1α,25(OH)2D3 and its 3-epimer promote rat lung alveolar epithelial-mesenchymal interactions and inhibit lipofibroblast apoptosis

R. Sakurai,1 E. Shin,1 S. Fonseca,1 T. Sakurai,1 A. A. Litonjua,2 S. T. Weiss,2 J. S. Torday,1 and V. K. Rehan1

1Department of Pediatrics, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California; and 2Brigham and Women’s Hospital, Harvard School of Medicine, Boston, Massachusetts

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Sakurai R, Shin E, Fonseca S, Sakurai T, Litonjua AA, Weiss ST, Torday JS, Rehan VK. 1α,25(OH)2D3 and its 3-epimer promote rat lung alveolar epithelial-mesenchymal interactions and inhibit lipofibroblast apoptosis. Am J Physiol Lung Cell Mol Physiol 297: L496–L505, 2009. First published July 2, 2009; doi:10.1152/ajplung.90539.2008.—Although alveolar wall thinning has been attributed to apoptosis of interstitial lung lipofibroblasts (LFs), the underlying molecular mechanism(s) remains unknown. Although the physiological vitamin D steroid hormone 1α,25(OH)2D3 (1,25D) has been suggested as a local paracrine/autocrine effector of fetal lung maturation and is known to affect fibroblast apoptosis, its effects on LF apoptosis are unknown. We determined the role of 1,25D and its metabolite, C-3-epimer (3-epi-1,25D), on LF and alveolar type II (ATII) cell differentiation, proliferation, and apoptosis. Embryonic day 19 Sprague-Dawley fetal rat lung LFs and ATII cells were treated with 1,25D or 3-epi-1,25D (1 × 10−10 to 1 × 10−8 M) for 24 h, and cell proliferation, apoptosis, and differentiation were assessed. Both 1,25D and 3-epi-1,25D exhibited dose-dependent increases in expression of the key homeostatic epithelial-mesenchymal differentiation markers, increased LF and ATII cell proliferation, and decreased apoptosis. Furthermore, rat pups administered 1,25D from postnatal days 0 to 14 showed increased expression of key LF and ATII cell differentiation markers, increased Bcl-2-to-Bax ratio as an index of decreased spontaneous alveolar LF and ATII cell apoptosis, increased alveolar count, and a paradoxical increase in septal thickness. We conclude that spatial- and temporal-specific actions of vitamin D play a critical role in perinatal lung maturation by stimulating key alveolar epithelial-mesenchymal interactions and by modulating LF proliferation/apoptosis. These data not only provide the biological rationale for the presence of an alveolar vitamin D paracrine system, but also provide the first integrated molecular mechanism for increased surfactant synthesis and alveolar septal thinning during perinatal lung maturation.

Lung development

ALVEOLAR EPITHELIAL-MESENCHYMAL interactions play a critical role in perinatal pulmonary maturation (32), which is characterized by an increase in surfactant synthesis (15) and alveolar wall thinning (39). Alveolar wall thinning has recently been attributed to apoptosis of the lipid-laden interstitial lung fibroblasts, or lipofibroblasts (LFs; Ref. 3), which are normally present in abundance during the perinatal period (12) but, for unknown reasons, become highly apoptotic over the first 1–2 wk of postnatal life in rats (3). The mechanism underlying this perinatal alveolar wall thinning is unknown. Although the physiological vitamin D steroid hormone 1α,25(OH)2D3 (1,25D) has been suggested as a local paracrine/autocrine effector for fetal lung maturation (17, 21, 22, 28), its effects on alveolar interstitial LF proliferation and/or apoptosis are unknown. We (28) have previously shown that 1,25D is metabolized to its C-3-epimer (3-epi-1,25D) in alveolar type II (ATII) cells. Compared with the parent compound 1,25D, the 3-epi-1,25D has significantly reduced vitamin D receptor affinity, but it has high metabolic stability, thereby explaining its high in vitro transcriptional activity (4a, 28). Interestingly, relatively high circulating concentrations of 3-epi-1,25D metabolites have recently been reported in infants (33). Therefore, we reasoned that 1,25D and 3-epi-1,25D might affect perinatal pulmonary maturation by modulating ATII cell and LF proliferation and differentiation. In the current study, we have determined the role of 1,25D and 3-epi-1,25D in key alveolar epithelial-mesenchymal interactions that are known to be critical for alveolar development and alveolar septal thinning during perinatal pulmonary maturation.

MATERIALS AND METHODS

Animal treatments and isolation of fetal rat LFs and ATII cells. Pregnant Sprague-Dawley rat dams were obtained from Charles River Laboratories (Hollister, CA). For isolating fetal rat lung LFs and ATII cells, dams were killed on embryonic day 19 (e19), and cells were isolated and cultured using standard methods (27). At near confluence, cells were treated with 1,25D or 3-epi-1,25D (1 × 10−10 to 1 × 10−8 M) under specified conditions. To determine the effect of vitamin D supplementation on LF apoptosis in vivo, newborn pups were administered 1,25D (10 ng/kg body wt) or 3-epi-1,25D (50 ng/kg body wt) once daily in 50-µl volumes intraperitoneally, starting from the day of delivery (day 0). Control rats received equivalent volumes of ethanol in normal saline (1:500 dilution) intraperitoneally. The pups were allowed to breast feed ad libitum. The animals were killed on days 1, 7, and 14, and lungs were collected for further analysis. All animal procedures were performed following National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Los Angeles Biomedical Research Institute Animal Care and Use Committee.

Cell proliferation. Cell proliferation was assayed using thymidine incorporation assay. Briefly, 5 × 103 cells/well were plated in 6-well culture plates in DMEM + charcoal-stripped 10% FBS. At 70–80% confluence, cells were synchronized by overnight serum deprivation by changing the medium to DMEM + 0.1% FBS. Cells were then exposed to specified treatment conditions (control, 1,25D or 3-epi-1,25D) in charcoal-stripped 10% FBS in the presence of 1 µCi/ml [3H]thymidine. 1,25D and 3-epi-1,25D 1 mM stock solutions in 100% ethanol were diluted with DMEM to a final ethanol dilution of 1:1,000. After 24 h, medium was aspirated and cells were washed twice with 1 ml of ice-cold PBS solution and once with 1 ml of 5% TCA and then incubated in 1 ml of fresh 5% TCA at 4°C for 30 min. The TCA-insoluble material was washed twice with 95% ethanol, and the fixed cellular material was counted twice with 95% ethanol, and the fixed cellular material was counted...
solubilized in 0.5 ml of 10.25 N NaOH. Solubilized solution (400 μl) was used to determine [³H]thymidine incorporation with liquid scintillation spectrometry. The data were normalized as counts per minute per microgram protein and are presented as percent control. Western blotting for phosphorylated (p-) Akt, a major cell survival pathway, which promotes cell survival by inhibiting apoptosis through several downstream targets, was used as an additional marker to assess cell proliferation.

Apoptosis assay. Cell apoptosis was assayed to determine the role of vitamin D in protection against apoptosis under both in vitro and in vivo conditions. Cultured cells were pretreated with 1,25D or 3-epi-1,25D for 1 h before treatment with the proapoptotic agent actinomycin D (20 ng/ml) for 16 h. Apoptosis was determined using Hoechst 33342 according to the manufacturer’s protocol (Invitrogen, Eugene, OR).

Markers of alveolar epithelial-mesenchymal interactions. Parathyroid hormone-related protein (PTHrP) receptor, peroxisome proliferator-activated receptor-γ (PPARγ), adipocyte differentiation-related protein (ADRP), and leptin were used as differentiation markers for LFs (38); surfactant protein B (SP-B) and leptin receptor were used as differentiation markers for ATIIs (38). These markers were assayed by Western blotting using the following antibodies: PTHrP receptor (1:100 dilution; Upstate, Temecula, CA), PPARγ (1:2,000 dilution; Alexis Biochemicals, San Diego, CA), SP-B and SP-C (1:2,000 dilution for each; Chemicon, Temecula, CA), leptin receptor (1:350 dilution; Santa Cruz Biotechnology), and ADRP [1:3,000 dilution; a kind gift from Dr. Constantine Londos, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)], Bcl-2 (1:500 dilution; Santa Cruz Biotechnology), and Bax (1:1,000 dilution; Santa Cruz Biotechnology). The densities of the specific protein bands were quantified using a scanning densitometer (Eagle Eye II still video system; Stratagene, La Jolla, CA). The blots were subsequently stripped and reprobed with anti-GAPDH antibody (Chemicon), and specific protein values were normalized to GAPDH.

Triolein uptake. The method used to quantitate triglyceride uptake by cultured fibroblasts has previously been described (37). Briefly, culture medium was replaced with DMEM containing 20% adult rat serum mixed with [³H]triolein (1 μCi/ml). The cells were incubated at 37°C in 5% CO₂-air balance for 4 h. At the termination of the incubation, the medium was decanted, and the cells were rinsed twice with 1 ml of ice-cold PBS and then thoroughly homogenized. An aliquot of the cell homogenate was taken for protein assay, and the remaining suspension was extracted for neutral lipid content.

Immunohistochemistry. Immunohistochemistry (IHC) was performed with UniTect ABC kit (Calbiochem, EMD Biosciences). Slides affixed with tissue were incubated with primary antibody overnight at 4°C in a humidified chamber. For Bcl-2 and Bax IHC, rabbit anti-Bcl-2 and -Bax antibodies (Santa Cruz Biotechnology) were used as primary antibodies at 1:100 dilutions. Following PBS washes at room temperature, the next day tissues were incubated

Fig. 1. Effect of 1α,25(OH)₂D₃ (1,25D) and C-3-epimer (3-epi-1,25D) on embryonic day 19 (e19) lipofibroblast (LF) differentiation. Twenty-four-hour treatment of cultured e19 LFs with either 1,25D or 3-epi-1,25D (1 × 10⁻¹₀ to 1 × 10⁻⁸ M) significantly increased parathyroid hormone-related protein receptor (PTHrP R; A), peroxisome proliferator-activated receptor-γ (PPARγ; B), and adipocyte differentiation-related protein (ADRP; C) protein levels, which was also accompanied by increased triolein uptake (D; *P < 0.05 vs. control; n = 4). VD, vitamin D.
Fig. 2. Effect of 1,25D and 3-epi-1,25D on e19 LF proliferation. Twenty-four-hour treatment of cultured e19 LFs with either 1,25D or 3-epi-1,25D (1 × 10^{-10} to 1 × 10^{-8} M) significantly increased thymidine incorporation (A), which was accompanied by increased phosphorylated (p-) Akt-to-total (T-) Akt ratio (B). Both 1,25D and 3-epi-1,25D significantly inhibited LF apoptosis (C), as reflected by increased Bcl-2-to-Bax ratio. Furthermore, actinomycin D (AD; 20 ng/ml)-induced LF apoptosis was at least partially blocked by pretreatment with either 1,25D or 3-epi-1,25D (D); *P < 0.05 vs. control and #P < 0.05 vs. actinomycin D group; n = 4.
sequentially with biotinylated secondary antibody, ABC reagent, diaminobenzidine (DAB) substrate in the dark. Sections were rinsed with water and counterstained with hematoxylin. Finally, tissue slices were dehydrated in xylene and permanently mounted with VectaMount. Immunostained sections were examined under a microscope (Axioskop 40; Zeiss) at \( \times 200 \) magnification.

**Lung morphometry.** An investigator unaware of the treatment group of each animal sample performed lung morphometry, which was objectively assessed by determining the alveolar count and alveolar septal thickness. For the alveolar count determination, 50 randomly selected nonoverlapping fields from sections obtained from 12 blocks (2 blocks per animal) from each treatment group were examined. Each field was viewed at 200-fold magnification, photographed, and projected onto a computer screen, and then the number of alveoli were counted visually and expressed per square millimeter. For septal thickness measurement, each slide was overlaid by random orientation with a meander scan so that at least 30 fields were analyzed (area fraction analyzed ranged from 9 to 45% of the total cross-sectional area). Analysis was performed at \( \times 852 \) magnification with a line segment grid, and all points intersecting septal tissue (minimum of 106 points/slide) as well as all intersections with the epithelial surface (minimum of 329 per slide) were counted. The arithmetic mean septal wall thickness (AMWT) was calculated according to the formula AMWT = (length per point \( \times \) sum of points)/(2 \( \times \) sum of intersections). At least 2 sections from each pup were analyzed.

**Statistical analysis.** Analysis of variance with Newman-Keuls post hoc test was used to analyze the experimental data. \( P < 0.05 \) was considered to indicate statistically significant differences between the control and experimental groups.

**RESULTS**

Both 1,25D and 3-epi-1,25D treatments of cultured LFs and ATII cells resulted in dose-dependent increases in the expression of key markers for homeostatic epithelial-mesenchymal interactions. We first examined the effects of 1,25D and its 3-epimer on LF differentiation. Treatment of LFs significantly increased the amounts of PTHrP receptor, PPAR\( \gamma \), and ADRP (Fig. 1, A–C). The enhanced LF differentiation with 1,25D and 3-epi-1,25D was also corroborated by the accompanying increased triolein uptake, a functional marker of LF capacity to recruit triglycerides from circulation (Fig. 1D).

With respect to the effect of 1,25D and its 3-epimer on cell LF proliferation, both caused significant increases in LF proliferation based on thymidine incorporation, with a maximal effect at 1 nM for both 1,25D and 3-epi-1,25D (Fig. 2A). Based on the p-Akt-to-total Akt ratio assay for cell survival, 1,25D significantly enhanced LF survival in a dose-dependent manner, with a maximal effect at 1 nM (Fig. 2B); by comparison, the 3-epimer significantly enhanced LF survival in a dose-dependent manner but with a maximal effect at 10 nM (Fig. 2B). The increased LF proliferation with 1,25D and its 3-epimer were corroborated by their effects on LF apoptosis; 1,25D significantly inhibited apoptosis based on the Bcl-2-to-Bax ratio in a dose-dependent fashion between 0.1 and 1 nM (Fig. 2C); when actinomycin D-induced LF apoptosis was challenged with 1 nM 1,25D or the 3-epimer pretreatment, both partially blocked the apoptotic effect of the actinomycin D, reflecting the antiapoptotic properties of both vitamin D metabolites (Fig. 2D).

We then examined the effects of 1,25D and its 3-epimer on ATII cell maturation. With respect to differentiation, treatment of ATII cells with either 1,25D or 3-epi-1,25D significantly increased the amounts of both SP-B and leptin receptor (Fig. 3, A and B). Similar to their effects on LFs, both 1,25D and 3-epi-1,25D stimulated ATII proliferation significantly, with maximal effects of each at 1 nM (Fig. 4A). Again, similar to their effect on LF survival, both 1,25D and 3-epi-1,25D significantly increased the p-Akt-to-Akt ratio at both 1 and 10 nM (Fig. 4B). In line with this observation, both 1,25D and 3-epi-1,25D were effective in increasing the Bcl-2-to-Bax ratio at 1 nM (Fig. 4D). As was the case for LFs, actinomycin-induced apoptosis, based on the Bcl-2-to-Bax ratio, was at least partially blocked by 1 nM 1,25D or 3-epi-1,25D (Fig. 4D).

To determine whether our in vitro findings for the effects of 1,25D and 3-epi-1,25D on lung maturation, and in particular for LF and ATII cell apoptosis, would be reproduced in vivo, we next determined the effects of parenteral administration of 1,25D and 3-epi-1,25D on markers of spontaneous lung maturation, including LF apoptosis that occurs during the period of alveolarization in the newborn rat lung, i.e., during the first 2 wk of postnatal life. Compared with controls, the expression of LF (PPAR\( \gamma \) and ADRP) and ATII cell (SP-B and SP-C) differentiation markers was increased significantly by either 1,25D (Fig. 5, A–D) or 3-epi-1,25D (Fig. 6, A–D) treatment and more so at 2 wk postnatally. Furthermore, the spontaneous stepwise decrease in the Bcl-2-to-Bax ratio in the whole rat lung tissue...
between postnatal day 1 and 2 wk was blocked by the parenteral administration of either 1,25D or 3-epi-1,25D, as shown by Bcl-2 and Bax Western hybridization of the whole lung lysates (Fig. 7, A and B) and by immunohistochemical staining (Fig. 7, C and D) of lungs from 1,25D-treated animals. On lung morphometry, at 2 wk, with 1,25D treatment, there was an increase in the alveolar count (Fig. 8A), accompanied by a paradoxical increase in alveolar septal thickness (Fig. 8B).

Last, to determine the paracrine nature of the effects of vitamin D in the lung, we compared the effect of 1,25D on triolein uptake by cultured fibroblasts alone vs. when fibroblasts were cocultured with ATII cells or on treatment of fetal lung explants with 1,25D. Whereas 24-h treatment of cultured e19 fetal rat lung fibroblasts with 1,25D at $10^{-8}$ M did not affect triolein uptake, treatment of cocultured fibroblasts and ATII cells with 1,25D at a similar concentration resulted in a significant increase in triolein uptake, clearly pointing to a paracrine effect. This was further confirmed by treatment of fetal rat lung explants with $10^{-7}$ M 1,25D, which resulted in an even greater increase in triolein uptake (Fig. 9).

DISCUSSION

Previous studies have implicated vitamin D and its 3-epimer in lung development (17, 21, 22, 28). The current study was designed to determine their specific effects on the growth and differentiation of both the LFs and ATII cells. Both 1,25D and its 3-epi-1,25D significantly inhibited LF apoptosis (C), as reflected by increased Bcl-2-to-Bax ratio. Furthermore, actinomycin D (20 ng/ml)-induced ATII cell apoptosis was at least partially blocked by pretreatment with either 1,25D or 3-epi-1,25D ($D; *P < 0.05$ vs. control and $#P < 0.05$ vs. actinomycin D group; $n = 4$).

Growth and differentiation of ATII cells and LFs are characteristic of lung development up until the time of birth (3–4a, 34), at which time LFs have been shown to become highly apoptotic. It is during the first 3 wk of postnatal life that the alveoli develop and become fully formed in the rat (5). Although we do not have data for 1,25D and its 3-epi-1,25D levels during this time period in the rat,
humans it has been found that the vitamin D 3-epimer levels remain elevated in preterm infants (33) in association with decreased alveolarization (12), suggesting a possible role of vitamin D and its metabolites in alveolarization. An association between normal vitamin D levels and lung development is also corroborated by the high frequency of alveolar abnormalities and respiratory distress in rat and human neonates born to vitamin D-deficient mothers (8, 9).

During the process of lung development, it is critically important that along with increased surfactant synthesis, the alveolar interstitium thins out for effective gas exchange (7). Following bulk alveolarization, a large percentage of interstitial lipofibroblasts undergo apoptosis, resulting in a substantial reduction in interstitial volume (3, 31, 35). In the fetal rat lung, whereas apoptotic epithelial cells appear at 18 days of gestation, apoptotic mesenchymal cells appear much earlier, at days 14–18 of gestation (13, 30). Bruce et al. (5) and others (35) have shown that after alveolar formation, the number of apoptotic lung fibroblasts increases sharply, reaching maximal levels that exceed 20% during the third postnatal week. This well-orchestrated removal of the bulk of LFs allows for the formation of intercellular contacts between endothelial cells of adjacent capillary layers, thereby promoting the formation of the lung vasculature, an integral part of which is the transformation of a double to a single capillary layer in the alveolar wall (31).

Although many cellular and molecular mechanisms have been implicated separately in driving surfactant maturation and septal wall thinning, the process remains incompletely understood. In particular, none of the previously implicated molecules has been shown to coordinately regulate both processes simultaneously. Furthermore, although surfactant maturation during lung development has been studied for decades, the role of apoptosis in septal thinning has only recently been recognized. Our findings of a decreased Bcl-2-to-Bax ratio by Western hybridization in the whole lung lysates and decreased Bcl-2 and increased Bax immunostaining from postnatal day 1 and at 2 wk postnatally support these observations. However, more importantly, blockage of LF apoptosis by 1,25D and 3-epi-1,25D in vitro and in vivo during the period of most active alveolarization suggests a physiological role for vitamin D in modulating alveolar septal thinning.

Fig. 5. Effect of parenteral administration of 1,25D on markers of alveolar LF and ATII cell differentiation. Two weeks of daily administration of 1,25D (10 ng/kg body wt ip once daily) from postnatal days 6 to 14 resulted in significantly increased expression of PPARγ and ADRP (LF differentiation markers; A and B) and SP-B and SP-C (ATII cell differentiation markers; C and D); *p < 0.05 vs. control at the corresponding stage of development; n = 4. 1D, 1 day; 1W, 1 wk.
In addition to the role of vitamin D in stimulating surfactant synthesis, its effects on LF apoptosis further underscores the biological rationale for vitamin D production by fibroblasts and its receptor expression by the adjoining ATII cells, i.e., the existence of an alveolar vitamin D paracrine system (22). Our data suggest temporally specific effects of vitamin D during alveolar development. Overall, based on the studies included here, we speculate that in the corners of the alveolus, i.e., the sites for ATII cell-LF units, locally produced vitamin D promotes epithelial-mesenchymal interactions, resulting in increased ATII cell and LF differentiation and proliferation (Fig. 10). However, the lack of this paracrine system away from the alveolar corners, i.e., in the alveolar septum, leads to LFs apoptosis, resulting in the observed shift from the earlier predominance of LFs, to the later predominance of non-LF interstitial fibroblasts in the alveolar septum during the process of alveolarization (3).

Contrasting effects of vitamin D and its metabolites on fibroblast apoptosis and differentiation have been described previously. For example, although in C3H10T1/2 murine fibroblasts vitamin D inhibited vitamin E succinate-induced apoptosis, in 3T3-L1 adipocytes it induced apoptosis and inhibited differentiation (2, 40). The effect of vitamin D on apoptosis has also been shown to be dose-dependent. For example, in 3T3-L1 cells, physiological doses (0.1–10 nM) of 1,25D protected against uncoupling protein 2 overexpression-induced apoptosis, whereas a high dose (100 nM) stimulated apoptosis (36). Although the mechanism of 1,25D-mediated inhibition of lung LF apoptosis remains unclear, previous studies have implicated the PI3K/PKB/Akt pathway in C3H10T1/2 murine fibroblasts (2). This is consistent with our finding of increased p-Akt expression by the cultured LFs on vitamin D stimulation. Our results are also supported by a recent study by Ormerod et al. (23), who observed mitogenic effects of 1,25D and its synthetic analog, e.g., EB1089, on alveolar fibroblasts both in vitro and in vivo. However, treatment with EB1089, a potent noncalcemic synthetic analog of vitamin D, resulted in localized regions of septal thickening due to fibroblast hyperproliferation, further underscoring the significance of biological
Fig. 7. Effect of parenteral administration of 1,25D and 3-epi-1,25D on whole lung apoptosis. Neonatal pups were administered 1,25D (10 ng/kg body wt) or 3-epi-1,25D (50 ng/kg body wt) intraperitoneally once daily from postnatal days 0 to 14. The spontaneous stepwise decrease in Bcl-2-to-Bax ratio in the whole rat lung tissue between postnatal day 1 (PND1) and 2 wk was completely blocked by the parenteral administrations of 1,25D and 3-epi-1,25D, as shown by Bcl-2 and Bax Western hybridizations of the whole lung lysates (A and B; *P < 0.05 vs. control at the corresponding stage of development; n = 4) and by immunohistochemical staining on lung tissue from 1,25D-treated animals (C and D, magnification, ×40).
fine tuning of physiological levels of vitamin D during perinatal lung maturation. It is likely that differences in the study design and the dose of vitamin D used account for the differences in the results in our study and the study by Ormerod et al. (23). The dose of 1,25D (10 ng/kg body wt) we used was estimated based on the plasma levels of 1,25D (25 pg/ml at postnatal day 14) and the calculated blood volume in the neonatal rat pup (1, 19). Since previous studies had suggested lower 3-epi-1,25D activity compared with its parent compound, we used a five times higher dose of 3-epi-1,25D (50 ng/kg body wt) to study its effects in vivo. Taken together, our data imply an important physiological role of vitamin D in perinatal pulmonary maturation and for the first time show its coordinated effects on alveolar epithelial and mesenchymal proliferation and differentiation.

We conclude that temporally specific effects of vitamin D and its metabolites on key alveolar epithelial-mesenchymal interactions and LF survival are critical for normal lung development. The American Academy of Pediatrics recently recommended doubling the daily vitamin D dose for neonates, citing the traditional skeletal and other nonpulmonary effects; however, it will be interesting to study the impact of this change on human lung development given the widespread evidence of vitamin D deficiency in pregnant women (38a) and the recent large body of evidence that perinatal vitamin D deficiency may explain a significant portion of the recent asthma epidemic (16).

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VITAMIN D AND ITS 3-EPIMER PROMOTE RAT LUNG MATURATION

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