Rat Preputial Sebocyte Differentiation Involves Peroxisome Proliferator-Activated Receptors[1]

Robert L. Rosenfield, Alex Kentsis, Dianne Deplewski, and Nancy Ciletti

Departments of Pediatrics and Medicine and The Committee on Developmental Biology, University of Chicago Children's Hospital, Chicago, Illinois, U.S.A.

The hallmark of sebaceous epithelial cell (sebocyte) differentiation is the accumulation of fused neutral fat droplets. Very little sebocyte differentiation occurs, however, in primary or organ culture, even upon incubating with androgens, which are required for maturation *in vivo*. We hypothesized that sebocyte cell culture systems lack activators of the peroxisome proliferator-activated receptors that are involved in adipocyte differentiation. We here report that activation of peroxisome proliferator-activated receptor γ and α by their respective specific ligands, a thiazolidinedione and a fibrate, induced lipid droplet formation in sebocytes but not epidermal cells. Linoleic acid and carbaprostacyclin, both peroxisome proliferator-activated receptor δ and α ligand-activators, were more effective but less specific, stimulating lipid formation in both types of cells. Either was more effective than the combination of peroxisome proliferator-activated receptor γ and α activation, suggesting that peroxisome proliferator-activated receptor δ is involved in this lipid formation. Linoleic acid 0.1 mM stimulated significantly more advanced sebocyte maturation than any other treatment, including carbaprostacyclin, which suggests a distinct role of long chain fatty acids in sebocyte differentiation. Peroxisome proliferator-activated receptor γ1 mRNA was demonstrated in sebocytes, but not in epidermal cells; it was more strongly expressed in freshly dispersed than in cultured sebocytes. In contrast, peroxisome proliferator-activated receptor δ mRNA was expressed to a similarly high extent before and after culture in both sebocytes and epidermal cells. These findings are compatible with the concepts that peroxisome proliferator-activated receptor γ1 gene expression plays a unique role in the differentiation of sebocytes, while peroxisome proliferator-activated receptor δ activation and long chain fatty acids finalize sebocyte maturation and are capable of stimulating epidermal lipid formation. These findings have implications for the development of new modalities of treatment for acne vulgaris.
Keywords: androgen/epidermis/fatty acids/thiazolidinediones. J Invest Dermatol 112:226–232, 1999

Sebaceous cells (sebocytes) are specialized epithelial cells which terminally differentiate by accumulating neutral fat droplets until they burst, giving rise to the lipid-rich holocrine secretion, sebum (Wheatley, 1986). The *in vivo* growth and development of sebaceous glands, including the rat preputial gland, is absolutely dependent on the action of androgens like dihydrotestosterone (DHT) (Sherins and Bardin, 1971; Ebling *et al*, 1975; Mesquita-Guimaraes and Coimbra, 1981; Yarbrough *et al*, 1990). Preputial sebocytes form submicroscopic lipid droplets (Rosenfield and Deplewski, 1995) and express androgen receptors when grown in monolayer culture (Miyake *et al*, 1994); however, androgens have not had a clear effect on sebocyte differentiation in a variety of *in vitro* culture systems (Akamatsu *et al* 1992; Rosenfield *et al*, 1993; Zouboulis *et al*, 1994; Guy *et al* 1996). Therefore, the role of androgen in promoting lipogenesis has been questioned (Guy *et al*, 1996). These considerations led us to suspect that a downstream signal transduction pathway involved in the regulation of lipid metabolism was not being expressed in cultured sebocytes.

We hypothesized that mechanisms involved in lipogenesis during adipocyte differentiation may be similarly utilized in sebocyte differentiation. The role of peroxisome proliferator-activated receptors (PPAR) was of particular interest as they have been shown to be important in the regulation of lipid metabolism in fat and liver cells (Green, 1995; Schoonjans *et al*, 1996). Recently, it was demonstrated that expression and activation of a PPAR is necessary and sufficient to induce preadipocyte cell lines to form fat and thus undergo terminal differentiation into adipocytes (Tontonoz *et al*, 1994b). The most potent subtype is PPARγ, specifically the γ2 isoform. PPARα is less adipogenic (Tontonoz *et al*, 1994a). PPARδ, also termed FAAR (Amri *et al*, 1995) and highly homologous to hNUC-1 and less so to xPPARβ (Kliewer *et al*, 1994; Xing *et al*, 1995), does not activate adipogenesis (Brun *et al*, 1996).

PPAR were discovered as a subfamily of "orphan receptors" within the nonsteroid receptor family of nuclear hormone receptors (Mangelsdorf and Evans, 1995). The specific ligands which activate mammalian PPAR have recently been elucidated by means of gene

Manuscript received May 29, 1998; revised July 31, 1998; accepted for publication October 16, 1998.

Reprint requests to: R. L. Rosenfield, Departments of Pediatrics and Medicine and The Committee on Developmental Biology, University of Chicago Children's Hospital, 5841 S. Maryland Ave. (MC 5053), Chicago, IL 60637-1470.

Abbreviations: DHT, dihydrotestosterone; LIN, linoleic acid; PPAR, peroxisome proliferator-activated receptor.

[1]Preliminary reports were presented at IV International Dermatology Symposium, April 11, 1997, Berlin; Biomedicine '97, April 25, 1997, Washington, DC; and The Midwest Society for Pediatric Research, Chicago, September 25, 1997.

reporter systems. The natural ligand-activator of PPARγ is a prostaglandin J_2 metabolite (Forman *et al*, 1995; Kliewer *et al*, 1995). The thiazolidinedione BRL-49653 (BRL) is a specific PPARγ ligand up to 10^{-5} M (Kliewer *et al*, 1995; Lehmann *et al*, 1995; Brun *et al*, 1996; Forman *et al* 1997). WY-14643 (WY) is a clofibrate analog, which is a specific PPARα ligand-activator up to 10^{-5} M (Brun *et al*, 1996; Forman *et al*, 1995). The prostacyclin analog carbaprostacyclin and the C18:2 essential fatty acid linoleic acid (LIN) are among the most potent ligand-activators of PPARδ. Carbaprostacyclin and LIN activate PPARδ and PPARα to a comparable extent and several-fold more than PPARγ (Kliewer *et al*, 1994; Brun *et al*, 1996; Forman *et al* 1997). Other agents such as LY-171883 (LY), a leukotriene antagonist which activates PPARγ and PPARα, seem to do so indirectly (Kliewer *et al*, 1994).

PPAR regulate multiple lipid metabolic genes in peroxisomes, microsomes, and mitochondria by acting on PPAR response elements (Schoonjans *et al* 1996). All of these organelles are prominent in sebocytes (Goldenberg *et al*, 1975; Mednieks *et al*, 1991). Some PPAR subtypes have been detected in rat sebaceous glands and epidermis (Braissant *et al*, 1996), but *in vivo* studies have not shown the PPAR activators clofibric acid (Venencie, 1995) or eicosatetraynoic acid (Strauss *et al* 1967) to stimulate sebum formation. In spite of these inconclusive data, we have found in a preliminary study that the PPARγ activator BRL induced preputial sebocytes to differentiate as lipid-forming colonies in culture (Rosenfield *et al*, 1998). Furthermore, when serum was omitted from the culture medium, the BRL effect was enhanced and an effect of DHT, WY, and LIN became apparent.

In this study we present PPAR activator dose–response studies and PPAR gene expression data on cultured sebaceous and epidermal cells in serum-free medium. These studies demonstrate that PPARγ is specifically involved in sebocyte maturation and indicate that PPARδ activation affects both types of cells. Our results point to important and distinct roles of PPARγ1, PPARδ, and long chain fatty acids in sebocyte maturation. They also suggest that PPARδ activators and long chain fatty acids have the potential to stimulate the formation of epidermal lipids.

MATERIALS AND METHODS

Cell culture Preputial and epidermal epithelial cells were obtained from adult male Sprague–Dawley rats, and single-cell suspensions were plated on 35 mm polystyrene dishes which contained a feeder layer of 3T3-J2 fibroblasts that had been pretreated with mitomycin C (Laurent *et al* 1992). They were initially grown for 3 d in Dulbecco's modified Eagles medium with 10% fetal bovine serum supplemented with 10^{-10} M cholera toxin, 10^{-6} M hydrocortisone, and antibiotics (Laurent *et al* 1992). Cellgro Complete (Mediatech, VA), a serum-free chemically defined cell culture medium containing bovine serum albumin and a proprietary nonanimal protein source, and 10^{-6} M insulin were substituted for Dulbellco's modified Eagles medium/fetal bovine serum on day 3, and the various treatments were added in triplicate in ethanol or dimethylsulfoxide concentrations (≤0.1% by volume) that did not affect lipid formation. Cultures were stopped after 6 + d, prior to confluence among colonies. At this time there were ≈150 colonies per well, each averaging about 4500 cells. BRL, LY, WY, and carbaprostacyclin were obtained from Biomol (Plymouth Meeting, PA); troglitazone from Parke-Davis (Morris Plains, NJ); and other chemicals from Sigma (St Louis, MO), except as otherwise noted.

Analysis of lipid droplet formation Cultured cells were fixed and stained with Oil Red O (Rosenfield, 1989). Lipid accumulation in epithelial cell colonies was quantitated by light microscopy at 25–40× in four groups according to the number of stained cells per colony: 0, 1–5, 6–50, and >50. A lipid-forming colony was defined as a colony containing over five cells positive for Oil Red O staining (cells equivalent to at least mid-differentiation of sebocytes, see below), in order to distinguish clearly specific cytoplasmic staining from the amorphous staining of deteriorating cells. Colonies containing one to five lipid-stained cells averaged <10% of colonies on any treatment.

Lipid accumulation was also quantitated after culture by differential counts and fluorescence-activated cell scanning of single cell suspensions. After removing 3T3 cells by a brief incubation with 0.02% ethylenediamine tetraacetic acid, cultured cells were dispersed by incubation with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid for 10 min, and then washed in phosphate-buffered saline. For differential counts, cell smears were fixed on poly L-lysine coated slides, stained with Oil Red O, and counterstained with Gill's hematoxylin. A modification of the method of Tosti was used to determine the percentage of cells at each successive stage of differentiation (Rosenfield, 1989), undifferentiated (UN), early (perinuclear lipid droplets), mid (fused lipid droplets), late (large droplets) and mature (nucleus distorted by large cytoplasmic lipid droplets). For fluorescence-activated cell scan, dispersed cells were stained for 20 min with 0.1 μg per ml Nile Red in 0.01% methanol, washed, and resuspended in phosphate-buffered saline. Fluorescence-activated cell scan excitation wavelength was 488 nm, and emission was monitored through band-pass filters at 585 ± 15 nm (FL2), and ⩾ 630 nm (FL3) (Greenspan *et al* 1985; Smyth and Wharton, 1992). The FL1 (525 ± 15 nm) region was gated out after preliminary studies with propidium iodide showed a negligible proportion of nonviable cells. The detector voltage gain settings were calibrated to minimize the autofluorescence signal of unstained cells, and the compensation settings were adjusted to minimize the overlapping Nile Red emission spectrum in the FL2 and FL3 regions. Analysis was performed using Lysis 2.0 software (Becton Dickinson, San Jose, CA).

Riboprobes and RNase protection assay A 275 bp cDNA fragment of rat PPARγ, comprising the 90 bp region specific to PPARγ2 and extending into the A/B domain of cDNA common to both PPARγ1 and γ2, was obtained by reverse transcription coupled to polymerase chain reaction using primers homologous to the mouse PPARγ2 cDNA sequence (Tontonoz *et al*, 1994a). The cDNA strand was synthesized from 5 μg of total RNA from rat epididymal fat pad using the SUPERSCRIPT II RNase H^- Reverse Transcriptase (Gibco BRL, Gaithersburg, MD). The primers consisted of γ-up 5′-AGTCATGGAACACGGACAGG-3′, and γ-down 5′-CTCCATGTTGAGGCTGCCAC-3′. The rat cDNA fragment was then sequenced by the dideoxy termination method (Sambrook *et al* 1989a) for determination of homologies to mPPARγ. This area of the cDNA allowed differentiation between the γ1 and γ2 isoforms using a single riboprobe, as the signal for γ2 is at 275 bp and that for γ1 is 90 bp less, at 185 bp. The 275 bp riboprobe was made by linearizing the plasmid with the restriction enzyme *Hin*dIII and then transcribing using T3 RNA polymerase in the presence of α(^{32}P)-UTP and three other nucleotide triphosphates (Sambrook *et al* 1989b). A 109 bp internal control probe was created by transcribing linearized pT7 18S rRNA (Ambion, Austin, TX) similarly with T7 RNA polymerase (Sambrook *et al* 1989b).

PPARδ was a gift from Dr. S. Liao (University of Chicago, Chicago, IL). It consisted of a 1 kb cDNA fragment inserted into the *Eco*R1 site of the polylinker of pBluescript SK. A 230 bp riboprobe, corresponding to base pairs 1166–1396 within the ligand binding domain (Xing *et al*, 1995), was made by linearizing the plasmid with *Msc*I and then transcribing with T7 RNA polymerase as above.

RNase protection assay was performed by hybridizing total RNA (10 μg) to the riboprobes, digesting unprotected RNA with RNase A and T1, and separating the protected RNA hybrids on a 5% polyacrylamide/8 M urea gel, which was then autoradiographed (Miyake *et al*, 1994).

Statistical analysis One-way analysis of variance followed by Fisher's protected least differences *post hoc* testing was used to compare the various treatments, after square root or logarithmic transformation as necessary to normalize variances among treatments. Statistical analyses were performed using Statview or Stata 4.0 statistical software. p values are two-tailed.

RESULTS

Thiazolidinediones are potent inducers of sebocyte differentiation The PPARγ ligand BRL induced lipid-forming colonies, in a broad, dose–response manner commencing at 10^{-10} M, with BRL 10^{-6} M yielding a maximum response (**Fig 1*a***) ($p < 0.01$). BRL 10^{-4} M had a cytotoxic effect. In 26 successive experiments, BRL 10^{-6} M induced 60.4% ± 3.8% (SEM) of colonies to differentiate as lipid-forming colonies, as compared with 5.2% ± 0.7% of untreated controls. The thiazolidinedione troglitazone had a similar dose–response effect, but was approximately one-tenth as potent as BRL, inducing 60% ± 10% lipid-forming colonies at the maximally tolerated dose of 10^{-5} M (n = 4).

Androgen and thiazolidinedione effects are additive DHT 10^{-6} M had a small but significant effect on lipid-forming colony development (**Fig 1*a***). Larger DHT doses or administration of the more potent, nonmetabolizable androgen mibolerone led to no greater effect (not shown). DHT 10^{-6} M was additive to the effect

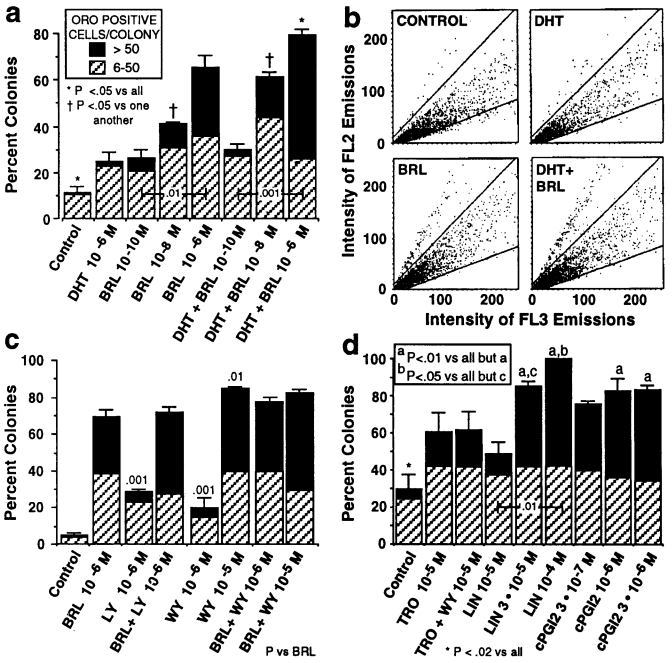

Figure 1. Differentiation of preputial sebocytes in primary culture after various treatments administered in triplicate in serum free medium from day 3 to day 9. Mean ± SEM of lipid-forming colonies are shown. ORO, Oil Red O. (*a*) Response to DHT and/or BRL (n = 5). DHT 10^{-6} M has a small but significant effect ($p < 0.05$). BRL has a dose–response effect over a broad range commencing at 10^{-10} M ($p < 0.01$ *versus* control, with 10^{-8} M BRL differing from the higher and lower doses at the p level shown). DHT is additive in its effect with BRL $\geqslant 10^{-8}$ M, and the effect of DHT + BRL 10^{-6} M is the greatest of all. *$p < 0.05$ *versus* other treatments. (*b*) Quantitation of neutral lipid in cultured preputial cells by fluorescence-activated flow cytometry. Results of a representative experiment are shown in which cultured preputial cells were dispersed, incubated with Nile Red, and fluorescence emission was evaluated for the yellow-gold band characteristic of neutral lipids (FL2) and for the red band characteristic of amphipathic lipids (FL3). The neutral lipid fluorescence of cells seen above the upper gate averaged 0.26%, 0.36%, 1.8%, and 2.5% of cells for control, DHT, BRL, and DHT plus BRL treatments, respectively, each 10^{-6} M. (*c*) Response to WY or LY, at maximally tolerated doses, with or without BRL (n = 5). Both WY and LY stimulate sebum formation, but are less potent than BRL. Nevertheless, WY 10^{-5} M stimulates lipid-forming colonies slightly but significantly more than BRL 10^{-6} M. Neither WY nor LY is additive with BRL (or DHT, not shown). (*d*) Effect of PPAR ligand-activators on lipid colony formation (n = 4). LIN and carbaprostacyclin (cPGI2) were effective over a relatively narrow range of doses; cPGI2 had minimal effect at 10^{-7} M (not shown). High doses of LIN and cPGI2 were more effective than maximal activation of PPARγ and PPARα by treatment with the combination of troglitazone (TRO) and WY. LIN 10^{-4} M consistently stimulated over 95% of sebocyte colonies to differentiate as lipid-forming colonies.

of BRL. In 26 successive experiments, DHT caused 14.8% ± 1.3% of colonies to develop as lipid-forming colonies ($p < 0.01$ *versus* controls) and DHT plus BRL (each 10^{-6} M) brought about 70.3% ± 3.1% lipid-forming colonies ($p < 0.001$ *versus* BRL alone). Most stikingly, the percentage of highly differentiated colonies (those containing over 50 lipid-positive cells per colony) was 1.7% ± 0.5% with DHT alone, 30% ± 3.2% with BRL alone, and 54%± 3.3% with DHT + BRL, all significantly different ($p < 0.001$).

The formation of neutral lipids by DHT and BRL was also evaluated by fluorescence-activated cell scan of the Nile Red fluorescence spectrum. **Figure 1(*b*)** shows the results of one of five experiments. Relative to controls, DHT brought about a 1.4 ± 0.1-fold increase in neutral fats ($p < 0.05$); BRL caused a 7.0 ± 0.3-fold increase and DHT plus BRL a 9.4 ± 0.3-fold increase ($p < 0.001$ *versus* all others).

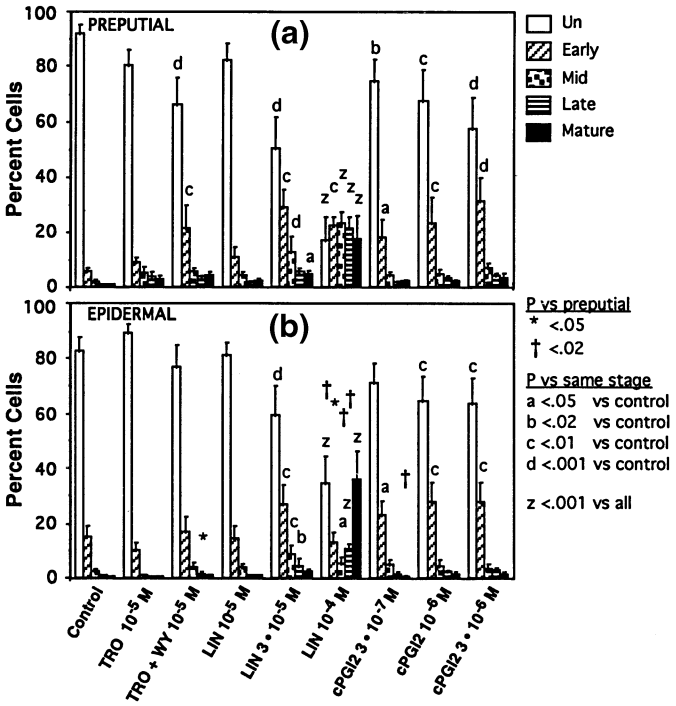

Figure 2. Effect of PPAR ligand-activators on lipid accumulation by cultured epithelial cells. Cells were classified according to the successive stages of sebocyte maturation, from undifferentiated (UN) to mature (n = 6). (*a*) Preputial cells. LIN is the most effective inducer of sebocyte differentiation, inducing formation of a significantly higher percentage of late and mature sebocytes than any other PPAR activator, including carbaprostacyclin (cPGI2). Carbaprostacyclin, which like LIN activates PPARδ, induced a significant increase in early differentiated sebocytes. This experiment did not have the statistical power to demonstrate the effect of the thiazolidinedione troglitazone that is readily apparent when the results of the initial subset of the same experiments were analyzed in terms of lipid-forming colonies (**Fig 1*d***). (*b*) Epidermal cells. Only LIN and carbaprostacyclin induce lipid droplet formation in epidermal cells like that in sebaceous epithelial cells, but significantly less in some regards, as indicated (*, †).

PPARα activators induce sebocyte differentiation If thiazolidinediones induce sebocyte differentiation via a PPAR-mediated pathway, one might expect other PPAR activators to be effective. Furthermore, the pharmacologic profile would be expected to be informative of the PPAR subtypes specifically involved in sebocyte differentiation. Therefore, we compared BRL to WY, a PPARα ligand. WY 10^{-6} M stimulated 19.8% ± 5.1% lipid-forming colonies, and WY 10^{-5} M stimulated significantly more (83% ± 1.1%) (**Fig 1*c***). WY 10^{-4} M had a toxic effect. We also tested a maximally tolerated dose of LY, an activator of both PPARα and PPARγ. **Fig 1(*c*)** shows that 10^{-6} M LY stimulated lipogenesis (28.3% ± 1.3% lipid-forming colonies, $p < 0.01$ *versus* control). Neither thiazolidinediones nor DHT (not shown) had an additive effect with WY or LY on lipid-forming colonies; however, differential counts showed a tendency of PPARγ plus PPARα stimulation to induce more early differentiated sebocytes than PPARγ stimulation alone: troglitazone plus WY (both 10^{-5} M) induced 22% ± 8.6% and troglitazone alone 9.0% ± 2.1% ($p < 0.05$) (**Fig 2**), yet the stimulation of early differentiation by WY alone (17% ± 7.0%, $p < 0.05$ *versus* control, not shown) was not significantly different from either of these treatments.

PPARδ activators induce the most sebocyte differentiation LIN was then tested to activate PPARδ in addition to PPARα. LIN yielded a steep dose–response effect on lipid-forming colonies ($p < 0.001$): no effect was discerned at 10^{-6} M, a slight effect occurred at 10^{-5} M, ED50 was 3×10^{-5} M, and the maximal response was found at 10^{-4} M, at which dose it consistently

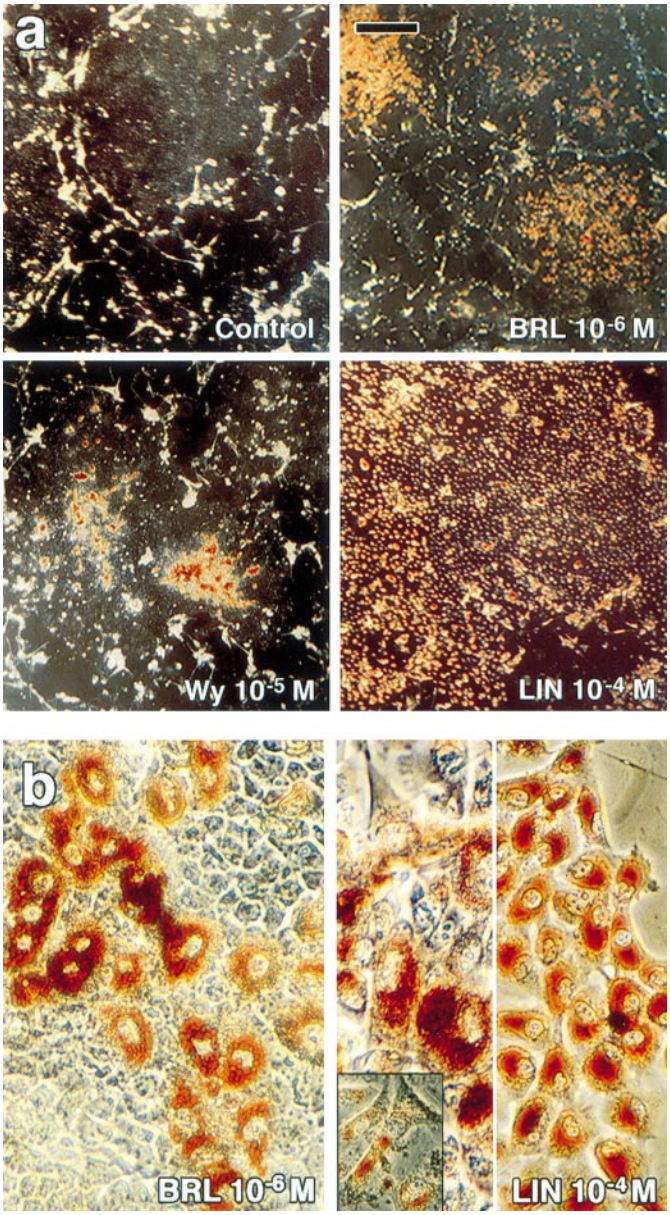

Figure 3. Effect of PPAR activators on preputial cell morphology in monolayer culture. Oil Red O stain. (*a*) Scanning power view of sebocyte colonies in response to PPAR activators. BRL and WY treatments cause lipids (reddish gold stain) to accumulate in the center of colonies, but LIN causes lipid staining throughout colonies (as does carbaprostacyclin, not shown) and the colony morphology is less compact. *Scale bar*: 400 μm. (*b*) High power view of preputial cells after treatment with BRL (*left*) and LIN (*right*). BRL treatment (like WY, not shown) leads to lipid accumulation in only a small fraction of sebocytes. In contrast, most sebocytes differentiate upon treatment with LIN, the spectrum ranging from perinuclear lipid droplets in healthy cells to disintegrating mature sebocytes. Only LIN was capable of inducing extensive late differentiation of the cells proliferating at colony edges (*right panel*). LIN also induced lipid formation in the 3T3-cell feeder layer (inset). *Scale bar*: 50 μm.

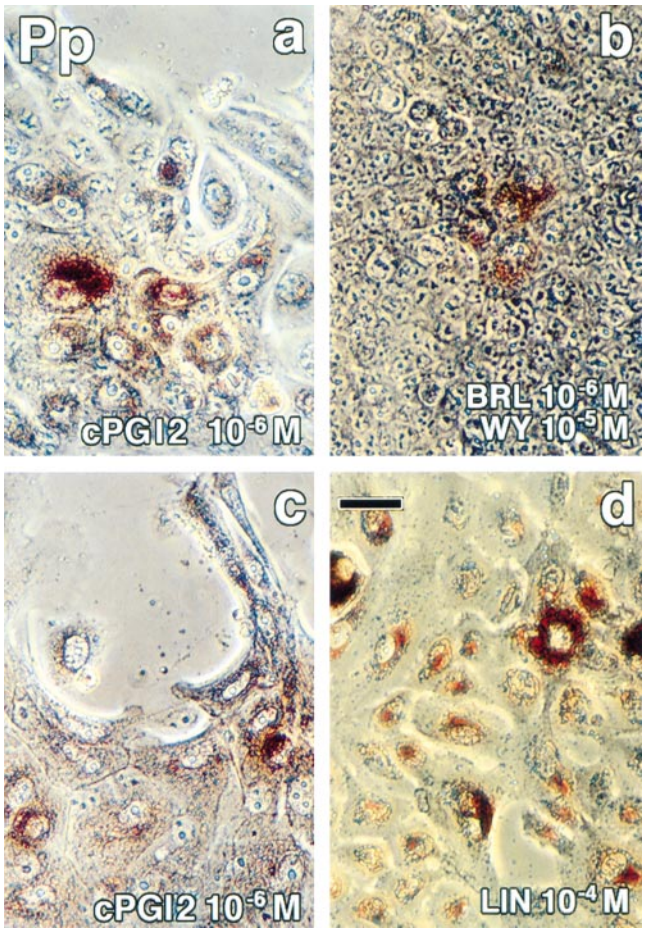

Figure 4. Effect of PPAR activators on the morphology of preputial cells (*a*) in comparison to epidermal cells (*b–d*) in monolayer culture. Oil Red O stain. *Scale bar*: 50 μm. (*a*, *b*) High power view of cultured preputial cells (Pp) (*a*) after treatment with carbaprostacyclin (cPGI2) 10^{-6} M and epidermal cells after treatment with BRL + WY (*b*). Note that cPGI2 causes lipid droplet accumulation in many cells out to the edge of colonies in both cell types, a property shared by LIN (**Fig 3*b***). On BRL plus WY, however, only an occasional epidermal cell contains an amorphous accumulation of lipid, as shown here. (*c*, *d*) High power view of cultured epidermal cells after treatment with cPGI2 (*c*) or LIN (*d*). Both stimulate widespread lipid droplet accumulation in cultured epidermal cells, in contrast to the lack of an effect of BRL plus WY (*b*).

induced over 95% lipid-forming colonies (n = 9) (**Fig 1*d***). Carbaprostacyclin, which has a PPAR binding and activity profile similar to LIN, also had a steep dose–response effect ($p < 0.01$): 10^{-7} M had a marginal effect (not shown), ED50 was 2×10^{-7} M (interpolated), and 10^{-6} M yielded a maximal response only slightly less than LIN (n = 4) (**Fig 1*d***). LIN was toxic to cells at 10^{-3} M, carbaprostacyclin at 10^{-4} M.

Over 80% of individual preputial cells differentiated in response to LIN 10^{-4} M, 2-fold or more than in response to any other PPAR activator, including carbaprostacyclin or the combination of WY with BRL (not shown) or troglitazone ($p < 0.001$) (**Fig 2**). Fully mature cells averaged 17% ± 8.5% of the total, and late differentiated cells averaged 21% ± 4.1% on this dose of LIN. Mid-differentiation of sebocytes increased significantly in response to LIN, commencing at 3×10^{-5} M. Carbaprostacyclin was less effective than LIN, but it was more effective at 3×10^{-6} M in stimulating early sebocyte differentiation than troglitazone or WY 10^{-5} M ($p < 0.05$). No interaction of LIN or carbaprostacyclin with DHT, BRL, or WY was seen at maximal doses (not shown).

BRL and WY caused advanced differentiation only in the central zone of colonies where the older cells reside (**Fig 3*a***); however, LIN (**Fig 3*b***) and carbaprostacyclin (**Fig 4*a***) caused a high degree of differentiation of cells throughout colonies, including the outer zone of colonies where immature cells proliferate. Only LIN caused a loss of intercellular cohesiveness that led to a loosening of colony morphology (**Fig 3*a***).

PPAR activation specificity for sebaceous epithelium The fused lipid droplet formation characteristic of sebocyte mid-differentiation was rarely discerned upon treatment of epidermal cells with the PPARγ or PPARα activators BRL, troglitazone, WY, LY, or

```
mPPARgamma2  CCCAGTGTGA ATTACAGCAA ATCTCTGTTT TATGCTGTTA TGGGTGAAAC TCTGGGAGAT TCTCCTGTTG ACCCA   75
rPPARgamma2  ---------- ---------- ---------- --------TA TGGGTGAAAC TCTGGGAGAT CCTCCTGTTG ACCCA   37

mPPARgamma2  GAGCATGGTG CCTTCGCTGA TGCACTGCCT ATGAGCACTT CACAAGAAAT TACCATGGTT GACACAGAGA TGCCA  150
rPPARgamma2  GAGCATGGTG CCTTCGCTGA TGCACTGCCT ATGAGCACTT CACAAGAAAT TACCATGGTT GACACAGAGA TGCCA  112

mPPARgamma2  TTCTGGCCCA CCAACTTCGG AATCAGCTCT GTGGACCTCT CGGTGATGGA AGACACTCGC CATTCCTTT GACAT  224
rPPARgamma2  TTCTGGCCCA CCAACTTCGG AATCAGCTCT GTGGACCTCT CGGTGATGGA AGACACTCGC CATTCCTTT GACAT  187

mPPARgamma2  CAAGCCCTTT ACCACAGTTG ATTTCTCCAG CATTTCTGCT CCACACTATG AAGACATTCC ATTCACAAGA AGCTG  298
rPPARgamma2  -AAACCCTTT ACCACAGTTG ATTTCTCCA- CATTTCTGCT CCACACTATG AAGACATTCC GTTCACAAGA AGCTG  260

mPPARgamma2  ACCCAATGGT TGCTGATTAC AAATATGACC TGAAGCTCCA AGAATACCAA AGTGCGATCA AAGTAGAACC TGCAT  373
rPPARgamma2  ACCCAATGGT TGCTGAT--- ---------- ---------- ---------- ---------- ---------- -----  277
```

Figure 5. Homology between rat and mouse PPARγ in the A/B domain. There is 95% homology (boxed) between the mouse and rat cDNAs in this area of DNA. The start codon for γ2 is at bp39 and for γ1 is at bp129 of the mouse PPAR.

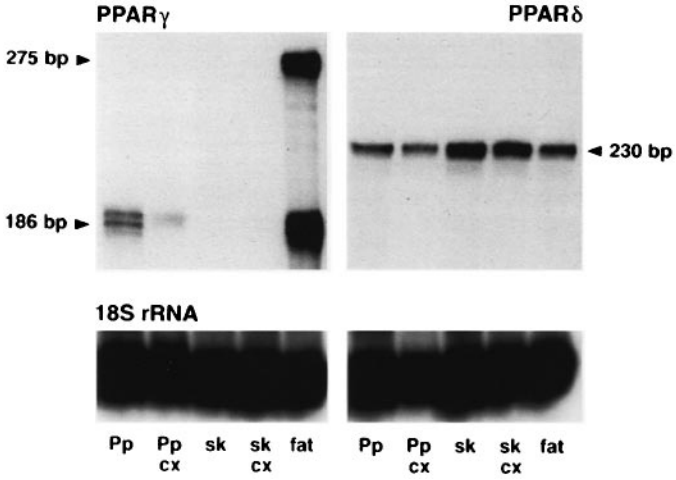

Figure 6. PPARγ and PPARδ expression in rat tissues according to RNase protection assay. PPARγ is expressed in both freshly dispersed and cultured rat preputial sebocytes (Pp). Sebocytes express only the γ1 isoform (186 bp), in contrast to fat homogenates in which γ2 (275 bp) is also expressed. PPARγ is not found in dispersed epidermal cells (sk). PPARδ (230 bp) was expressed in all tissues, and more so in cultured (cx) Pp than PPARγ1, which can only faintly be seen. 185 rRNA used as internal control.

combinations thereof (**Figs 2**, **4*b***). LIN and carbaprostacyclin, however, stimulated lipid formation by both types of cells (**Figs 2**, **4*c***, ***d***), and LIN was more effective than carbaprostacyclin on each. There was a tendency of epidermal cells to be less responsive than sebocytes to each of these treatments (**Fig 2**). LIN had the additional effect of inducing lipid droplet formation in the 3T3-J2 cells of the feeder layer (**Fig 3*b***).

Demonstration of PPAR gene expression As the activation of sebocyte lipogenesis by a thiazolidinedione suggested a prime role of PPARγ, which had not been detectable in sebocytes previously, we sought its expression in sebocytes by RNase protection assay (RPA). PPARδ gene expression was studied as a control. A 275 bp cDNA fragment for rat PPARγ1 and γ2 was prepared using primers homologous to the A/B domain of mouse PPARγ (Tontonoz *et al*, 1994a). Sequencing of this segment showed 95% homology of rat to mouse PPARγ (**Fig 5**).

We then demonstrated PPARγ and PPARδ in freshly dispersed sebocytes, using rat PPAR riboprobes. **Figure 6** confirms our previous finding of PPARγ1 mRNA expression in the sebaceous gland (Rosenfield *et al*, 1998), and it also shows that sebaceous cells do not express the γ2 isoform found in fat cells. PPARγ1 was detectable in cultured sebocytes at a lower level of abundance than in freshly dispersed sebocytes. This study also demonstrated that PPARδ mRNA was similarly expressed in sebocytes and epidermal cells, whether freshly dispersed or cultured, and was more prominent in cultured sebocytes than is PPARγ (**Fig 6**).

DISCUSSION

All PPAR activators reproducibly induced sebocyte lipogenesis at concentrations that have been shown to activate specific PPAR-reporter gene constructs (Kliewer *et al*, 1994; Yu *et al*, 1995; Forman *et al* 1997). PPARγ gene expression has not previously been detected in the sebaceous gland (Braissant *et al*, 1996), probably because the methodology was not as sensitive as that used here. Our studies, however, suggest that expression of PPARγ1 is involved in the unique ability of sebocytes to undergo the transition from an early differentiated stage, at which they resemble epidermal cells and form perinuclear lipid droplets, to the mid-differentiated stage at which fused lipid droplets are formed. This concept arises from the following observations. Thiazolidinediones, specific PPARγ ligands, activated lipogenesis only in sebocytes, and only in the central zone of these colonies where the oldest cells reside. Furthermore, PPARγ1 mRNA was detectable at only a low level in cultured sebocytes, which are predominantly immature (Rosenfield and Deplewski, 1995), but was expressed in abundance in freshly dispersed sebocytes, which are predominantly well-differentiated. In contrast, PPARγ1 gene expression was undetectable in epidermal epithelial cells. The modest *in vitro* differentiative effect of androgen on sebocytes was additive with that of BRL at maximum dose, but not with activators of other PPAR. This suggests that androgen influences an early step in sebocyte differentiation, which is related to but distinct from that influenced by PPARγ1.

Activation of PPARα by WY led to a similar pattern of lipogenesis in sebocytes as PPARγ activation, characterized by central zonation. We could not detect a significant additive effect of maximal PPARγ and PPARα activation. This is compatible with PPARα activation amplifying the same early lipid biosynthetic pathways in sebocytes as PPARγ. PPARα activator treatment did not induce lipid-forming colonies in epidermal cells; however, PPARα gene expression has previously been detected in similarly modest amounts in both epidermis and sebaceous glands (Braissant *et al*, 1996). A recent report indicates that the PPARα activators clofibric acid and LIN, but not PPARγ activators, accelerate the development of the epidermal water barrier in fetal skin explants, and electron microscopy demonstrated an accompanying development of mature lipid lamellar units in the interstitial spaces (Hanley *et al*, 1997). Our studies do not rule out the possibility of such a submicroscopic effect of PPARα activation on the discrete pathways involved in initiating the biosynthesis of lamellar lipids in adult rat epidermal cells.

PPARδ appears to be important in the late stages of sebocyte maturation. This contrasts to the lack of an effect of PPARδ activation on fat cell differentiation (Brun *et al*, 1996), which has led to the physiologic relevance of PPARδ being questioned. Carbaprostacyclin, a ligand-activator of both PPARδ and PPARα, was not only more potent than the PPARα activator WY and the PPARγ-activating thiazolidinediones, alone or together, but it brought a significantly higher proportion of sebocytes to the late differentiated phase. Carbaprostacyclin and LIN, which has a similar PPAR activation profile, also brought about a distinctly different pattern of lipogenesis in sebocyte colonies than the selective PPARα and PPARγ activators. They induced lipogenesis promiscuously, throughout colonies, even at the periphery of colonies where the youngest cells are proliferating. Thus, one can deduce that PPARδ mediates this effect. In addition, this zonation pattern suggests that PPARδ is expressed earlier in sebocyte differentiation than the

other PPAR subtypes. Consistent with this conclusion, PPARδ mRNA appears to be constitutively expressed, as it was found in similar amounts in both freshly dispersed and cultured sebocytes and epidermal cells, unlike PPARγ.

Our data also suggest that fatty acid effects are to some extent PPAR independent. Although LIN was inactive at the micromolar concentration at which BRL and carbaprostacyclin were maximally effective, 100 μM LIN was the most effective treatment for the induction of sebocyte differentiation. This LIN concentration appears to be within the physiologic range for fatty acids in the sebaceous gland (Wheatley, 1986) and brought about more complete seboctye differentiation than any other PPAR activator and more than reported to spontaneously occur in a human sebocyte secondary cell culture system (Zouboulis *et al*, 1994). LIN also was unique in its property of inducing a loss of intercellular cohesiveness, a change that seems to indicate the development of apoptosis, which occurs at the most mature stage of sebocyte maturation (Tamada *et al*, 1994). The different effects of LIN and carbaprostacyclin, both ligand-activators of PPARδ and PPARα, suggest a distinct role of LIN or a LIN metabolite in the final, mature stage of sebocyte terminal differentiation at which these cells burst and extrude lipid. LIN differs from other PPAR activators in being an essential fatty acid, necessary for the synthesis of ω-6 long chain fatty acids and a component of more complex sebum lipids.

Lipid droplet formation by epidermal cells in response to carbaprostacyclin and LIN was initially unexpected, because PPARα activation did not induce lipid-forming colonies and because PPARδ had not previously been detected in skin (Braissant *et al*, 1996). Our study, however, indicates considerable expression of PPARδ mRNA in skin keratinocytes. Although the unnatural accumulation of lipid droplets in cultured epidermal cells in response to LIN would seem explicable by the unphysiologic nature of the high concentration of free fatty acid in the milieu of these cells, these findings may be relevant to known effects of LIN on epidermal differentiation. LIN is required for formation of the epidermal water barrier (Swarzendruber *et al*, 1989; Schurer and Elias, 1991), discussed above. In addition, the high concentration of fatty acids in sebum have been proposed to play a part in evoking hyperkeratosis of the follicular duct epithelium (Kligman *et al* 1970).

These findings have implications for the therapy of acne vulgaris, the most common skin disorder of adolescents (Phillips and Dover, 1992). Increased sebum secretion is an important element in the pathogenesis of acne (Kligman, 1974; Downing *et al*, 1986). PPAR appear to mediate the accumulation of cytoplasmic fat droplets that characterizes sebocyte differentiation. Our working hypothesis is that PPARα plays a part in the initial stage of lipogenesis common to sebaceous and epidermal cells; PPARγ mediates the transition of sebocytes to the mid-differentiated stage at which they form fused lipid droplets, a process augmented by androgen; PPARδ amplifies the lipogenic process in response to activation by the accumulating fatty acids; and the subsequent high intracellular free fatty acid levels successively activate sebocyte apoptosis and cause reactive changes in the sebaceous ducts and follicles, which contribute to comedogenesis. As PPAR regulate the terminal pathways of lipid biosynthesis that are necessary for these processes, it would seem worthwhile to develop PPAR antagonists to reduce sebum formation. By using a PPARγ1 or PPARδ antagonist, it would theoretically seem possible to interfere selectively with sebum production without invoking the side-effects of currently available treatment modalities, which include suppression of androgen levels, use of antiandrogens, or use of retinoic acid analogs, all of which have major drawbacks that limit their usefulness.

These studies were supported in part by USPHS grant HD-O6308 (RLR) and a Howard Hughes Medical Institute Undergraduate Education Initiative grant to the University of Chicago (AK). We are indebted to Drs. Reed Graves, Shutsung Liao, John Kokontis, and Richard Hiipakka for their advice.

REFERENCES

Akamatsu H, Zouboulis C, Orfanos CE: Control of human sebocyte proliferation in vitro by testosterone and 5-alpha-dihydrotestosterone is dependent on the localization of the sebaceous glands. *J Invest Dermatol* 99:509–511, 1992

Amri E-Z, Bonino F, Ailhaud G, Abumrad NA, Grimaldi PA: Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. *J Biol Chem* 270:2367–2371, 1995

Braissant O, Foufelle F, Scotto C, Dauca, M, Wahli W: Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat. *Endocrinology* 137:354–366, 1996

Brun RP, Tontonoz P, Forman BM, Ellis R, Chen J, Evans, RM, Spiegelman BM: Differential activation of adipogenesis by multiple PPAR isoforms. *Gene Dev* 10:974–984, 1996

Downing DT, Stewart ME, Wertz PW, Strauss JS: Essential fatty acids and acne. *J Am Acad Dermatol* 14:221–225, 1986

Ebling FJ, Ebling E, Randall V, Skinner J: The effects of hypophysectomy and of bovine growth hormone on the responses to testosterone of prostate, preputial, harderian and lachrymal glands and of brown adipose tissue in the rat. *J Endocrinol* 66:401–406, 1975

Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman, BM, Evans RM: 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell* 83:803–812, 1995

Forman BM, Chen J, Evans RM: Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Proc Natl Acad Sci USA* 94:4312–4317, 1997

Goldenberg H, Hüttinger M, Ludwig R, Kramar, R, Böck P: Catalase-positive particles ("microperoxisomes") from rat preputial gland and bovine adrenal cortex. *Exp Cell Res* 93:438–442, 1975

Green S: PPAR: a mediator of peroxisome proliferator action. *Mutat Res* 333:101–109, 1995

Greenspan P, Mayer EP, Fowler SD: Nile red: a selective fluorescent stain for intracellular lipid droplets. *Cell Biol* 100:965–973, 1985

Guy R, Ridden C, Kealey T: The improved organ maintenance of the human sebaceous gland: modeling in vitro the effects of epidermal growth factor, androgens, estrogens, 13-*cis* retinoic acid, and phenol red. *J Invest Dermatol* 106:454–460, 1996

Hanley K, Jiang Y, Crumrine D, *et al*: Activators of the nuclear hormone receptors PPARα and FXR accelerate the development of the fetal epidermal permeability barrier. *J Clin Invest* 100(3):705–712, 1997

Kliewer SA, Forman BM, Blumberg B, *et al*: Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci* 91:7355–7359, 1994

Kliewer SA, Lenhard JM, Wilson TM, Patel I, Morris DC, Lehmann JM: A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* 83:813–819, 1995

Kligman AM: An overview of acne. *J Invest Dermatol* 62:268, 1974

Kligman AM, Wheatley VR, Mills OH: Comedogenicity of human sebum. *Arch Dermatol* 102:267–275, 1970

Laurent SJ, Mednieks MI, Rosenfield RL: Growth of sebaceous cells in monolayer culture *in vitro*. *Cell Dev Biol* 28A:83–89, 1992

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkinson WO, Willson, TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma. *J Biol Chem* 270:12953–12956, 1995

Mangelsdorf DJ, Evans RM: The RXR heterodimers and orphan receptors. *Cell* 83:841–850, 1995

Mednieks MI, Laurent SJ, Hand AR, Rosenfield RL: Cyclic AMP-receptor protein activity in rat preputial cells. *J Invest Dermatol* 97:517–523, 1991

Mesquita-Guimaraes J, Coimbra A: The effect of sexual hormones on the lipid and proteinaceous secretion of the rat preputial sebaceous gland. *Arch Dermatol Res* 270:325–331, 1981

Miyake K, Ciletti N, Liao S, Rosenfield RL: Androgen receptor expression in the preputial gland and its sebocytes. *J Invest Dermatol* 103:721–725, 1994

Phillips TJ, Dover JS: Recent advances in dermatology. *N Engl J Med* 326:167–176, 1992

Rosenfield RL: Relationship of sebaceous cell stage to growth in culture. *J Invest Dermatol* 92:751–754, 1989

Rosenfield RL, Deplewski D: Role of androgens in the developmental biology of the pilosebaceous unit. *Am J Med* 98:80S–88S, 1995

Rosenfield RL, Miyake K, Ciletti N, Liao S: Androgen receptor (AR) and paradoxical response to androgen of cultured preputial sebocytes. *Clin Res* 41:257A, 1993

Rosenfield RL, Deplewski D, Kentsis A, Ciletti N: Mechanisms of androgen induction of sebocyte differentiation. *Dermatology* 196:43–46, 1998

Sambrook J, Fritsch E, Maniatis T (eds): Sequencing by the Sanger dideoxy-mediated chain-termination method. In: *Molecular Cloning: a Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, 1989a, pp. 13.42–77

Sambrook J, Fritsch EF, Maniatis T (eds): Synthesis of RNA probes by in vitro transcription of double-stranded DNA templates by bacteriophage DNA-dependent RNA polymerases. In: *Molecular Cloning: a Laborarory Manual*. New York: Cold Spring, Harbor Laboratory Press, 1989b, pp. 10.27–37

Schoonjans K, Staels B, Auwerx J: The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochim Biophys Acta* 1302:93–109, 1996

Schurer NY, Elias PM: The biochemistry and function of stratum corneum lipids. *Adv Lipid Res* 24:27–56, 1991

Sherins RJ, Bardin CW: Preputial gland growth and protein synthesis in the

androgen-insensitive male pseudohermaphroditic rat. *Endocrinol* 89:835–841, 1971

Smyth MJ, Wharton W: Differentiation of A31T6 proadipocytes to adipocytes: a flow cytometric analysis. *Exp Cell Res* 199:29–38, 1992

Strauss JS, Pochi PE, Whitman EN: Suppression of sebaceous gland activity with eicosa-5:8:11:14-tetraynoic acid. *J Invest Dermatol* 48:492–493, 1967

Swarzendruber DC, Wertz PW, Kitko DJ, Madison KC, Downing DT: Molecular models of the intercellular lipid lamellae in mammalian stratum corneum. *J Invest Dermatol* 92:251–257, 1989

Tamada Y, Takama H, Kitamura T, Hokochi K, Nitta Y, Ideya T, Matsumoto Y: Identification of programmed cell death in normal human skin tissues by using specific labelling of fragmented DNA. *Br J Dermatol* 131:521–524, 1994

Tontonoz P, Hu E, Graves RA, Budavari A, Spiegelman BM: mPPARgamma2: tissue-specific regulator of an adipocyte enhancer. *Gene Dev* 8:1224–1234, 1994a

Tontonoz P, Hu E, Spiegelman BM: Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* 79:1147–1156, 1994b

Venencie PY: Modification of lipogenesis in the isolated hamster flank organ through clofibric acid. *Skin Pharmacol* 8:203–206, 1995

Wheatley VR: The sebaceous glands. In: Jarrett A (ed.). *Physiology, Pathophysiology of Skin*, Vol. 9. New York: Academic Press, 1986

Xing G, Zhang L, Zhang L, *et al*: Rat PPARδ contains a CGG triplet repeat and is prominently expressed in the thalamic nuclei. *Biochem Biophys Res Commun* 217:1015–1025, 1995

Yarbrough WG, Quarmby VE, Simental JA, *et al*: A single base mutation in the androgen receptor gene causes androgen insensitivity in the testicular feminized rat. *J Biol Chem* 265:8893–8900, 1990

Yu K, Bayona W, Kallen CB, *et al*: Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J Biol Chem* 270:23975–23984, 1995

Zouboulis ChC, Krieter A, Gollnick H, Mischke D, Orfanos CE: Progressive differentiation of human sebocytes in vitro is characterized by increasing cell size and altering antigen expression and is regulated by culture duration and retinoids. *Exp Dermatol* 3:151–160, 1994