Endocrine, *vol. 8, no. 2, 143–151, April 1998* 0969–711X/98/8:143–151/$10.25

Parathyroid Hormone-Related Protein Expression and Secretion in a Skin Organotypic Culture System

Eric A. G. Blomme,[1] James R. Werkmeister,[1] Hong Zhou,[2] Vicky Kartsogiannis,[2] Charles C. Capen,[1] and Thomas J. Rosol[1]

[1]Department of Veterinary Biosciences, The Ohio State University, Columbus, OH; and [2]Department of Medicine, University of Melbourne, Victoria, Australia

Parathyroid hormone-related protein (PTHrP), an important factor in the pathogenesis of humoral hypercalcemia of malignancy, is produced by many normal tissues, including the epidermis, where it is thought to play a role in the regulation of keratinocyte growth and differentiation. Most in vitro studies of normal keratinocytes use monolayer cell cultures, which have limitations, including the inability to reproduce the stratified structure of the epidermis. The objective of this study was to investigate PTHrP production and secretion, and mRNA expression in skin organotypic cultures. The cultures consisted of an artificial dermis with differentiating keratinocytes grown at the air–liquid interface. Immunohistochemical assessment of cytokeratins 14 and 10/13, involucrin, and proliferative cell nuclear antigen (PCNA) demonstrated that keratinocytes differentiated in a manner similar to keratinocytes in normal epidermis. PTHrP expression was demonstrated in all viable layers of the epidermis, as well as in some fibroblasts of the collagen lattice by immunohistochemistry and *in situ* hybridization. Since most fibroblasts expressed α-smooth muscle actin, these cells were interpreted to be consistent with myofibroblasts. PTHrP expression by myofibroblasts suggests a possible role for PTHrP in the regulation of contractibility of these cells. PTHrP was also detected in conditioned media for 50 days. In conclusion, because of its superior tissue morphology and ability to induce organized keratinocyte differentiation, this culture system will be an excellent model to study the role of PTHrP in pathologic and physiologic processes involving the epidermis in vitro.

Key Words: Keratinocyte; fibroblast; differentiation; growth; PTHrP; organotypic culture.

Received November 7, 1997; Revised January 12, 1998; Accepted January 12, 1998.

Author to whom all correspondence and reprint requests should be addressed: Dr. Thomas J. Rosol, The Ohio State University, 1925 Coffey Rd., Columbus, OH 43210. E-mail: rosol.1@osu.edu

Introduction

Parathyroid hormone-related protein (PTHrP) was initially purified from human tumors associated with the syndrome of humoral hypercalcemia of malignancy, and has been shown to play a central role in the pathogenesis of this paraneoplastic syndrome in human beings and animals *(1,2)*. Subsequently, PTHrP has been shown to be also produced by many normal tissues, where it acts as a paracrine/autocrine factor with many potential functions, including regulation of cell growth and differentiation, regulation of smooth muscle cell relaxation, and transepithelial calcium transport *(3)*.

PTHrP is expressed at high levels in the epidermis, where its major function is suspected to be the regulation of keratinocyte growth and differentiation *(4,5)*. For instance, PTHrP has been reported to inhibit keratinocyte proliferation in vitro *(4,6)*. In addition, systemic administration of PTHrP antagonists to hairless mice was associated with hair growth and increased keratinocyte proliferation *(6)*. The major role played by PTHrP in the maintenance of epidermal homeostasis was demonstrated by several in vivo studies using transgenic mice. Transgenic mice overexpressing PTHrP in their basal keratinocytes had defects in hair follicle development, whereas PTHrP "knock out" mice developed surface hyperkeratosis and sebaceous gland hypoplasia *(3,7,8)*. PTHrP acts on keratinocytes by binding to and activating an as yet uncharacterized receptor, which uses calcium ion as a second messenger *(9)*.

The skin is a two-compartment system, the epidermis and the dermis. Multiple, reciprocal, complex, and poorly characterized interactions between epidermal keratinocytes and dermal mesenchymal cells are involved to maintain the balanced homeostasis between growth and differentiation of both tissue compartments *(10)*. PTHrP produced by keratinocytes may interact with and regulate the function of dermal fibroblasts. Dermal fibroblasts express the classic PTH/PTHrP receptor, but usually do not express PTHrP *(3,11)*. It has been suggested that PTHrP is one of the cytokines involved in dermal-epidermal cooperativity *(3)*. This is supported by the findings of abnormal dermal

features in both PTHrP "knock out" mice and PTHrP-overexpressing transgenic mice *(3,7,8)*.

Most in vitro studies to investigate the potential role of PTHrP in the epidermis have been performed using monolayer cultures of normal or transformed keratinocytes *(3,5,12)*. These in vitro experiments have limitations to study the potential paracrine effect of PTHrP. Moreover, depending on the culture conditions (e.g., calcium concentration, presence of serum, nature of the substratum), these cultures are associated with different degrees of keratinocyte differentiation *(12–15)*. Finally, monolayer cultures do not reproduce the well-organized structure of a mature squamous cornifying epithelium *(10)*. The objective of organotypic cultures is to develop cellular models that exhibit as many properties of the organ from which they were derived as possible *(16,17)*. In the case of skin, this entails the formation of a dermal component consisting of dermal fibroblasts embedded in extracellular matrix overlaid with a stratified, cornified epidermis *(16)*. The extracellular matrix and dermal fibroblasts have major influences on keratinocyte proliferation and differentiation *(10,12)*. Air exposure is an additional important factor in the differentiation and proliferation of keratinocytes *(18–21)*. Cultures at the air–liquid interface, a condition thought to be more physiologic, facilitate keratinocyte differentiation and lead to the formation of a multilayered tissue, which, in many aspects, resembles the living epidermis *(16,20,22–24)*. In this study, we utilized a skin organotypic culture system, and characterized it by evaluating the expression of several epidermal and proliferation markers. We also used this system to evaluate the production and secretion of PTHrP and mRNA expression by immunohistochemistry, radioimmunoassay, and *in situ* hybridization.

Results

Histology of Reconstructed Skin

The histologic morphology of the reconstructed skin was evaluated at different time-points. After 3 d at the air–liquid interface, the epidermis was composed of a basal layer and thin spinous and cornified layers. The dermal equivalent consisted of loose collagen containing dispersed fibroblasts. After 6 d at the air–liquid interface, the histologic appearance was similar to that of normal epidermis with basal, spinous, and granular layers in adequate proportions. The cornified layer was more compact than normal and did not have the typical basket-weave appearance (Fig. 1A). The morphology was still satisfactory after 9 d at the air–liquid interface, although the cornified layer appeared proportionally thicker than normal, and scattered keratinocyte nests could be seen infiltrating the dermal equivalent with formation of rete ridge-like structures.

Immunohistochemistry of Reconstructed Skin

Immunohistochemistry was performed on cultures after 6 d at the air–liquid interface, since this time-point was associated with the optimal morphology. Involucrin and cytokeratins 10/13 were detected in the upper spinous layers (Fig. 1B,C). Cytokeratin 14 was expressed diffusely throughout all viable layers of the epidermis, although expression was greatest in the basal layer (Fig. 1D). Expression of proliferative cell nuclear antigen (PCNA) was frequent in basal cells, but could also be detected in few suprabasal cells (Fig. 1E). Approximately half of the fibroblasts expressed α-smooth muscle actin, which was especially prominent in the fibroblasts from the superficial part of the dermal equivalent (Fig. 1F).

PTHrP Expression and Secretion by Reconstructed Skin

PTHrP was demonstrated in all viable layers of the reconstructed epidermis with both N-terminal and midregion polyclonal antibodies. With the midregion antibody, there was no difference in staining intensity among the epidermal layers (Fig. 2A). However, when the N-terminal antibody was used, basal cells and the superficial spinous layer were stained with greater intensity (Fig. 2B). Nests of keratinocytes infiltrating the dermal equivalent were usually intensely stained, and staining of most fibroblasts was also evident (Fig. 2A,B).

In situ hybridization using a poly-T probe was associated with staining of dermal fibroblasts and all viable layers of the epidermis, confirming adequate preservation of mRNA in the samples (Fig. 3A). PTHrP mRNA could be detected in all keratinocytes composing the basal, spinous, and granular layers of the epidermis (Fig. 3B). Expression was slightly stronger in the basal cells and the superficial part of the spinous layer. Keratinocytes infiltrating the dermal equivalent were usually intensely positive, and there was faint staining of most fibroblasts in the dermal equivalent. PTHrP was not detected in conditioned medium from contracted collagen lattices without keratinocytes. However, PTHrP was detected in conditioned medium for up to 50 d after keratinocytes were seeded on contracted collagen lattices. PTHrP secretion remained high during this entire period (Fig. 4).

Discussion

The skin organotypic cultures were similar histologically to normal epidermis with an optimal morphology 6 d after lifting the cultures to the air–liquid interface. Keratinocytes differentiated in an organized manner in this culture system. Involucrin is often used as a specific marker of terminal differentiation of keratinocytes *(25)*. In normal epidermis, involucrin is first expressed in differentiating keratinocytes of the upper stratum spinosum. Cytokeratin 10 is a type I keratin expressed in differentiating layers of the normal epidermis, and its expression is indicative of differentiation *(13,14,26)*. Involucrin and cytokeratin 10 expression in our cultures suggested that the keratinocytes were undergoing differentiation in a manner similar to that of normal epidermis.

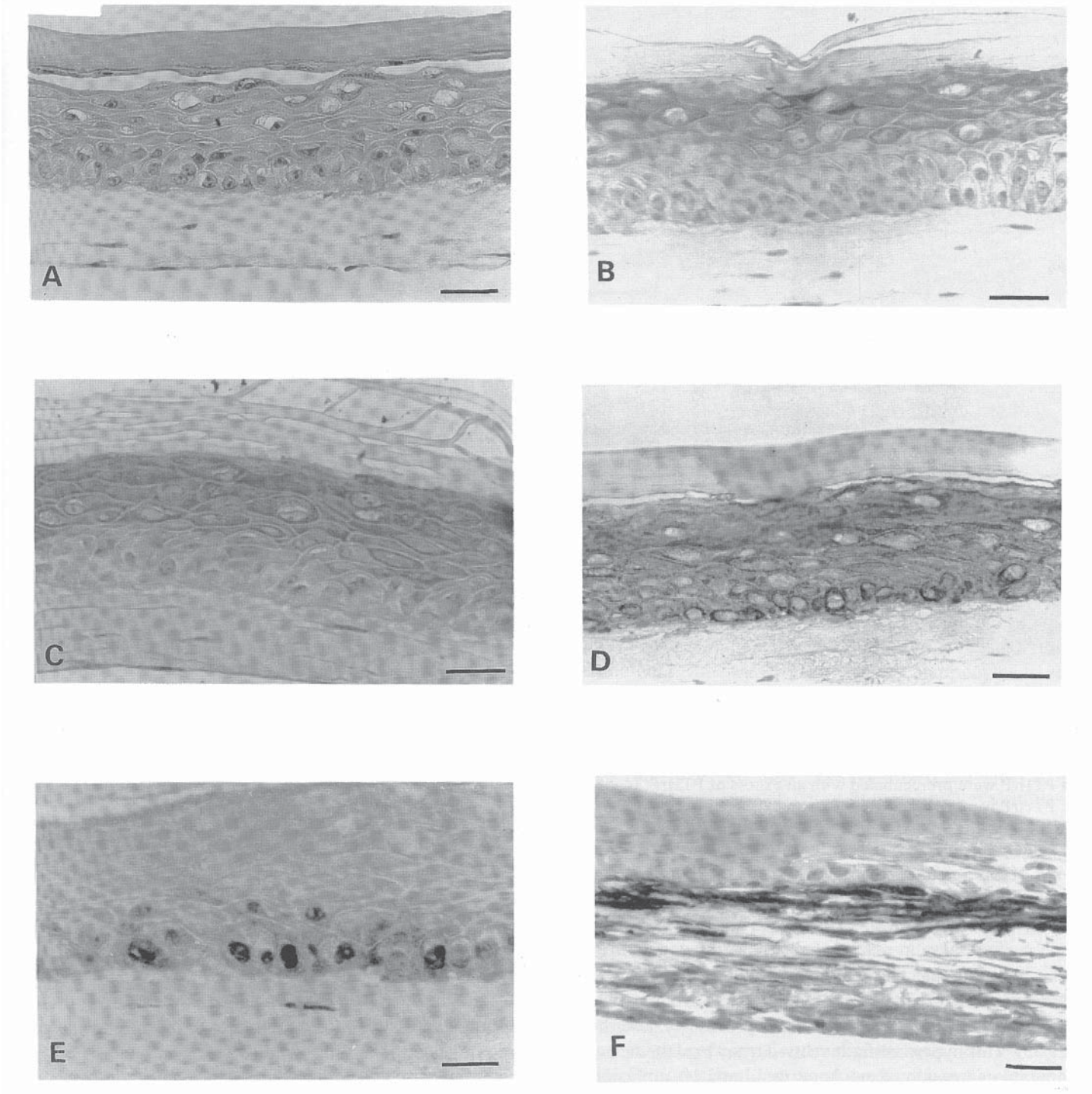


Fig. 1. Human skin organotypic cultures. **(A)** Histologic appearance showing the presence of basal, spinous, granular, and cornified layers. **(B)** Involucrin immunohistochemistry. Note the staining of the upper spinous layer. **(C)** Cytokeratin 10/13 immunohistochemistry showing staining of the upper spinous layer. **(D)** Cytokeratin 14 immunohistochemistry. Cytokeratin 14 was expressed diffusely throughout all viable layers of the reconstructed epidermis. **(E)** PCNA immunohistochemistry. Nuclei of frequent basal cells and few suprabasal cells were positively stained. **(F)** α-Smooth muscle actin immunohistochemistry. The cytoplasm of many fibroblasts of the dermal equivalent were positively stained. Scale bar, 100 μm (A, B, C, D, and F), 67 μm (E).

Despite a close histologic similarity to normal epidermis with evidence of organized differentiation, these organotypic cultures did not completely mimic normal epidermis. Cytokeratin 14 is a type I keratin expressed in basal cells of normal epidermis *(26)*. The protracted expression of cytokeratin 14 in our cultures in suprabasal cells indicates that the differentiation process was not as complete as suggested by expression of the differentiation markers, involucrin and cytokeratin 10 *(22)*.

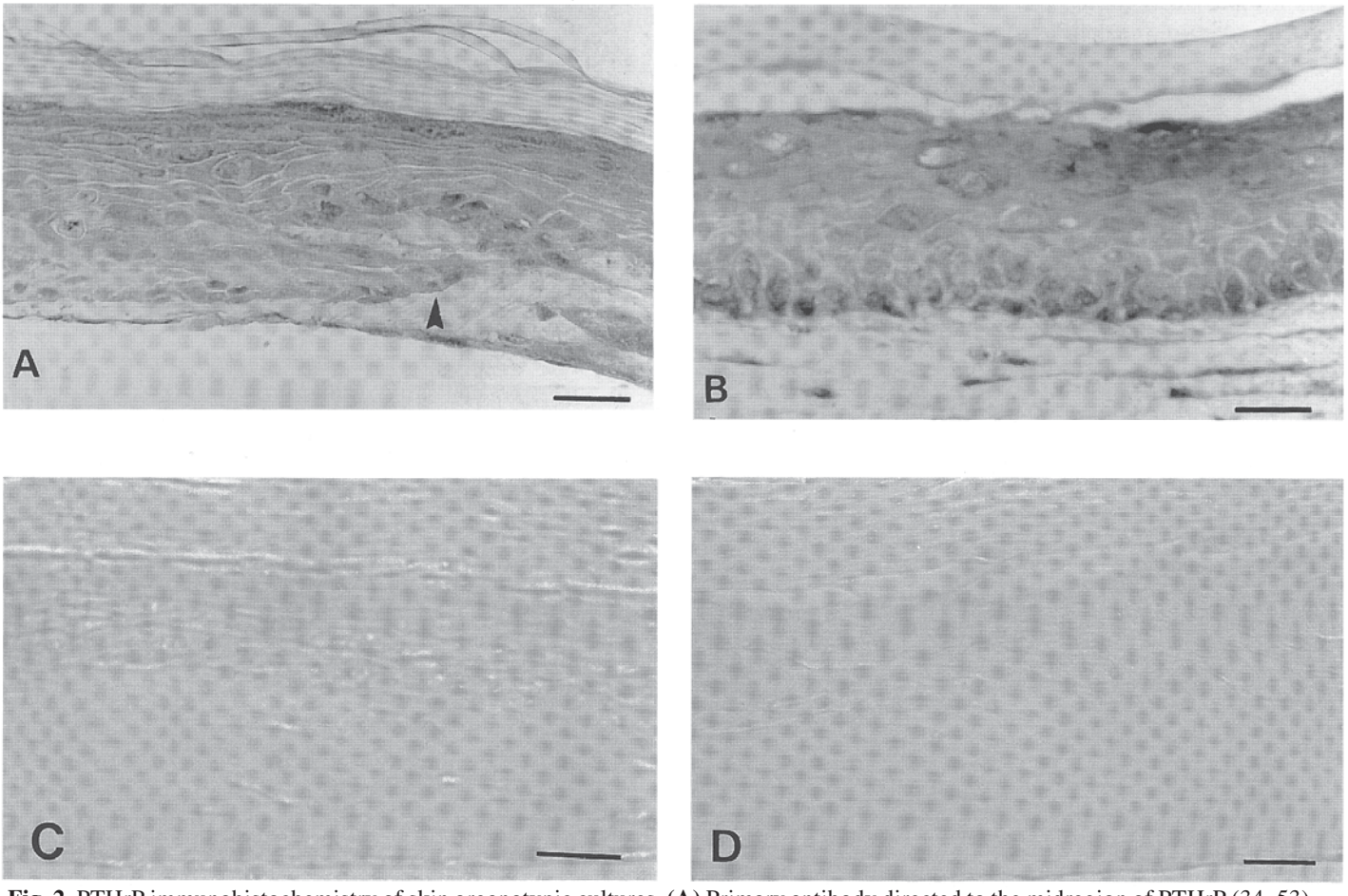
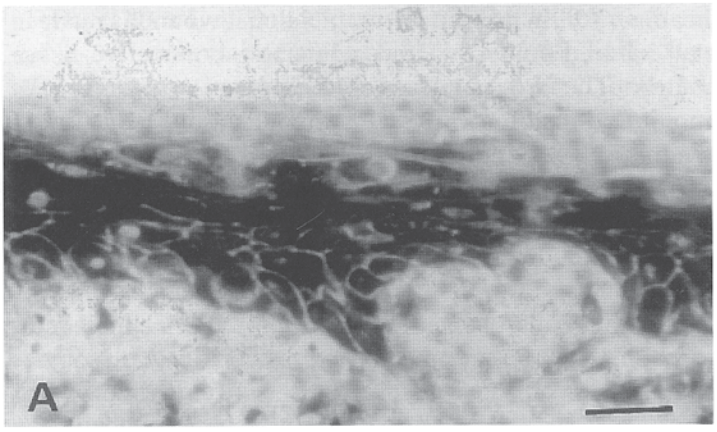
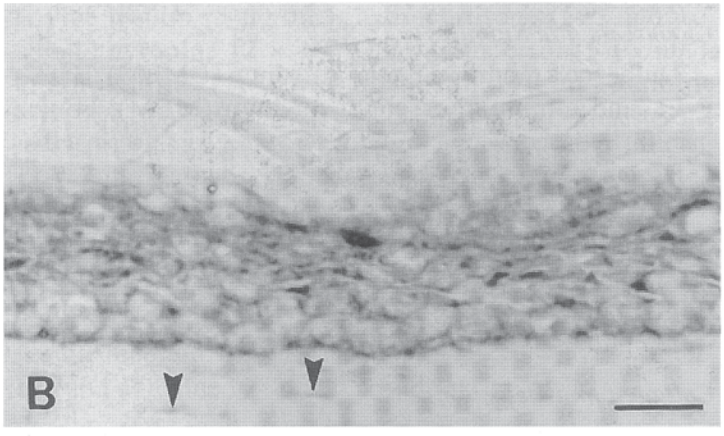


Fig. 2. PTHrP immunohistochemistry of skin organotypic cultures. **(A)** Primary antibody directed to the midregion of PTHrP (34–53). Note the staining of all viable epidermal layers and fibroblasts, and the more intense staining of keratinocytes infiltrating the dermal equivalent (arrowhead). **(B)** Primary antibody directed to the N-terminal region of PTHrP (1–36). Basal cells and the upper spinous layer were stained with greater intensity. Note the staining of dermal fibroblasts. **(C)** Negative control: anti-PTHrP antibodies to the midregion of PTHrP were preincubated with an excess of PTHrP(34–53). **(D)** Negative control: anti-PTHrP antibodies to the N-terminal region of PTHrP were preincubated with an excess of PTHrP(1–36). Scale bar, 100 μm.

PCNA is a marker expressed by cells in the proliferative fraction of a tissue. PCNA is expressed in only few basal cell nuclei of normal skin *(27)*. In the skin equivalent cultures, PCNA expression in many basal cells and few suprabasal cells indicates that the keratinocytes of the reconstructed epidermis were in a hyperproliferative mode as compared to keratinocytes of normal epidermis, as previously described *(22,23)*. This hyperproliferative mode may explain the concomitant expression of cytokeratins 14 and 10, a phenomenon not seen in vivo. Although not evaluated in this study, several other differences have been reported between similar types of skin cultures and normal human skin. This includes expression of keratins associated with hyperproliferation (keratins 6, 16, or 19), different lipid content and composition, and increased permeability of the stratum corneum *(10,16,22–24)*. However, the biological response of such cultures to many different chemicals has been reported to have similarities to normal skin. Therefore, these models are useful tools for in vitro research and testing, as long as their limitations are properly recognized *(16,23,24)*.

PTHrP was demonstrated in all viable layers of the reconstructed epidermis using two polyclonal antibodies directed toward different epitopes, as previously reported in normal skin *(28)*. Slight differences in the spatial intensity of expression were noted between the two antibodies. With the antibody directed toward the midregion of the peptide, expression appeared greater in the spinous layer. In contrast, with the antibody directed toward the N-terminus of the peptide, staining of the basal cells was particularly intense, especially at the dermo-epidermal junction. This feature has been reported previously with these antibodies in canine epidermis *(1)*. The reason for this difference is not certain. PTHrP is a protein subjected to posttranslational processing *(3)*. In keratinocytes, at least three distinct peptide species have been shown to be produced *(3)*. Keratinocytes are capable of cleaving PTHrP at Arg-37 and, consequently, produce both PTHrP(1-36) and a midregion fragment beginning at amino acid 38 *(29)*. The difference noted between the two antibodies might reflect different posttranslational processing between keratino-





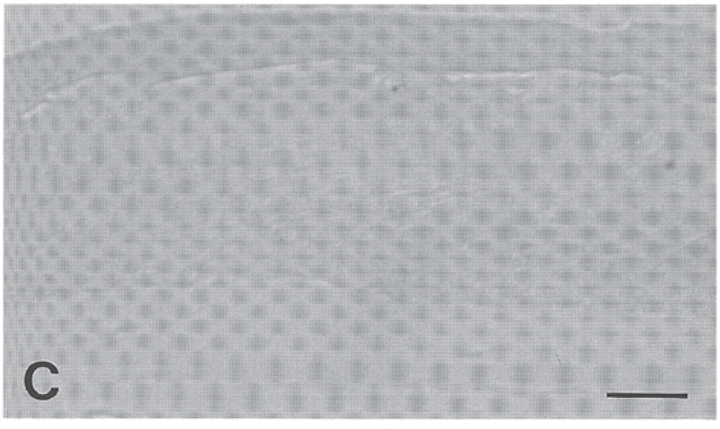


Fig. 3. *In situ* hybridization of skin organotypic cultures. **(A)** Using a poly-T probe, there was intense staining of dermal fibroblasts and viable epidermal layers for mRNA. **(B)** Using a PTHrP-specific probe, PTHrP mRNA was detected in keratinocytes of the basal, spinous, and granular layers. Expression was slightly greater in the basal cells and the upper spinous layer. Dermal fibroblasts were faintly stained (arrowheads). **(C)** Negative control: sections were hybridized with a sense riboprobe. Treatment with RNase A prior to prehybridization also resulted in loss of staining. Scale bar, 100 μm.

cytes at different stages of differentiation, as suggested for the human prostate *(30)*. PTHrP mRNA was also detected in all viable layers of the reconstructed epidermis, a pattern

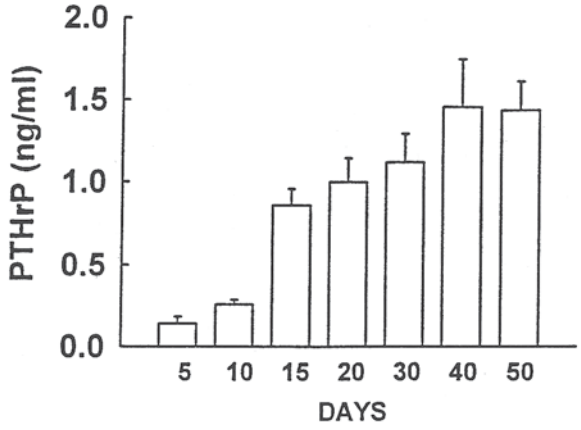


Fig. 4. PTHrP secretion into conditioned medium. PTHrP was detected in conditioned medium for up to 50 d after keratinocytes were seeded on contracted collagen lattices. However, PTHrP was not detected in conditioned medium from contracted collagen lattices with no keratinocytes. Mean ± SD, $n = 3$.

similar to the normal human epidermis *(31)*. The similarity between protein and mRNA expression strongly supports the concept that in keratinocytes PTHrP acts as an autocrine or intracrine factor. The intense staining of keratinocytes infiltrating the dermis was particularly interesting. The significance of this finding is not certain. It seems unlikely that this phenomenon was a staining artifact, as *in situ* hybridization confirmed this result. These keratinocytes have a migratory phenotype *(23)* and PTHrP might, therefore, have a potential role in the migration of keratinocytes. This possibility is supported by the finding that squamous cell carcinomas, with infiltrative properties, frequently overexpress PTHrP and can cause humoral hypercalcemia of malignancy in human beings *(31,32)*. Alternatively, the increased PTHrP expression may have been secondary or incidental to the migratory phenotype of the keratinocytes. For instance, it is likely that infiltrating keratinocytes, because of their different tissue location, were subject to different signals from dermal fibroblasts and the collagenous matrix. Further studies are needed to answer this question.

The detection of high levels of PTHrP in conditioned media for up to 50 d contrasted with results using monolayer cultures of normal and malignant keratinocytes, where PTHrP production and secretion has been shown to be decreased at cell confluence and at the onset of cellular differentiation *(5,12,33)*. This difference of pattern indicates that the culture system has a major influence on PTHrP secretion and production by keratinocytes. PTHrP production and secretion by squamous carcinoma cells have been shown to be induced by paracrine factors originating from interactions with fibroblasts *(34)*. The prolonged PTHrP secretion in organotypic cultures was probably owing, at least in part, to the regulatory effects of fibroblasts on PTHrP production by normal keratinocytes. However, other factors may also have influenced PTHrP secretion.

For instance, we have documented that the nature of the extracellular matrix influenced the phenotype of and PTHrP production by normal human keratinocytes *(12)*. Because of their closer similarity to normal physiologic conditions, skin organotypic cultures may provide more meaningful data for PTHrP expression and secretion in vitro.

The positive staining of fibroblasts for PTHrP was unexpected, since dermal fibroblasts are among the few cell types reported not to express PTHrP *(3)*. Positive staining was seen by immunohistochemistry (with two different antibodies) and by *in situ* hybridization, and therefore, is unlikely to be nonspecific. However, PTHrP was not detected in conditioned medium from contracted collagen lattices with no keratinocytes, suggesting that although fibroblasts expressed PTHrP, they did not secrete significant amounts of the peptide into the culture medium. We have observed PTHrP expression by dermal fibroblasts using immunohistochemistry and *in situ* hybridization in cells that have acquired a contractile phenotype, such as granulation tissue myofibroblasts (data not published). Therefore, we evaluated α-smooth muscle actin expression by the fibroblasts of the dermal equivalent. α-Smooth muscle actin is the actin isoform present in smooth muscle cells and myoepithelial cells, and its expression is considered to be characteristic of the myofibroblastic phenotype *(35)*. Many of the fibroblasts in the dermal equivalent expressed α-smooth muscle actin and were consistent with myofibroblastic cells. The reason for this is not certain, since many aspects of myofibroblast biology are unknown, such as the factors regulating the expression of α-smooth muscle actin *(36)*. It has been reported that fibroblastic cells isolated from the dermis are a heterogenous population, which contains between 7 and 20% of cells expressing α-smooth muscle actin *(37)*. In addition, heparin and transforming growth factor-β1 have previously been shown to induce α-smooth muscle actin expression by dermal fibroblasts, but it is likely that other factors may be involved as well *(36,38)*. In organotypic cultures, fibroblasts are actively involved in the contraction of the collagen lattice and, therefore, play a role similar to the retractile role of myofibroblasts in scars and other fibrotic lesions *(36,39)*. Based on this apparent association between the myofibroblastic phenotype and PTHrP expression, it can be speculated that PTHrP participates in the regulation of contraction of these cells, as it does in smooth muscle cells of the vascular, gastrointestinal, or uterine system for instance *(3)*. Further studies are necessary to confirm this potential function.

In conclusion, the skin organotypic culture model appears similar to normal human skin with respect to its histologic appearance, differentiation process, and expression of PTHrP mRNA and protein expression. This type of culture should prove valuable to gain new insights into the function of PTHrP in the epidermis. For instance, a composite organotypic coculture wounding model has previously been used to study the role of cytokines in the process of wound healing *(40)*. In this model, an incisional wound is made in the cultured epidermis and supporting connective tissue matrix. The process of re-epithelialization can then be monitored histologically. This model should be useful to evaluate the spatial and temporal expression of PTHrP mRNA and protein during re-epithelialization in an environment devoid of the multiple cytokines derived from the blood and migratory inflammatory cells. Moreover, treatment of these cultures with various cytokines, pharmaceutical compounds, or chemicals will help to define molecules affecting PTHrP secretion and expression by keratinocytes organized in a properly structured cornifying epidermis.

Materials and Methods

Chemicals and Supplies

Dulbecco s modified Eagle's medium (DMEM), Ham's F-12, and minimum essential medium (MEM), bovine insulin, hydrocortisone, triiodothyronine, transferrin, ethanolamine, *o*-phosphorylethanolamine, adenine, glutamine, MEM essential amino acids, MEM nonessential amino acids, MEM vitamins, and progesterone were purchased from Sigma (St. Louis, MO). Fetal bovine serum, 100X sodium pyruvate, and keratinocyte serum-free medium were purchased from Gibco-BRL (Grand Island, NY).

Collagen and Cells

Sterile, acid-extracted type I collagen was derived from rat tail tendon as previously described *(39)* and used at 1.5 mg/mL in 0.1 % acetic acid, as determined by the BCA protein assay (Pierce, Rockford, IL). Human dermal fibroblasts were derived from trypsin-collagenase dissociation of newborn foreskin and propagated in B medium supplemented with 10% fetal bovine serum. B medium consisted of MEM supplemented with MEM essential amino acids (20 mL/L), MEM nonessential amino acids (10 mL/L), MEM vitamins (5 mL/L), and 100X sodium pyruvate (10 mL/L). Human epidermal keratinocytes were derived from newborn foreskin by trypsinization and maintained in keratinocyte serum-free medium as previously described *(41)*.

Formation of the Dermal Lattice

Dermal lattices were made using 30 mm diameter, 0.4 μm pore-sized, culture plate inserts (Millipore, Bedford, MA) suspended in a maintenance tray (Organogenesis, Canton, MA) *(17)*. Briefly, a mixture of 2.6 mL 10X B medium, 2.5 mL fetal bovine serum, and 700 μL buffer (4.77g HEPES, 2.2 g sodium bicarbonate, 0.05 *N* sodium hydroxide/100 mL) was prepared. This mixture (5.5 mL) was added to 18 mL of collagen solution on ice. The final mixture (1 mL) was spread over the surface of the culture insert, and gelation was allowed to occur at room temperature for at least 10 min. B medium (2 mL) containing 2.5×10^5 fibroblasts/mL was mixed in the rest of the mixture, and 3 mL of the resulting cell suspension were spread over the gelled acellular layer

of each insert. After an incubation of 10 min at room temperature, 13 mL of B medium were added to the outside of the insert in the maintenance tray. The collagen lattice was then allowed to contract in the incubator at 37°C for 4–7 d.

Formation of the Reconstructed Epidermis

Human foreskin keratinocytes (50 µL, 3×10^6 cells/mL) at low passage (2 to 5) were pipeted on the surface of the contracted collagen lattices after removing the medium from the maintenance tray. After an incubation period of at least 1 h at 37°C, 13 mL of growth medium were added to the outside of the insert, submerging the cultures. The growth medium was adapted with modifications from established protocols *(17)*. It consisted of a 3:1 mixture of DMEM and Ham's F-12 medium supplemented with bovine insulin (5 µg/mL), hydrocortisone (500 ng/mL), triiodothyronine (2×10^{-11} *M*), transferrin (5 µg/mL), ethanolamine (10^{-7} *M*), *o*-phosphorylethanolamine (10^{-7} *M*), adenine (1.8 m*M*), glutamine (7 m*M*), progesterone (2×10^{-15} *M*), and 0.3% fetal bovine serum.

After 5 d of being submerged in growth medium, the medium was removed, and cornification medium was added so that the reconstructed epidermis was at the air–liquid interface. The cornification medium was changed every 3 d. The cornification medium was similar to the growth medium, but was not supplemented with progesterone and contained 2% fetal bovine serum *(17)*.

Morphological and Immunohistochemical Analysis

Inserts containing the cultures were fixed in 4% paraformaldehyde for 2 h and were routinely processed and embedded in paraffin or were frozen in liquid nitrogen and embedded in O.C.T. Compound (Miles, Naperville, IL). Paraffin sections (5 µm) were used for histology, immunohistochemistry, and *in situ* hybridization, except for cytokeratin 10/13 where frozen sections (5 µm) were used.

For immunohistochemistry, sections were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol, and endogenous peroxidase was blocked with 1.5% hydrogen peroxide for 15 min. All primary antibodies were diluted in phosphate-buffered saline (PBS). Primary antibodies were used to involucrin (Biomedical Technologies, Stoughton, MA; working dilution 1:200), cytokeratin 10/13 (Novocastra Laboratories, Newcastle, UK; working dilution 1:50), cytokeratin 14 (Novocastra Laboratories; working dilution 1:20), PCNA (Oncogene Sciences, Manhasset, NY, working dilution 1:200), α-smooth muscle actin (Boehringer Mannheim, Indianapolis, IN; working dilution 1:100), and PTHrP. For PTHrP, two primary antibodies to different epitopes were used. These included a rabbit polyclonal antibody (Oncogene Sciences; working dilution 1:100) directed to the midregion of the molecule (amino acids 34–53), and a chicken polyclonal antibody directed to the N-terminus of the molecule (amino acids 1–36) prepared in our laboratory as previously described *(1)*. Universal Secondary Antibody (Research Genetics, Huntsville, AL) was used as secondary antibody for cytokeratin 10/13, cytokeratin 14, and α-smooth muscle actin detection, whereas biotin-labeled goat antirabbit IgG antibody (Zymed, San Francisco, CA), and biotinylated rabbit antichicken IgG antibody (Zymed) were used for detection of involucrin and PTHrP. Slides were incubated with avidin–biotin–horseradish peroxidase complex (ImmunoPure Ultra-Sensitive ABC Staining Kit, Pierce), and peroxidase activity was detected by incubating the sections for up to 4 min in Stable DAB (Research Genetics). Normal human foreskin or breast reduction tissue was used as positive controls. Primary anti-PTHrP antibodies were preincubated with PTHrP (1–36) or PTHrP (34–53) (500 µg/mL) as negative controls to demonstrate specificity of immunohistochemical staining.

Preparation of Riboprobe

A PTHrP riboprobe corresponding to the common coding exon VI was obtained by subcloning a 422-bp genomic PCR fragment, corresponding to the region +102 to +524 of the human PTHrP gene *(42)*, into the *Eco*RI site of pAM-19 (Amersham International, Buckinghamshire, UK) in both orientations. The plasmid was linearized with *Bam*HI and transcribed with either T7 or Sp6 RNA polymerase to yield antisense and sense riboprobes, respectively. The riboprobes were labeled with digoxigenin (DIG) during RNA transcription using an RNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions.

In Situ *Hybridization*

In situ hybridization for total mRNA was performed using the Microprobe System (Fischer Scientific, Pittsburgh, PA) to confirm the retention of mRNA in the tissue sections. Briefly, sections were deparaffinized with Auto Dewaxer (Research Genetics), rehydrated with Auto Alcohol (Research Genetics), buffered with Universal Buffer (Research Genetics), and digested with Pepsin Solution (Research Genetics) for 3 min at 105°C. Hybridization was performed at 45°C for at least 60 min using Poly T Probe (Research Genetics). Posthybridization washing was performed at 45°C for 5 min using Post Hybe Wash (Research Genetics). Streptavidin AP Detection System (Research Genetics) was applied for 5 min at 50°C and alkaline phosphatase activity was visualized by incubation for 20 min at 50°C in BCIP/NBT solution substrate (Amresco, Solon, OH).

In situ hybridization for PTHrP mRNA was performed as previously described with minor modifications *(43)*. Sections were dewaxed with xylene and rehydrated with decreasing concentrations of ethanol before rinsing in diethylpyrocarbonate-treated water (DEPC-H2O). Deproteinization was carried out with 0.2 *M* HCl for 20 min at room temperature followed by digestion with proteinase K at 5–10 µg/mL in 0.1 *M* Tris buffer (pH 8.0)/50 m*M* EDTA for 30 min at 37°C. Proteinase K was inactivated by the

addition of 2 mg/mL glycine in PBS for 5 min. Tissue sections were postfixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Prehybridization was performed at 37°C for 1 h in hybridization buffer (50% formamide, 5X SSC, 2% blocking reagent [Boehringer Mannheim], 0.1% *N*-lauroyl-sarcosine, and 0.02% sodium dodecyl sulfate). Hybridization was performed at 42°C for 16–18 h in a humidified chamber with hybridization buffer containing the DIG-labeled probe at a final concentration of 1–4 ng/μL. After hybridization, slides were washed in 2X SSC at 37°C for 15 min, treated with 25 μg/mL RNase A (Sigma) in 2X SSC for 30 min at 37°C to remove nonhybridized RNA, and successively washed once in 2X SSC, twice in 1X SSC, and twice in 0.1X SSC at 37°C for 15 min each. The hybridized probe was detected using alkaline phosphatase-coupled anti-DIG antibody (Boehringer Mannheim) after blocking for nonspecific binding with a combination of 30% rabbit serum, 3% bovine serum albumin (Sigma), and 0.1% Triton X-100 (Sigma) in PBS for 30 min. Alkaline phosphatase activity was detected with BCIP/NBT solution substrate (Boehringer Mannheim) and sections were counterstained with nuclear fast red.

Negative controls consisted of sections hybridized with a sense riboprobe or treated for 2 h at 37°C with 100 μg/mL RNase A (Sigma) in 2X SSC prior to prehybridization.

PTHrP Nonequilibrium Radioimmunoassay (RIA)

The RIA for PTHrP was performed as described by our laboratory using chicken polyclonal anti-PTHrP-(1–36) antibody *(44)*. [Tyr^{36}]-PTHrP-(1–36) (Bachem, Torrance, CA) was radioiodinated with ^{125}I and Iodogen (Pierce) and purified using reverse-phase HPLC. Polyclonal chicken anti-PTHrP-(1–36) antibodies (10 mg/mL in PBS, 1:1000 dilution) were added to borosilicate tubes containing standards or medium samples (100 μL) and assay buffer (20 m*M* sodium phosphate, pH 7.4, 140 m*M* NaCl, 50 m*M* EDTA, 0.5% BSA, 0.1 sodium azide) and were incubated for 48 h at 4°C. Iodinated PTHrP (1–36) was added (10,000 cpm/tube) and dextran-coated charcoal (5 mg/mL activated charcoal, 0.5 mg/mL dextran) was utilized to separate the bound from free PTHrP after 48 h. The supernatant was measured in a γ-radiation counter and PTHrP content quantified by log-logit transformation using Securia 1.0 (Packard Instruments, Downers Grove, IL). The RIA was sensitive to 0.1 ng PTHrP (1–36)/mL of medium.

Acknowledgments

The investigation was supported by the National Institutes of Arthritis, Musculoskeletal, and Skin Diseases and of Dental Research (AR40220, AR01923, DE00321). E. A. G. Blomme is supported by a fellowship from Rhône-Poulenc-Rorer Pharmaceuticals, Inc.

References

1. Groene, A., Werkmeister, J. R., Steinmeyer, C. L., Capen, C. C., and Rosol, T. J. (1994). *Vet. Pathol.* **31,** 308–315.
2. Rosol, T. J. and Capen, C. C. (1992). *Lab. Invest.* **67,** 680–702.
3. Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Holt, E., Orloff, J., Yang, K. H., et al. (1996). *Physiol. Rev.* **76,** 127–173.
4. Kaiser, S. M., Sebag, M., Rhim, J. S., Kremer, R., and Goltzman, D. (1994). *Mol. Endocrinol.* **8,** 139–147.
5. Werkmeister, J. R., Merryman, J. I., McCauley, L. K., Horton, J. E., Capen, C. C., and Rosol, T. J. (1993). *Exp. Cell Res.* **208,** 68–74.
6. Holick, M. F., Ray, S., Chen, T. C., Tian, X., and Persons, K. S. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 8014–8016.
7. Wysolmerski, J. J., Philbrick, W. M., Zhou, J., Broadus, A. E., and Milstone, L. M. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 1133–1137.
8. Foley, J., Wysolmerski, J. J., Longley, J. B., Dreyer, B. E., Broadus, A. E., and Philbrick, W. M. (1997). *J. Bone Miner. Res.* **12(Suppl. 1),** S163.
9. Orloff, J., Ganz, M. B., Ribaudo, A. E., Burtis, W. J., Reiss, M., Milstone, L. M., and Stewart, A. F. (1992). *Am. J. Physiol.* **262,** E599–E607.
10. Fusenig, N. E. (1994). In: *The Keratinocyte Handbook*. Leigh, I. M., Lane, E. B., and Watt, F. M. (eds.). Cambridge University Press: London, pp. 71–94.
11. Hanafin, N. M., Chen, T. C., Heinrich, G., Segre, G. V., and Holick, M. F. (1995). *J. Invest. Dermatol.* **105,** 133–137.
12. Blomme, E. A. G., Weckmann, M. T., Capen, C. C., and Rosol, T. J. (1997). *Exp. Cell Res.* **238,** 204–215.
13. Van Muijen, G. N. P., Warnaar, S. O., and Ponec, M. (1987). *Exp. Cell Res.* **171,** 331–345.
14. Poumay, Y. and Pittelkow, M. R. (1995). *J. Invest. Dermatol.* **104,** 271–276.
15. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980). *Cell* **19,** 245–254.
16. Parenteau, N. L., Bilbo, P., Nolte, C. J. M., Mason, V. S., and Rosenberg, M. (1992). *Cytotechnology* **9,** 163–171.
17. Parenteau, N. L. (1994). In: *The Keratinocyte Handbook, Methods Section*. Leigh, I. and Watt, F. (eds.). Cambridge University Press: London, pp. 45–55.
18. Sugihara, H., Toda, S., Miyabara, S., Kusaba, Y., and Minami, Y. (1991). *In Vitro Cell Dev. Biol.* **27A,** 142–146.
19. Font, J., Braut-Boucher, F., Pichon, J., Noel-Hudson, M. S., Muriel, M. P., Bonnet, M., et al. (1994). *Cell. Biol. Toxicol.* **10,** 353–359.
20. Williams, M. L., Brown, B. E., Monger, D. J., Grayson, S., and Elias, P. M. (1988). *J. Cell. Physiol.* **136,** 103–110.
21. Asselineau, D., Bernhard, B., Bailly, C., and Darmon, M. (1985). *Exp. Cell. Res.* **159,** 536–539.
22. Wilson, J. L., Dollard, S. C., Chow, L. T., and Broker, T. R. (1992). *Cell Growth Differ.* **3,** 471–483.
23. Ponec, M. (1995). *ATLA* **23,** 97–110.
24. Bilbo, P. R., Nolte, C. J. M., Oleson, M. A., Mason, V. S., and Parenteau, N. L. (1993). *J. Toxicol.-Cut Ocular Toxicol.* **12,** 183–196.
25. Watt, F. M. and Phil, D. (1983). *J. Invest. Dermatol.* **81,** 100s–103s.
26. Steinert, P. M. (1993). *J. Invest. Dermatol.* **100,** 729–734.
27. Furukawa, F., Imamura, S., Fukita, M., Kinoshita, K., Yoshitake, K., Brown, W. R., et al. (1992). *Arch. Dermatol. Res.* **284,** 86–91.
28. Atillasoy, E. J., Burtis, W. J., and Milstone, L. M. (1991). *J. Invest. Dermatol.* **96,** 277–280.
29. Soifer, N. E., Dee, K. E., Insogna, K. L., Burtis, W. J., Matovcik, L. M., Wu, T. L., et al. (1992). *J. Biol. Chem.* **267,** 18,236–18,243.

30. Cramer, S. D., Chen, Z., and Peehl, D. M. (1996). *J. Urol.* **156,** 526–531.
31. Danks, J. A., McHale, J. C., Clark, S. P., Chou, S. T., Scurry, J. P., Ingleton, P. M., et al. (1995). *J. Histochem. Cytochem.* **43,** 5–10.
32. Burtis, W. J., Brady, T. G., Orloff, J., Ersbak, J. B., Warrel, R. P., Olson, B. R., et al. (1990). *N. Engl. J. Med.* **322,** 1106–1112.
33. Loewik, C. W., Hoekman, K., Offringa, R., Groot, C. G., Hendy, G. N., Papapoulos, S. E., et al. (1992). *J. Invest. Dermatol.* **98,** 198–203.
34. Hoekman, K., Loewik, C. W. G. M., v.d. Ruit, M., Kempenaar, J., Bijvoet, O. L. M., and Ponec, M. (1990). *Cancer Res.* **50,** 3589–3594.
35. Skalli, O., Schurch, W., Seemayer, T., Lagace, R., Montandon, D., Pittet, B., et al. (1989). *Lab. Invest.* **60,** 275–285.
36. Desmouliere, A., Geinoz, A., Gabbiani, F., and Gabbiani, G. (1993). *J Cell Biol.* **122,** 103–111.
37. Desmouliere, A., Rubbia-Brandt, L., Abdiu, A., Walz, T., Macieira-Coelho, A., and Gabbiani, G. (1992). *Exp. Cell. Res.* **201,** 64–73.
38. Desmouliere, A., Rubbia-Brandt, L., Grau, G., and Gabbiani, G. (1992). *Lab. Invest.* **67,** 716–726.
39. Bell, E., Ivarsson, B., and Merrill, C. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 1274–1278.
40. Garlick, J. A. and Taichman, L. B. (1994). *J. Invest. Dermatol.* **103,** 554–559.
41. Boyce, S. T. and Ham, R. G. (1985). *J. Tissue Cult. Methods.* **9,** 83–93.
42. Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., et al. (1987). *Science* **237,** 893–896.
43. Zhou, H., Choong, P., McCarthy, R., Martin, T. J., and Ng, K. W. (1994). *J. Bone Miner. Res.* **9,** 1489–1499.
44. Rosol, T. J., Steinmeyer, C. L., McCauley, L. K., Merryman, J. I., Werkmeister, J. R., Groene, A., et al. (1993). *Vet. Immunol. Immunopathol.* **35,** 321–337.