid plus 1% BSA). Cell cAMP was extracted twice with ethanol. Cyclic AMP was measured by radiocompetition [16]. The cAMP content is expressed as pmoles/mg protein.

2.5. Calcium measurement

Keratinocytes and fibroblasts were grown on rectangular glass coverslips for 4 days in medium supplemented with 10% FCS. Cells were transferred to serum-free medium 24 h before use. The cells were loaded with 1 mM Fura-2/AM for 30 min in Hanks' HEPES, pH 7.4 (137 mM NaCl, 5.6 mM KCl, 0.441 mM KH_2PO, 0.442 mM Na_2HPO_4, 0.885 mM $MgSO_4{\cdot}7H_2O$, 27.7 mM glucose, 1.25 mM $CaCl_2$, and 25 mM HEPES), at room temperature. The Fura-2 fluorescence response to the intracellular calcium concentration ($[Ca^+]$i) was calibrated from the ratio of the 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm, using a thermostatted (37 °C) Hitachi F-2500 spectrofluometer. [17]. Drugs and reagents were added directly to the cuvette under continuous stirring.

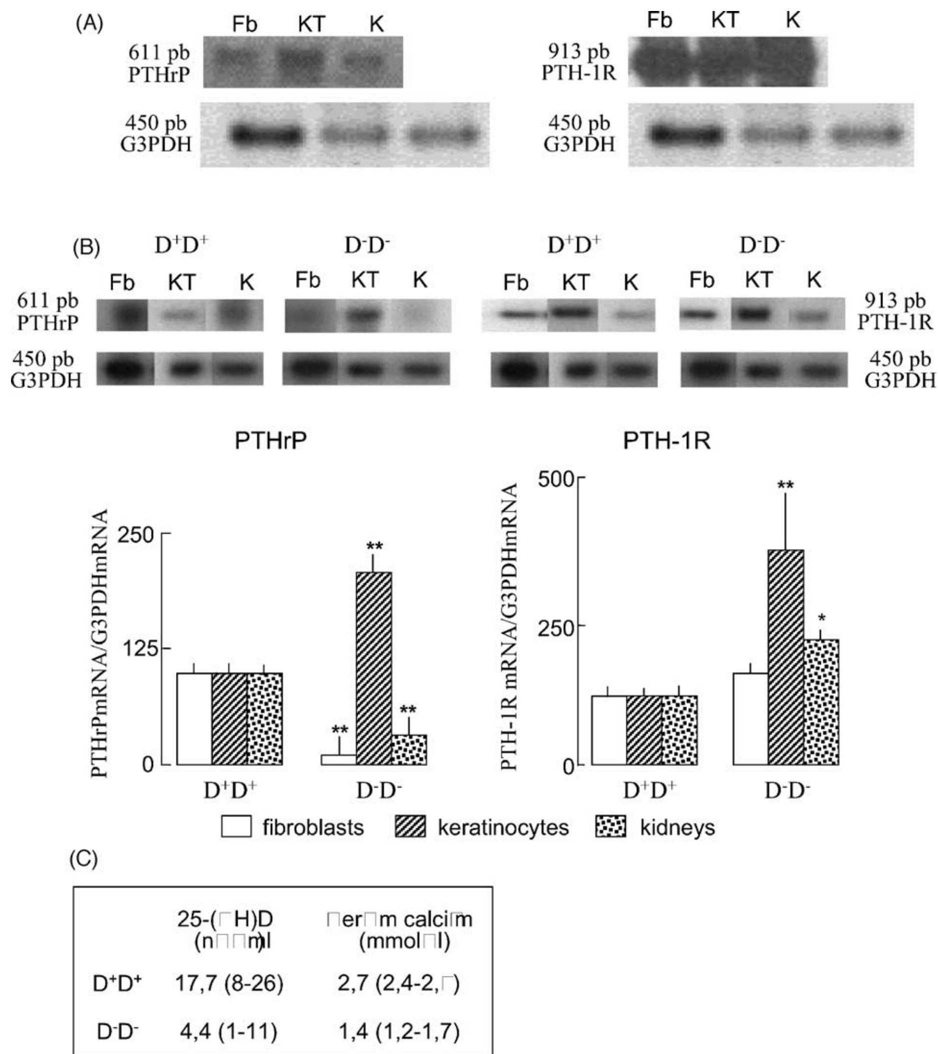
3. Results

3.1. PTHrP and PTH-1R mRNA in keratinocytes and fibroblasts of newborn rats and vitamin D-deficient rats

Both fibroblasts and keratinocytes of newborn rats exhibited PTHrP and PTH-1R (Fig. 1A). Vitamin D deficiency resulted in decreases in serum calcium and vitamin D metabolites, 25(OH)D and $1,25(OH)_2D_3$, (Fig. 1C), and caused severe hyperparathyroidism. The semi-quantitative analysis of RT-PCR product of PTHrP followed by a Southern blot showed a marked increase in the amount of PTHrP in vitamin D-deficient skin keratinocytes (Fig. 1B). In contrast, vitamin D deficiency decreased drastically PTHrP mRNA expression in both fibroblasts and kidney. Vitamin D deficiency increased the expression of PTH-1R mRNA in keratinocytes and, to a less extend, in kidneys, but had no effect on the PTH-1R mRNA expression in fibroblasts (Fig. 1B).

3.2. Effects of different fragments of PTHrP on intracellular calcium and cAMP

The basal $[Ca^{2+}]$i in newborn rat keratinocytes was 149 ± 5 nM, mean ± S.E., $n = 6$. PTHrP (1–34) triggered a rapid transient increase in $[Ca^{2+}]$i. The profile of the calcium response consisted of a sharp peak followed by a plateau that remained above the basal level (21 ± 2%, mean ± S.E.,



	25-([illegible]H)D (n[illegible]m)l	[illegible]er[illegible]m calci[illegible]m (mmol[illegible]l)
D+D+	17,7 (8-26)	2,7 (2,4-2,[illegible])
D-D-	4,4 (1-11)	1,4 (1,2-1,7)

Fig. 1. PTHrP and PTH-1R messenger RNA in keratinocytes and fibroblasts of newborn, normal and vitamin D deficient rats. Total RNA was extracted from kidney tissue, keratinocytes and fibroblasts, and analyzed by reverse transcriptase-polymerase chain reaction (PCR) using primers, which detect mRNA of 611 bp corresponding to exons 3 and 4 of the rat PTHrP cDNA, 910 bp corresponding to exons E2/M of the rat PTH-1R. (A) Newborn rats. (B) Normal and vitamin D deficient rats. The Southern blot of the 611 and 910 bp hybridized with a specific PTHrP or PTH-1R oligonucleotide. The intensities of the hybridizing signals of the PTHrP PCR products were quantified with densitometric scanning and normalized against the G3PDH signal. Values are means ± S.E.M. ($n = 5$), $^{*}P < 0.05$ vs. D^+D^+group, $^{**}P < 0.01$ vs. D^+D^+group.

$n = 6$, $P < 0.05$) for at least 1 min (Fig. 2A, left panel). This effect started at 10 nM, and was the greatest at 100 nM. Keratinocytes also responded to 100 nM C-terminal peptide (107–139) and to 100 nM bPTH (1–34), but not to the Mid-region (67–89) peptide. The three PTHrP peptides and bPTH (1–34) stimulated the $[Ca^{2+}]i$ in fibroblasts (Fig. 2A, right panel).

The cAMP content of keratinocytes was increased after 10-min incubation with 100 nM human PTHrP (1–34), bovine PTH (1–34), or Forskoline, but the C-terminal and Mid-region PTHrP peptides had no effect (Fig. 2B, left panel). Human PTHrP (1–34) and bPTH (1–34) (100 nM) increased cAMP after 5 min in fibroblasts (Fig. 2B, right panel) whereas Forskoline needed 10 min. The maximal cAMP response to PTHrP (1–34) was at 10 nM. Mid-region PTHrP (67–89) and C-terminal PTHrP (109–137) at 100 nM had no effect in fibroblasts.

4. Discussion

In this study, we have investigated: (i) whether the PTHrP produced by keratinocytes is an autocrine local regulator of the differentiation or a paracrine one, acting on underlying dermal fibroblasts that display PTH-1R and respond to PTHrP; and (ii) whether the PTHrP and its receptor in both cells are regulated by vitamin D. The adjacent dermal fibroblasts, isolated from the same skin of 1-day-old

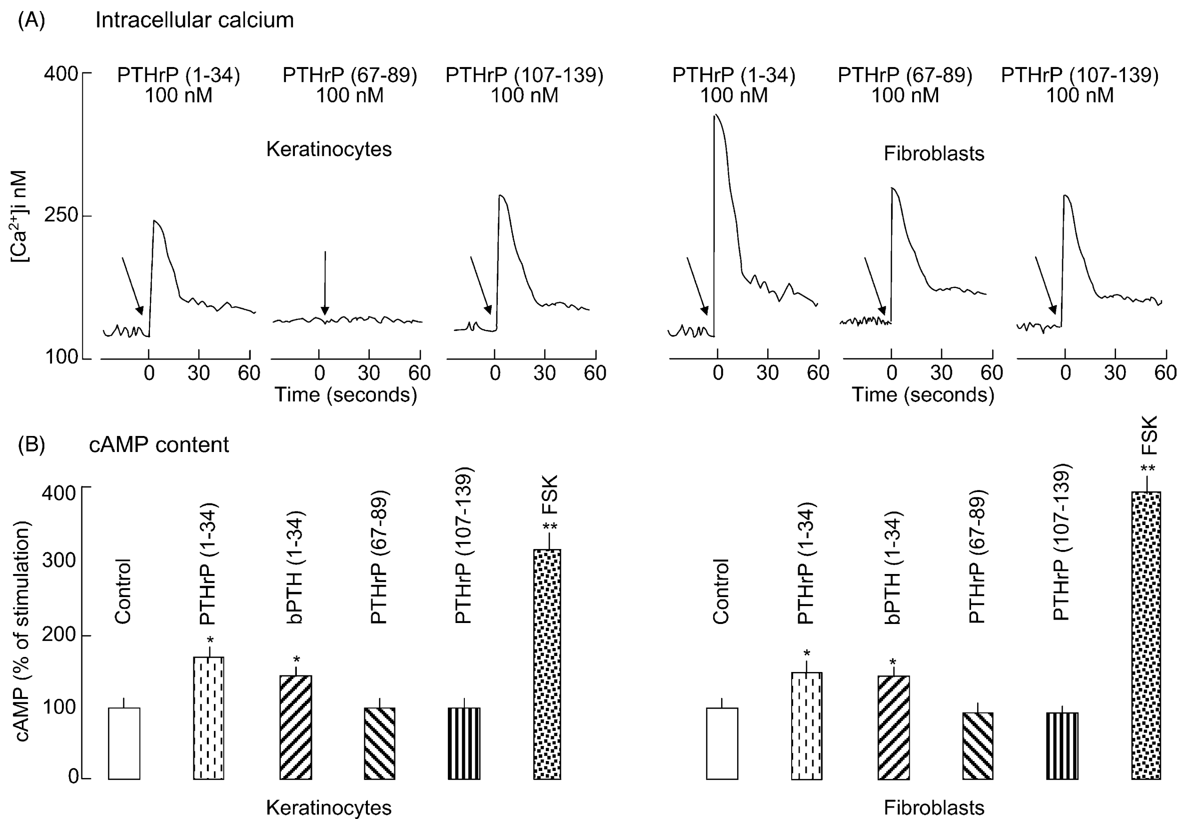


Fig. 2. Effects of PTHrP peptides on intracellular Ca^{2+} and cAMP in keratinocytes and fibroblasts. (A) Effects of various PTHrP peptides on intracellular calcium. Keratinocytes or fibroblasts from newborn skin were loaded for 30 min with 1 mM Fura-2/AM. The response profile consisted of a peak followed by a plateau phase. These results are representative of six experiments. (B) Effects of PTHrP peptides, bovine PTH (bPTH), and forskolin (FSK) on cellular cAMP content in keratinocytes and fibroblasts. Fibroblasts were incubated for 5 min, and keratinocytes for 10 min with 100 nM hPTHrP (1–34), hPTHrP (67–89), hPTHrP (107–139), bPTH (1–34). Forskolin (100 nM) served as positive control to ensure that cells had a functional adenylyl cyclase. The cAMP is expressed as pmoles/mg proteins, and the results are the percentage of the amount measured in vehicle-treated controls. The control level of cAMP was 40 pmoles/mg proteins in keratinocytes and fibroblasts. Values are means ± S.E. of data obtained in 10 separate experiments. Anova and Fischers PLSD were used for significance (significant at 95%). $^{*}P < 0.05$ vs. controls and $^{**}P < 0.01$ vs. controls.

rats, expressed PTH-1R, and surprisingly PTHrP, while it has been stated that skin fibroblasts usually do not secrete PTHrP. Our findings support an autocrine/paracrine function, involving the presence of functional PTH-1R in both cells. We have also shown that PTHrP in freshly isolated keratinocytes uses both transduction pathways, cAMP, and intracellular calcium. The rise in cAMP in response to PTHrP (1–34) has been found in RHEK-1 cells [8], but not in SQCC/Y1 or Balbc/MK-2 cells [9]. Both the N-terminal and C-terminal PTHrP, but not the Mid region, transiently increase the $[Ca^{2+}]$i. This suggests that distinct PTHrP receptor(s) may recognize specific fragments in keratinocytes. Although the PTH-1R identified in rat keratinocytes and dermal fibroblasts has common points with the rat type-I PTH/PTHrP receptor, they act differently. The cAMP time response to PTHrP (1–34) is shorter in fibroblasts (5 min versus 10 min), and the Mid-region PTHrP increases the $[Ca^{2+}]$i only in fibroblasts. Our results suggest that PTHrP acts as a paracrine and an autocrine factor, affecting the proliferation and differentiation of both cell types via distinct effects on two different signaling pathways.

Vitamin D induces subtle changes in the differentiation of the epidermis by regulating its own receptors [7,8]. The active form of vitamin D is a potent inhibitor of PTHrP production in vitro by keratinocytes and in tumor cell lines [14]. The negative gene regulation by vitamin D may be linked to the presence of a negative vitamin D response element (nVDRE) in the upstream region of the PTH and PTHrP genes [18]. We have demonstrated that the regulation of PTHrP and PTH-1R depends on the skin cell type. Vitamin D deficiency enhances the expression of PTHrP in keratinocytes, but decreases it in adjacent fibroblasts as in kidney. Moreover, vitamin D deficiency increases the PTH-1R expression in kerarinocytes, but not in fibroblasts. Such a differential regulation by vitamin D had been reported in other cell types [13]. In vivo treatment of mice by $1,25(OH)_2D_3$ reduces the expression of PTH-1R in osteoblasts, but not in chondrocytes in spite of the presence of an active P2 promoter in both cells [14]. In conclusion, keratinocytes and skin fibroblasts

are target cells for PTHrP and express PTH-1R. However, the two adjacent cell types differ as regards their intracellular signaling in response to PTHrP (1–34), and various PTHrP peptides. Since vitamin D regulates PTHrP and PTH-1R in a cell-specific manner, vitamin D deficiency may impair local function of the fibroblasts in the skin PTH-1R system.

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References

[1] J.J. Merendino, K.L. Insogna, L.M. Milstone, A.E. Broadus, A.F. Stewart, Parathyroid hormone related-like protein from cultured human keratinocytes, Science 231 (1986) 388–390.
[2] M.F. Holick, J. Nussbaum, K.S. Persons, PTH-like humoral hypercalcemic factor may be an epidermal differentiation factor, J. Bone Miner. Res. 3 (Suppl) (1988) S214.
[3] A. Errazahi, Z. Bouizar, M. Lieberherr, E. Souil, M. Rizk-Rabin, Functional Type I PTH/PTHrP receptor in freshly isolated newborn rat keratinocytes: identification by RT/PCR and immunohistochemistry, J. Bone Miner. Res. 18 (2003) 737–750.
[4] Z. Huang, Y. Chen, R.A. Nissenson, The N-terminal region of the third intracellular loop of the parathyroid hormone related peptide receptor is critical for coupling to cAMP and inositol phosphate Ca^{2+} signal transduction pathway, J. Biol. Chem. 270 (1996) 151–156.
[5] J.E. Henderson, K. Kremer, J.S. Rhim, D. Goltzman, Identification of adenylate cyclase receptors for parathyroid hormone like peptides on immortalized human keratinocytes, Endocrinology 130 (1992) 449–457.
[6] J.F. Whitfield, R. Balu, B.R. Chakravarthy, J.P. Durkin, R.J. Issacs, H. Jouishome, M. Sikorska, R.E. Williams, R.H. Rixon, Parathyroid hormone stimulates protein kinase C but not adenyl cyclase in mouse epidermal keratinocytes, J. Cell Physiol. 150 (1992) 299–303.
[7] M. Rizk-Rabin, Z. Rougui, Z. Bouizar, M. Garabedian, J.H. Pavlovitch, Synthesis of and response to 1,25 dihydroxycholecalciferol by subpopulations of murine epidermal keratinocytes: existence of a paracrine system for 1,25 dihydroxycholecalciferol, J. Cell Physiol. 159 (1994) 131–141.
[8] Z. Rougui, Z. Bouizar, O. Walrant, M. Rizk-Rabin, Distinct, tissue specific regulation of vitamin D receptor in the intestine, kidney and skin by dietary calcium and vitamin D, Endocrinology 139 (1998) 1844–1852.
[9] R. Kremer, A.C. Karaplis, J. Henderson, W. Gulliver, D. Banville, G.N. Hendy, D. Goltzman, Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. Effect of growth factors and 1,25 dihydroxyvitamin D3 on gene expression and secretion, J. Clin. Invest. 87 (1991) 884–893.
[10] S. Fwojcik, C. Capen, T.J. Rosol, Expression of PTHrP and the PTH/PTHrP receptor in purified alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts derived from the lactating rat mammary gland, Exp. Cell Res. 248 (1999) 415–422.
[11] M. Sebag, J.E. Henderson, J. Rhim, R. Kremer, Relative resistance to 1,25 dihydroxyvitamin D_3 in a keratinocyte model of tumor progression, J. Biol. Chem. 267 (1992) 12162–12167.
[12] J. Henderson, D. Goltzman, M. Sebag, J. Rhim, R. Kremer, Dysregulation of parathyroid hormone-like peptide expression in a keratinocyte model of tumor cell progression, Cancer Res. 51 (1991) 6521–6528.
[13] G. Turner, C. Coureau, M. Rizk-Rabin, B. Escoubet, M. Hruby, O. Walrant, C. Silve, Parathyroid hormone (PTH/PTH-related protein receptor messenger ribonucleic acid expression and PTH response in a rat model of secondary hyperparathyroidism associated with vitamin D, Endocrinology 136 (1995) 3751–3758.
[14] D. Goltzman, J. White, R. Kremer, Studies of the effects of 1.25-dihydroxyvitamin D on skeletal and calcium homeostasis and on inhibition of tumor cells, J. Steroid Biochem. Mol. Biol. 76 (2001) 43–47.
[15] S.H. Yuspa, C.C. Harris, Altered differentiation of mouse epidermal cells treated with retinyl acetate in vitro, Exp. Cell Res. 86 (1974) 95–105.
[16] W. Lust, E. Dye, A.V. Deaton, J.V. Passonneau, A modified cyclic AMP binding assay, Ann. Biochem. 72 (1976) 8–15.
[17] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new gneration of Ca^{2+} indicators with greatly improved fluorescent properties, J. Biol. Chem. 260 (1985) 3440–3450.
[18] J. Russel, S. Ashok, N.J. Koszewski, Vitamin, D receptor interactions with the rat parathyroid hormone gene: synergistic effects between two negative vitamin D response elements, J. Bone Miner. Res. 14 (1999) 1828–1837.