Transforming Growth Factor Beta 1 Binding and Receptor Kinetics in Fetal Mouse Lung Fibroblasts (44267)

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Abstract. **TGFβ1 inhibits fetal lung maturation *in vitro*. As TGFβ1 is present in fetal lung, mechanisms must exist to overcome this inhibition and allow late gestation maturation to progress. We studied the ontogeny of TGFβ1 binding, and TGFβ receptor kinetics and subtypes in primary cultures of fetal mouse lung fibroblasts from Day 16 to Day 18 of gestation. TGFβ1 specific binding in fetal lung fibroblasts declined with advancing gestation. The decrease occurred earlier, and was more pronounced in female fibroblasts (50% decrease) than in the male fibroblasts (29% decrease). Dihydrotestosterone treatment of Day 18 female fibroblasts resulted in a dose-dependent increase in TGFβ1 binding. Scatchard analysis revealed a decline in receptor number with advancing gestation (Day 16 female B_{max}: 7.3×10^{-16}; Day 18 female B_{max}: 5.5×10^{-16}) whereas binding affinities remained constant. Affinity labeling followed by chemical cross-linking and autoradiography showed the three known TGFβ receptor subtypes at both Days 16 and 18 of gestation. The relative abundance of nonsignaling Type III receptors in comparison to signaling Type II and Type I receptors was increased at Day 18 versus Day 16. Incorporation of [^{3}H]thymidine into DNA after treatment with TGFβ1 changed from Day 16 to Day 18, consistent with changes previously reported between fetal and adult lung fibroblasts. We conclude that as fetal mouse lung maturation progresses, TGFβ receptor number decreases in fibroblasts, the relative proportion of nonsignaling versus signaling receptor types increases, and the response to TGFβ1 stimulation changes. These changes may contribute to overcoming TGFβ1 inhibition of lung maturation.** [P.S.E.B.M. 1998, Vol 218]

The transforming growth factor beta (TGFβ) group of proteins are members of a family of polypeptides with multifunctional regulatory activities involved in the regulation of growth and differentiation. Several closely related isoforms of TGFβ have been found in vertebrates, three of which (TGFβ1, TGFβ2 and TGFβ3) are known to exist in humans. All three of these have been shown in a variety of studies to either stimulate or inhibit cellular proliferation, differentiation, or function depending on the cell type (1–5). A strong role for TGFβ1 in the control of various aspects of embryogenesis through modulation of cell proliferation and/or local control of extracellular matrix (ECM) production has been suggested (3, 6–8).

In the developing lung, TGFβ1, TGFβ2, and TGFβ3 genes are expressed at high levels and show distinct patterns of spatial and temporal distribution (7–9). TGFβ1 expression is strongest, appearing predominantly in the bronchial mesenchyme, especially underlying epithelial branch points (7). This pattern of expression of TGFβ1 in the fetal lung led to the suggestion that TGFβ1 participates in directing lung branching morphogenesis. There is also evidence that TGFβ1 inhibits lung maturation. TGFβ1 inhibits the synthesis of the specific surfactant-associated glycoprotein A (SP-A) in organ cultures of human fetal lung (10). Furthermore, TGFβ1 inhibits fibroblast-type II cell interactions. Type II cell differentiation, and surfactant synthesis by fetal Type II cells (10–14).

The activity of all the TGFβ isoforms in controlling cell proliferation and ECM production is mediated through binding to specific cell-surface TGFβ receptors (TGFβ-R).

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This work was supported by NIH grants HL 37930 and HL 43407, and Deutsche Forschungsgemeinschaft Da 3781/1-1.

Received September 3, 1996. [P.S.E.B.M. 1998, Vol 218]
Accepted January 8, 1998.

0037-9727/98/2181-0051$10.50/0

A number of TGFβ receptors have been described in a variety of target cells. Three types of TGFβ-R (Types I, II, and III) have been characterized in terms of their structure and biological function ((15–18); also reviewed in Ref. 19–21). TGFβ-R Types I and II are transmembrane serine-threonine kinases. TGFβ1 binds the Type II receptor; this allows recruitment of Type I receptors into a heterodimer complex. In the complex, the Type II receptor kinase phosphorylates Type I receptors, activating them. Signals are transduced by the heterodimerization of Type I and Type II receptors to ultimately control gene transcription. TGFβ1 activates at least two different signal transduction pathways for growth inhibition and growth stimulation, through the activation of different *ras* proteins and myelin basic protein kinases (22). Studies using mutant constructs of the Type I receptor that are constitutively active demonstrate that activation of this receptor is necessary and sufficient for downstream signal transduction. Thus, formation of Type II-Type I receptor heterodimers, with activation of the Type I receptor by the Type II receptor, appears to be the mechanism through which TGFβ initiates signal transduction (23). The Type III receptor is a transmembrane proteoglycan with a short, highly conserved cytoplasmic domain that has no apparent signal transduction motif (24, 25). It is suggested that the Type III receptor helps mediate cell-specific TGFβ signaling by controlling the availability of TGFβ molecules in the local environment (24). TGFβ activity may be dependent in part on relative amounts of TGFβ Type I, Type II, and Type III receptors.

The importance of understanding the regulation of TGFβ1 activity in the developing lung is emphasized by *in vitro* studies of the effects of exogenous TGFβ1 on embryonic lung branching morphogenesis. TGFβ1 in high doses (≥20 ng/ml) inhibits branching morphogenesis (26); at low doses (0.2 ng/ml) it acts synergistically with EGF to enhance branching morphogenesis (27); and at intermediate doses (2 ng/ml) it exhibits a mixture of inhibitory and stimulatory effects on different aspects of branching morphogenesis (28). These different results are likely explained by dose-dependent differences in the availability of TGFβ1 to a local environment. Higher treatment doses may overwhelm the ability of TGFβ receptors to control TGFβ-mediated events locally. A better understanding of local control through regulation of TGFβ receptors during lung development would help in understanding the role of TGFβ in modulating fetal lung growth and differentiation.

The fact that TGFβ1 and TGFβ receptors are present in the fetal lung throughout development, and the observations that TGFβ1 inhibits maturational processes involved in surfactant synthesis suggest that the inhibitory influence must be overcome to allow normal maturation to occur. It is probable that this inhibition is normally overcome at a point in gestation when growth and remodeling of structure is established and maturation of surfactant synthesis begins. The mechanisms that may be involved in overcoming this inhibition by TGFβ1 on lung maturation are not known.

In late gestation, maturation of the lung for surfactant synthesis is mediated by fibroblast–type II cell interactions. TGFβ1 acts on fetal lung fibroblasts to inhibit these interactions (12–14). Thus, a mechanism for removing the inhibitory influence of TGFβ1 on lung maturation should be active in fetal lung fibroblasts. We hypothesized that the mechanism through which the inhibition by TGFβ1 of fibroblast–type II cell communications controlling fetal lung maturation is overcome involves a maturational decline in cell-specific TGFβ1 binding in fibroblasts. We tested this hypothesis by studying the developmental ontogeny of TGFβ1 binding, TGFβ receptor kinetics, and TGFβ receptor subtypes in primary cultures of fetal mouse lung fibroblasts of different gestational ages.

Materials and Methods

Reagents. ^{125}I-labeled porcine TGFβ1 (specific activity 66-156 μCi/μg) was purchased from DuPont New England Nuclear (Cambridge, MA). Unlabeled TGFβ1 was purchased from R & D Systems (Minneapolis, MN). [^{3}H]Thymidine (specific activity 50 Ci/mmole) was purchased from ICN (Irvine, CA). Bovine serum albumin (BSA) was purchased from Calbiochem (La Jolla, CA). Hepes, deoxyribonuclease I, soybean trypsin inhibitor, Triton X-100, antipain, aprotinin, bestatin, leupeptin, pepstatin A, benzamidine hydrochloride, and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma Chemical Company (St. Louis, MO). Disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, IL). Dulbecco's modified eagle's medium (DMEM), Hanks balanced salt solution, and trypsin were purchased from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, Utah). Plastic tissue culture dishes were purchased from Costar (Cambridge, MA) and Falcon (Becton Dickinson Labware, Lincoln Park, NJ).

Animals. The animal study protocol was approved by the Tufts-New England Medical Center Animal Research Committee. Timed pregnancy Swiss Webster mice were obtained from Taconic Farms (Germantown, NY). Animals were placed together in the evening; the following morning, sperm-positive females were denoted as Day 1 of gestation. Litters were sacrificed at Day 16, Day 17, and Day 18 of gestation (delivery normally occurs on the afternoon of Day 19). Fetuses were separated by sex as identified by gonadal inspection (29).

Cell Cultures. Primary, nonpassaged cultures of fetal lung fibroblasts were used. Many studies have shown that these fibroblasts maintain a specific developmental phenotype in culture (29). The use of primary cell cultures avoided potential changes in phenotype that may be introduced by passage. Fibroblast cultures were prepared by differential adherence as we have described previously (29). Briefly, the lungs were removed aseptically and pooled according to sex in ice cold Hanks buffer, then minced with a razor blade. The cells were dissociated by trypsinization with 0.25% trypsin and deoxyribonuclease I (20 μg/ml) in

Hanks at 37°C for 15 min. The trypsinization reaction was stopped by adding DMEM with 10% stripped fetal calf serum. The resulting cell suspension was filtered through 15-μm Nitex filters and centrifuged at 640*g* for 10 min. The cell pellet was resuspended in DMEM with 10% stripped fetal calf serum and plated on six well plates. A 1-hr differential adherence step allowed fibroblasts to adhere to the bottom of the plates. These fibroblast cultures were grown to confluence in DMEM with 10% stripped fetal calf serum at 37°C in 95% air and 5% CO_2. Each individual culture experiment was conducted using fibroblasts isolated from the male or female fetal lungs pooled from three to five litters. In one group of experiments, female Day 18 fibroblasts were grown in the absence (control) or presence of dihydrotestosterone (DHT) in different concentrations ($10^{-10}M$–$10^{-6}M$) to identify the effect of androgen on expression of TGFβ binding.

TGFβ1 Binding Assay. The binding assay for confluent fibroblast monolayer cultures was a modification of the procedures of Massague (30) and Wakefield (31). Confluent fibroblast monolayers in six well plates were washed with dissociation buffer (DMEM containing 25 m*M* Hepes and 0.1% BSA) and incubated in dissociation buffer (0.5 ml) for 2 hr at 37°C to allow for dissociation and/or internalization of endogenously bound TGFβ1. The cells were then washed with ice cold binding buffer (128 m*M* NaCl, 5 m*M* KCl, 5 m*M* $MgSO_4$, 1.2 m*M* $CaCl_2$, 50 m*M* Hepes, 10 mg/ml BSA, ph 7.5) and preincubated in binding buffer for 30 min at 4°C. Fibroblasts in triplicate wells were incubated in 0.5 ml of binding buffer containing 25–125 pmoles of ^{125}I-TGFβ1 with or without a 50-fold excess of unlabeled TGFβ1 for equilibrium-binding experiments. All binding experiments were done at 4°C to minimize internalization and/or degradation of labeled TGFβ1. Initial experiments were performed from 15 min through 5 hr at 4°C with constant shaking at 150 rpm on a rotary shaker platform to identify the time of maximum binding. Thereafter, all binding experiments were carried out over 4 hr. At the end of this incubation period, the cultures were again washed with binding buffer to remove unbound labeled TGFβ1. The bound labeled TGFβ1 was solubilized with solubilizing buffer (1% vol/vol Triton X-100, 10% vol/vol glycerol, 25 m*M* Hepes, 10 mg/ml BSA) and counted in a gamma counter to determine total binding per well. Nonspecific binding was determined concurrently in triplicate wells by adding a 50-fold excess of unlabeled TGFβ1 before adding the ^{125}I-TGFβ1. The average nonspecific binding was subtracted from total binding to obtain specific binding. Additional wells were used to measure the amount of DNA per well, as previously described for TGFβ-binding studies in lung fibroblasts from older animals (32). Specific binding was expressed as cpm/nmole of DNA. The level of specific binding was always >65% of total binding. Initial experiments were performed with increasing doses of ^{125}I-TGFβ1 to identify a linear range for specific binding. Based on this we used 50 pmoles for our binding experiments. The specificity of TGFβ1 binding was demonstrated using nonspecific growth factors as described by Kalter and Brody (32). Fibroblasts were preincubated with 10 ng/ml or 62 ng/ml (an 8-fold and a 50-fold excess, respectively) of epidermal growth factor (EGF), keratinocyte growth factor (KGF), insulin, or TGFβ1 (as done to measure nonspecific TGFβ1 binding in all other experiments). Saturation binding analysis was performed by incubating fibroblasts with 25–400 pmoles of ^{125}I-TGFβ1 with and without a 50-fold excess of unlabeled TGFβ1, using in separate experiments Day 16 and Day 18 male or female fibroblast cultures. Competition binding analysis was performed by incubating fibroblast monolayers with 50 pmoles of ^{125}I-TGFβ1 and 0–4 nmoles/ml of unlabeled TGFβ1 for Day 16 and Day 18 male and female fibroblast cultures.

Affinity Labeling of Cell Monolayers. Affinity labeling of cells was a modification of the procedures of Massague (30) and Mitchell *et al.* (33). Confluent monolayers of Day 16 and Day 18 male and female fibroblasts in six well plates were incubated with 50, 100, or 200 pmoles of ^{125}I-TGFβ1 with or without unlabeled TGFβ1 in 0.5 ml of binding buffer/well for 3 hr at 4°C with continuous gentle agitation as described earlier. The 3-hr incubation was chosen in agreement with the methods previously described by Massague (30); this time is also appropriate based on our binding curve. Monolayers were washed three times with ice cold binding buffer and incubated with 1 m*M* DSS in binding buffer for 15 min at 4°C. Cells were washed briefly with cell detachment buffer (0.25 *M* sucrose, 10 m*M* Tris, 1 m*M* EDTA, pH 7.4; 0.3 m*M* PMSF) and scraped off the dishes with a Teflon scraper in the presence of 0.5–1.0 ml of cell detachment buffer. The cells were sedimented by centrifugation at 12,000*g* for 2 min in 1.5 ml microfuge tubes. The supernatant was discarded, and the pellet was solubilized in a minimal volume (60–100 μl) of solubilizing buffer (1% v/v Triton X-100; 10% v/v glycerol; 1 m*M* EDTA; 20 m*M* Tris-HCl, ph 7.4) supplemented with protease inhibitors (10 μl/ml each of cocktail I and cocktail II; cocktail I containing 1 mg/ml leupeptin, 1 mg/ml antipain, 5 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, 10 mg/ml benzamidine hydrochloride; and cocktail II containing 1 mg/ml pepstatin, 1 mg/ml bestatin, 30 m*M* PMSF in dimethyl sulfoxide) for 40 min at 4°C. The insoluble debris was removed by centrifugation for 15 min at 12,000*g*. The supernatant was mixed with 1/5 vol of 5× electrophoresis sample buffer (0.25 *M* Tris-HCl, pH 6.8, 5% w/v SDS, 10% v/v β-mercaptoethanol, 50% v/v glycerol, 0.0004% w/v bromphenol blue), heated to 100°C for 5 min and frozen until use for SDS-PAGE.

Gel Electrophoresis and Autoradiography. Discontinuous gel electrophoresis on 7.5% SDS-polyacrylamide gel was performed according to the procedures of Laemmli (34). The gels were fixed, stained in Brilliant Blue G, destained, and dried under vacuum. Dried gels were exposed to phosphorimage plates for 4 days, or to Kodak AR-Xomat film using Dupont Lightning Plus Screens and

stored at −70°C for 3–5 weeks. The films were developed, and the molecular weights of the resulting bands were determined from calibration curves of simultaneously electrophoresed molecular weight markers for each gel. The intensity of labeled bands was quantified by phosphorimage analysis or by densitometry analysis of the autoradiographs.

[^{3}H]Thymidine Incorporation. Sex-specific Day 16 and Day 18 fetal mouse lung fibroblasts were plated in 24-well plates in DMEM plus 10% stripped fetal calf serum. After 48 hr of incubation the cells were serum-starved for 24 hr in serum-free medium. Cultures were then incubated with serum-free DMEM as controls or in the presence of different doses of TGFβ1 (0.1 ng/ml-10 ng/ml) for 20 hr. After a 6-hr-pulse with 2 μCi/ml [^{3}H]thymidine, the cells were rinsed three times with phosphate buffered saline and lysed in Hanks buffer containing 0.8 m*M* EDTA and 0.1 m*M* trypsin. The incorporated amount of [^{3}H]thymidine was measured in a beta scintillation counter. DNA content of each well was measured as deoxyribose content (35).

Statistical Analysis. Competition and saturation binding assays were analyzed with the LUNDON receptor analysis program (Lundon, Chagrin Falls, OH). Statistical analysis used regression analysis, analysis of variance (ANOVA), and the two sample Student's *t* test. Corrections for multiple comparisons were made using the Tukey procedure (36).

Results

Specific Binding of ^{125}I-TGFβ1. Binding of ^{125}I-TGFβ1 increased with time up to a maximum at 2 hr and continued at this level through 5 hr (Fig. 1a), similar to results reported for passaged lung fibroblasts from infant rats (32). Specific binding was linear between 25–75 pmoles ^{125}I-TGFβ1 (Fig. 1b). Binding was specific for TGFβ, in that binding was not competed away by EGF, insulin, or KGF (Fig. 1c), similar to results described for passaged infant rat lung fibroblasts (32).

Ontogeny of Specific Binding of ^{125}I-TGFβ1. Specific TGFβ1 binding fell with advancing gestation (Fig. 2). Results were first grouped and evaluated without regard to fetal sex. Overall, specific binding fell 39% from Day 16 to Day 18 (Day 16: 951 ± 73; Day 17: 769 ± 63; Day 18: 576 ± 65; mean ± SEM of CPM ^{125}I-TGFβ1 bound per nmole DNA (n = 10–11 experiments, each experiment a mean of six to nine observations). One-way ANOVA showed that the decrease with advancing gestation was statistically significant (P = 0.0018). This decline with advancing gestation was further analyzed by fetal sex. Both males and females exhibited a progressive decline in specific binding of TGFβ1 in fetal mouse lung fibroblasts with increasing gestation. In female fibroblasts, specific binding was decreased by 28.4% on Day 17, and by 49.6% on Day 18, compared to Day 16. This decline in specific binding in female fibroblasts with gestational age was statistically significant (P = 0.003). Further analysis using ANOVA followed by the Tukey multiple comparison showed that Day 18 was significantly lower than Day 16 (P = 0.008). In male fibroblasts, the pattern of the decline was different. Specific binding was decreased only by 11.7% on Day 17, but then was 29.1% lower on Day 18, compared with Day 16. Thus, the initial decline seen between Day 16 and Day 17 in the female fibroblasts occurred between Day 17 and Day 18 in the male fibroblasts. In the male, the decline in TGFβ1 specific binding with increasing gestation fell short of statistical significance (P = 0.07).

Effect of DHT Treatment on TGFβ1 Binding. Day 18 female fetal lung fibroblasts were treated with DHT to learn if androgen exposure increased TGFβ binding. Binding was increased by DHT treatment in a dose-dependent manner (Fig. 3). The increase was highest at the lowest dose of DHT studied (10^{-10} *M*) and decreased with higher doses. Significant increases ($P < 0.01$) were observed with DHT doses just below or within the fetal physiologic range, but not at supraphysiologic doses.

Affinity and Number of TGFβ Receptors on Fetal Lung Fibroblasts. Estimations of receptor binding affinity (K_d) and number of binding (B_{max}) sites for ^{125}I-TGFβ1 were obtained by measuring equilibrium binding of labeled TGFβ1 to confluent fetal mouse lung fibroblast cultures at 4°C. Results of Scatchard analysis (average of three to four experiments each) were consistent with a single class of receptors with apparent K_d values of 98 p*M* and 78 p*M* for Day 16 males and females respectively, and 108 p*M* and 100 p*M* for Day 18 males and females respectively (Table I). A representative Scatchard plot is shown in Figure 4A. The number of binding sites was calculated as the x axis intercept in each experiment. The average B_{max} values (Moles/nmole DNA) for male and female fibroblasts showed a decrease from Day 16 to Day 18 (Table I). These average values for B_{max} binding sites are in agreement with the B_{max} values obtained from competition assay (Day 16: Male 5.3×10^{-16}, Female 5.5×10^{-16}; Day 18: Male 4.3×10^{-16}, Female 3.2×10^{-16}; Moles/nmole DNA). A representative competition binding curve is shown in Figure 4B.

Affinity Labeling of TGFβ1 Receptor Proteins. ^{125}I-TGFβ1 was chemically cross-linked to mouse fetal lung fibroblast receptor proteins at Day 16 and Day 18 of gestation for both male and female fibroblasts separately. The labeled proteins were extracted with Triton-X, separated by SDS-PAGE, and detected by autoradiography (Fig. 5A and B). The three TGFβ receptor subtypes were identified according to molecular weight. On 7.5% gels all three TGFβ receptor subtypes were visible, although the Type III receptor was located near the origin of the gel because of its large molecular weight. PAGE was also performed using 5% gels to allow better migration and identification of the Type III receptor. These gels confirmed the molecular size of the Type III receptor but the low molecular weight Type I receptor had migrated beyond the gel and was no longer visible (data not shown). Thus, the data were evaluated using 7.5% gels because they showed all three receptor subtypes. ^{125}I-TGFβ1 specifically labeled three major pro-

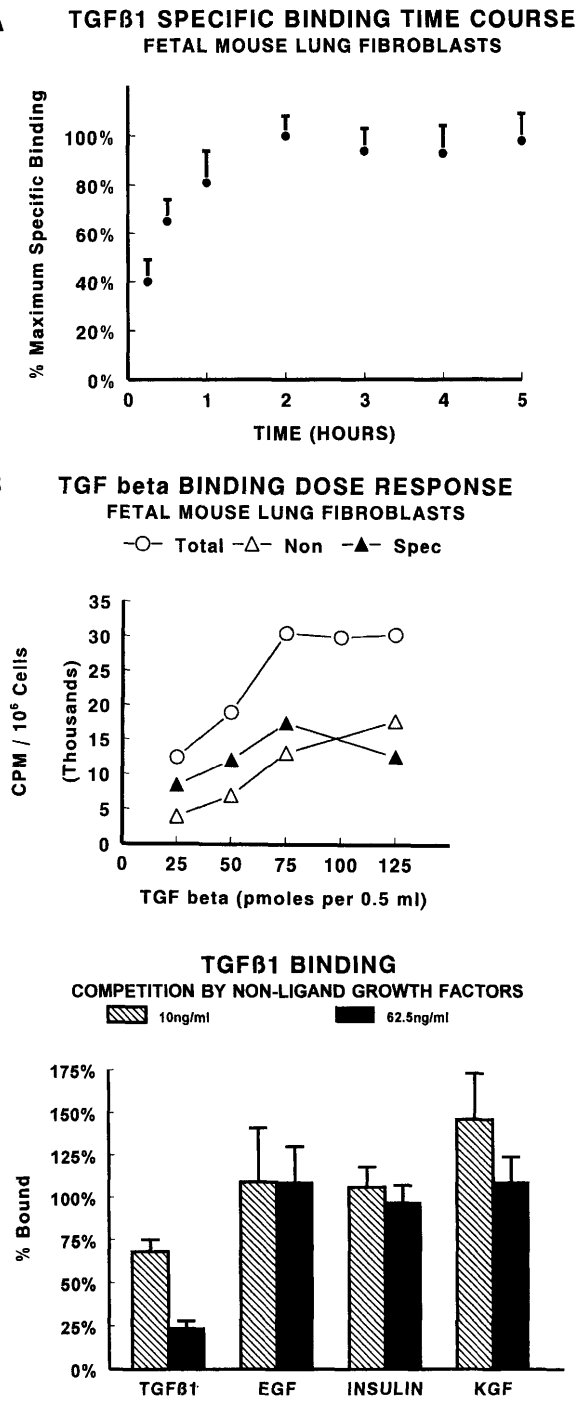


Figure 1A: TGFβ1 time binding curve showing the specific TGFβ1 binding to mouse fetal lung fibroblasts from 30 min through 5 hr incubation. The results of each experiment were converted to the percent of the maximal specific binding. The data are the means ± SEM of four to seven observations from seven experiments. Specific binding increased with incubation time up to 2 hr and was maximal from 2 through 5 hr of incubation.

Figure 1B: TGFβ1 equilibrium binding curve showing the total bound ^{125}I-TGFβ1 (open circles), nonspecific bound (open triangles) and specific bound ^{125}I-TGFβ1 (closed triangles). Values are expressed as cpm/million cells at increasing doses of ^{125}I-TGFβ1 from 25 pmoles to 125 pmoles in 0.5 ml of binding buffer. Specific binding was linear between 25 and 100 pmoles; therefore, the 50-pmole dose of labeled TGFβ1 was chosen for further experiments.

Figure 1C: TGFβ1 binding in the presence of competing doses (10 ng/ml, cross hatched bars; and 62.5 ng/ml, solid bars) of EGF (1.7 nmoles, 10.6 nmoles), insulin (1.7 nmoles, 10.6 nmoles), KGF (525 pmoles, 3280 pmoles), and TGFβ1 (400 pmoles, and 2.5 nmoles, 8- and 50-fold excesses, respectively; the latter amount of TGFβ was used in all subsequent binding experiments to identify nonspecific binding). These competing doses were previously used by others to demonstrate specific ^{125}I-TGFβ1 binding (32). Experiments were performed in triplicate in male and female fibroblasts, and the data combined as mean ± SEM. The y-axis represents the percent of the mean total ^{125}I-TGFβ1 bound in comparison to experiment-specific control cells. Excess amounts of either EGF, insulin, or KGF do not significantly compete for ^{125}I-TGFβ1 binding, and increasing amounts of TGFβ1 produces increasing competition for binding. This supports the conclusion that binding of ^{125}I-TGFβ1 in the cultured fibroblasts is ligand-specific.

tein bands with approximate molecular weights of 70, 97, and 280 kD, corresponding to the previously identified TGFβ Type I, Type II, and Type III receptors (15–19) at both Day 16 and Day 18 of gestation (the autoradiographs shown in Figures 5A and 5B were intentionally overexposed to better illustrate the three receptor types). In addition, bands were sometimes seen at approximate molecular weights of 36 kD and 120 kD, as has been described by other authors for TGFβ cross-linking studies in other tissues (15, 33, 37). The intensity of the Type III band appeared strongest at each gestation. To delineate further the three receptor bands, affinity labeling was done using 50, 100, and 200 pmoles per reaction of ^{125}I-TGFβ1 (Figure 5B shows a representative autoradiograph from Day-18 male cells). This demonstrated increased labeling with increased amount of ^{125}I-TGFβ1, and similarly revealed a relative preponderance of Type III TGFβ receptor. Differences in amount of protein loaded per lane precluded direct comparisons of receptor subtype across gestation. Also, as discussed by Anders *et al.*, cross-linking experiments are not reliable for making quantitative comparisons of receptor number between different conditions (38). Therefore, only the relative proportion of the receptors within each gestational age was evaluated. In the absence of any competitor,

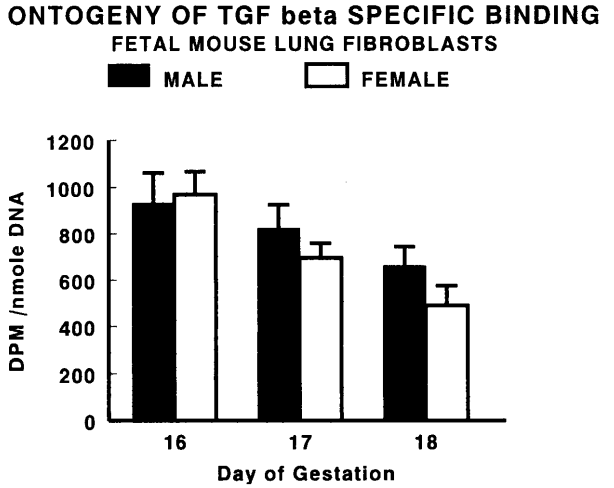


Figure 2. The ontogeny of TGFβ1 receptor binding at various gestations in fetal mouse lung fibroblasts, represented as specific binding in CPM per nmole of DNA, for males (filled bars) and females (open bars) at each day of gestation. Mean ± SEM of five to six experiments, each experiment representing a mean of six to nine observations. In females there is a progressive stepwise decline in specific ^{125}I-TGFβ1 binding with increasing gestation (P = 0.003). Day 18 females are significantly less than Day 16 females (P = 0.008). In males the decline in the TGFβ1 binding begins later, on Day 18 (P = 0.07).

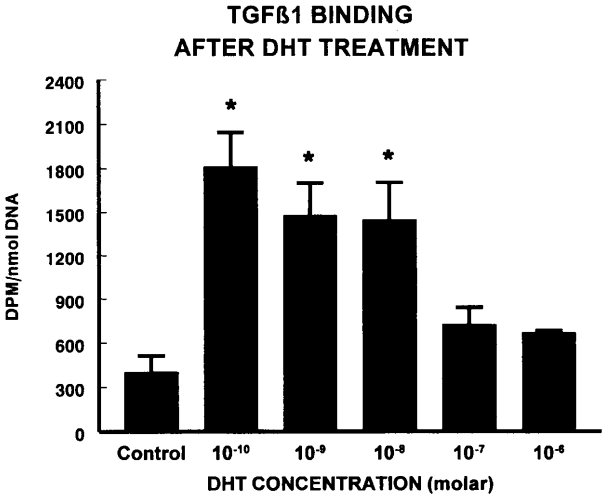


Figure 3. The effect of DHT (doses $10^{-10}M$–$10^{-6}M$) on TGFβ1 binding in Day 18 female fetal lung fibroblasts, represented as specific binding in CPM per nmole of DNA at each dose of DHT. Bars represent the mean ± SEM of two experiments, each with two to four observations. * = $P < 0.01$ compared to control. A dose-dependent increase in TGFβ1 binding was observed, which disappeared at supra-physiologic DHT concentrations.

^{125}I-TGFβ1 labeled the 280-kD Type III receptor more intensely than both Type I and Type II receptors taken together. Phosphorimage analysis of the three bands of labeled receptor proteins revealed that the relative proportion of Type I and Type II receptors compared to Type III receptor was significantly decreased on Day 18 as opposed to Day 16 for both sexes (Table II). The ratio of the total of labeled Type I plus Type II receptor proteins relative to the Type III receptor protein declined by 43% in the female from Day 16 to Day 18 (ratio of the proportions of Type I plus Type II compared to the proportion of Type III was 0.646 in Day-16 females and 0.375 in Day-18 females). In males this ratio declined by 38%. The labeling of the three receptor species was inhibited in a concentration dependent manner by the presence of increasing amounts of unlabeled TGFβ1 (Fig. 6). In summary, although there was a relative preponderance of Type III receptors at both Day 16 and Day

Table I. Results of the Scatchard Analysis

Gestation	$B_{max} \times 10^{-16}$ Mole/nmole DNA	$K_d \times 10^{-12}$ Mole
Day 16 Male	7.5 ± 2.4	98 ± 28
Day 16 Female	7.3 ± 1.5	78 ± 30
Day 18 Male	5.2 ± 0.7	108 ± 23
Day 18 Female	5.5 ± 1.0	100 ± 18

Note. (Mean ± SEM of 3–4 Experiments)

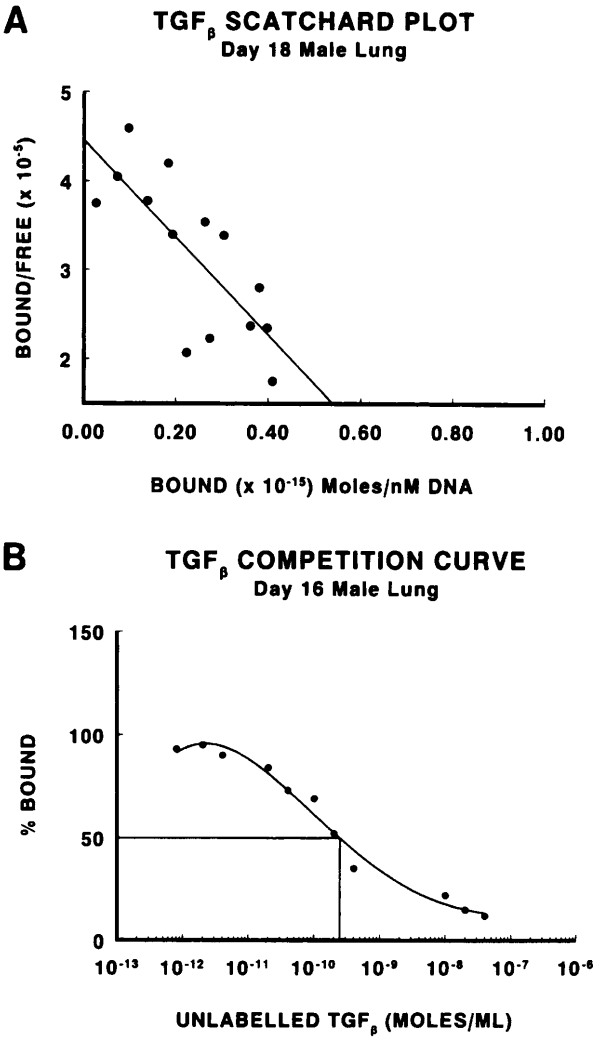


Figure 4A: A representative Scatchard plot of Day 18 male fetal mouse lung fibroblast saturation binding data using 25–300 pmoles of ^{125}I-TGFβ1 in the presence of a 50-fold excess of unlabeled TGFβ1.

Figure 4B: A representative competition curve of day 16 male fetal mouse lung fibroblasts. Competition analysis was performed by incubating fibroblast monolayers with 50 pmoles ^{125}I-TGFβ1 and 0–4 nmoles/ml of unlabeled TGFβ1.

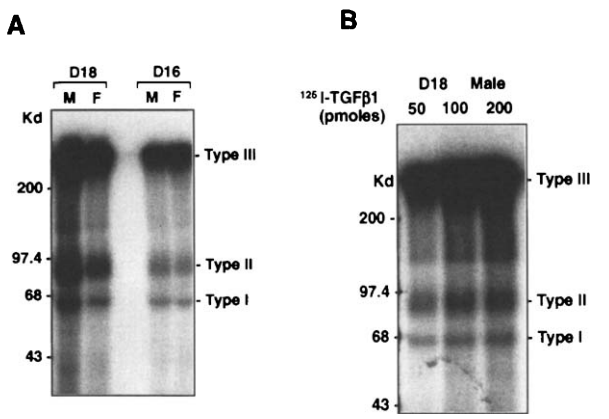


Figure 5A: Autoradiograph obtained after chemical cross-linking ^{125}I-TGFβ1 with male and female fetal mouse lung fibroblast receptor proteins at Days 16 and 18 of gestation. ^{125}I-TGFβ1 specifically labeled three major protein bands with approximate molecular weight of 70, 97, and 280 kD, corresponding to the Type I, Type II, and Type III receptors at both Days 16 and 18. This blot was intentionally overexposed to better show the three receptor subtypes.
Figure 5B: Representative autoradiograph showing affinity labeling of the Type I, Type II, and Type III receptor proteins at Day 18 gestation in male mouse lung fibroblasts using 50 pmoles, 100 pmoles, and 200 pmoles (each in 0.5 ml buffer) of ^{125}I-TGFβ1. Bands of increasing strength appeared with increasing ligand dose. This blot was intentionally overexposed to better show the three receptor subtypes at each dose.

Table II. Analysis of Relative Proportions of Receptor Subtypes in Female and Male Fetal Lung Fibroblasts at Day 16 and Day 18 Expressed as a Percentage of the Total in Each Lane

Receptor protein	Females		Males	
	Day 16	Day 18	Day 16	Day 18
Type III	61 ± 2%	72 ± 2%*	68 ± 1%	77 ± 2%†
Type II	21 ± 1%	15 ± 1%	18 ± 1%	13 ± 1%
Type I	19 ± 1%	12 ± 1%	15 ± 1%	10 ± 1%

Note. Blots were analyzed using phosphorimager analysis. Data are means ± SEM of the percent of the sum of the phosphorimage signal of the three receptors. n = 8 experiments except for Day 16 males where n = 2 experiments. Statistical analysis compared the means for Type III receptors at Day 16 versus Day 18 for each sex. * = $P < 0.001$. † = $P < 0.05$.

18 of gestation, at Day 18 the relative proportion of signaling Type I and Type II receptors compared to nonsignaling Type III receptors was decreased from that of Day 16.

Effect of TGFβ1 Treatment on Fibroblast Mitogenesis. In addition to the previously mentioned inhibitory effect of TGFβ1 on fetal lung maturation, TGFβ1 also inhibits proliferation of immature fetal lung fibroblasts but stimulates proliferation of adult lung fibroblasts (39). To learn if the observed changes in TGFβ1 binding were accompanied by changes in response to TGFβ1, the incorporation of [^{3}H]thymidine into DNA was measured using different amounts of TGFβ1 in sex-specific Day 16 (Fig. 7A) and Day 18 (Fig. 7B) fetal lung fibroblasts. On Day 16, female fibroblasts exhibited a small but statistically significant dose-dependent increase in [^{3}H]thymidine incorporation that reached a maximum of 178% of controls at a dose

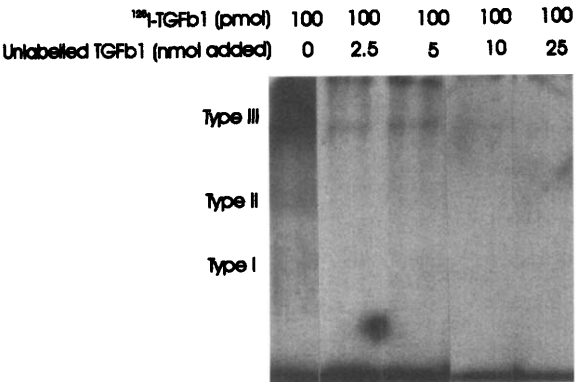
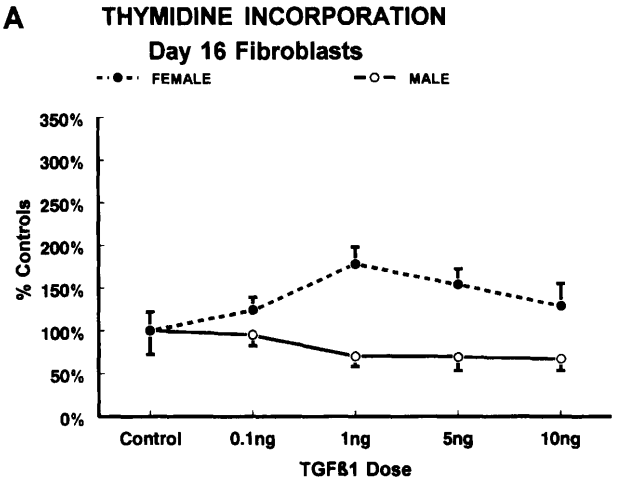


Figure 6. Autoradiograph demonstrating competitive inhibition of affinity labeling of the three receptor subtypes with increasing concentration of unlabeled TGFβ1 in female mouse lung fibroblasts. Affinity labeling of cell monolayers was done with 100 pmoles/ml of ^{125}I-TGFβ1 with and without increasing concentrations of unlabeled TGFβ1 (2.5 nmoles–25 nmoles/ml).

of 1 μg/ml (Kruskal-Wallis ANOVA; $P = 0.047$). In contrast, proliferation in Day 16 male fibroblasts decreased with increasing TGFβ1 concentration to a minimum of 69% of controls at the highest dose used (10 ng/ml), a change that was not statistically significant. The response to TGFβ1 treatment was dramatically different in Day 18 fibroblasts. TGFβ1 increased [^{3}H]thymidine incorporation at all concentrations in female Day 18 fibroblasts, and at the highest concentrations in Day 18 male fibroblasts. Baseline [^{3}H]thymidine incorporation was also higher in both male and female Day 18 fibroblasts than at Day 16 (data not shown). These results reflect the transition from inhibition by TGFβ1 treatment of lung fibroblast proliferation in very immature fetal lung fibroblasts to stimulation of proliferation in mature adult lung fibroblasts described by Zhao and Young (39). The male-female differences in response are consistent with the known male delay in lung maturation (29, 40–42). The data indicate that the changes in TGFβ receptor binding observed in these studies are accompanied not by complete loss of signal activity but by changes in the response to TGFβ1 ligand.

Discussion

Cell maturation leading to the induction of surfactant synthesis is a crucial event in the developing lung for preparation of the fetus for extrauterine life. Structural development and cellular maturation in the fetal lung may be coordinately regulated processes. It is likely that numerous growth factors act in synchrony in the fetal lung to regulate growth and differentiation. TGFβ1 is a negative regulator of the process of fetal lung maturation. For example, TGFβ1 inhibits the development of surfactant protein SP-A synthesis in human fetal lung explant cultures. This inhibition of maturation was not reversed by addition of epidermal growth factor or thyroxine, which alone or in combination enhanced the development of SP-A synthesis in the explant cultures (10). TGFβ1 inhibits Type II cell surfactant phospholipid and surfactant protein SP-C synthesis. It interferes



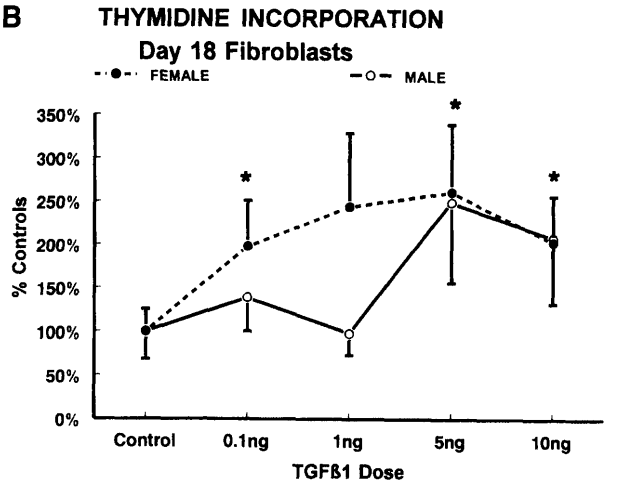


Figure 7A: Effect of TGFβ1 on ^{3}H-thymidine incorporation into DNA in Day 16 female (closed circles) and male (open circles) fibroblasts. Data were expressed as percent intraexperimental control. Points represent the mean ± SEM of 11–13 observations from 3 experiments. With increasing doses of TGFβ1 thymidine incorporation increased in female fibroblasts ($P < 0.05$, ANOVA), with a maximum 178% of control at 1 ng/ml TGFβ1, and decreased in male fibroblasts to a minimum 69% of control at 10 ng/ml.

Figure 7B: Effect of TGFβ1 on ^{3}H-thymidine incorporation into DNA in Day 18 female (closed circles) and male (open circle) fibroblasts. Data were expressed as percent intraexperimental control. Points represent the mean ± SEM of 11–13 observations from 3 experiments. * = $P < 0.01$ by comparison with control (Tukey multiple comparisons). With TGFβ1 treatment thymidine incorporation increased in female fibroblasts to a maximum 261% of control (at 5 ng/ml). Male fibroblasts showed no response at low doses, and then similarly reached a maximum of 249% of control at 5 ng/ml.

with fibroblast-Type II cell communication by blocking the production by the fibroblast of factors that stimulate Type II cell maturation, and by blocking the response of the Type II cell to such factors (12–15, 43). These data suggest that TGFβ1 inhibits maturation of the fetal lung by inhibiting fibroblast-Type II cell communications and also inhibits Type II cell surfactant phospholipid and protein production. Since TGFβ1 and the TGFβ receptors are present in the fetal lung, a mechanism for overcoming the inhibitory influences of TGFβ1 on maturation must be operative.

We chose to study the development of TGFβ1 binding in primary cultured fibroblasts (as opposed to homogenates of whole lung) in order to address cell-specific events. We focused our attention on fibroblasts because a major inhibitory effect of TGFβ1 in developing lung is the inhibition of fibroblast-Type II cell communication. Cells in primary culture, particularly fetal lung fibroblasts, have been repeatedly found to exhibit developmental characteristics relevant to *in vivo* lung development. Development-specific events are present in primary fibroblast cultures obtained from different gestational ages, including the developing ability to stimulate Type II cell surfactant synthesis *via* cell-cell communication (29, 44), male-female differences in the maturational progression of the developmental phenotype (40), and development of expression of other growth factors and growth factor receptors (45, 46). These *in vitro* events closely parallel similar observations made using fresh whole lungs. Thus, it is reasonable to conclude that gestational age-specific observations made *in vitro* reflect similar events occurring developmentally *in vivo*.

In this study, we found that during the crucial period of mesenchyme-directed fetal type II cell maturation, (Day 16 to Day 18 in the fetal mouse), there is a progressive decline in the total specific binding of TGFβ1 in fetal lung fibroblasts with advancing gestation. The earlier and significant decline in specific TGFβ1 binding in the female fetal lung fibroblasts is consistent with the earlier appearance of fibroblast-Type II cell communications, maturation of the Type II cells, and surfactant synthesis in the female fetus (29, 40–42). Male fibroblasts exhibited a later decline in specific binding. The level of specific binding in Day 18 male fibroblasts was similar to that in Day 17 females; however, consistent with the later maturation of surfactant synthesis reported in the male (29). Since the delay in surfactant system maturation in males is an androgen-dependent mechanism, we tested whether DHT affects TGFβ1 binding. Binding in Day 18 females, the most developmentally mature cells in this study, was significantly increased by DHT at levels just below or at the normal fetal physiologic range. In our data, the stimulatory effect of DHT on TGFβ1 binding was lost at doses above fetal physiologic concentrations. This may have been due to downregulation of the DHT receptor by the supraphysiologic DHT doses.

Our Scatchard analysis indicates that the decrease in TGFβ1 binding is due to a decline in the available TGFβ receptors with advancing gestation. There are no changes in the relative affinities of the TGFβ receptors for TGFβ1 with advancing gestation or between males and females. Similar results were obtained by competition binding assays, suggesting that the downregulation of TGFβ1 is due only to the decline in the available receptor sites. However, these equilibrium binding studies provide only the average affinity of all the TGFβ receptor types and do not reflect the affinities and the relative abundance of the individual receptor subtypes.

We used affinity labeling techniques to analyze the

receptor subtypes and to understand the dynamics of these receptor subtypes over the course of increasing gestation. Affinity labeling techniques revealed that the Type III receptor subtype was more abundant compared to the Type II and Type I receptors at both Day 16 and Day 18. There was a relative increase in the abundance of the Type III nonsignaling receptors compared to the signaling receptors (Types I and II) at Day 18 compared to Day 16. The physiologic function of Type III TGFβ receptors is not understood. They have been proposed to target TGFβ molecules to site-specific locations for restricted TGFβ activity, and also to sequester TGFβ away from signaling receptors as a negative control on signaling (19). In this study, the observed change in receptor dynamics with fewer total receptors and a relative decrease in the availability of the signaling Type I and Type II receptors occurs at a point in gestation when a change in TGFβ receptor activity would be necessary to reverse the inhibitory influences of TGFβ on fibroblast-Type II cell interactions controlling lung maturation, based on when this fibroblast-Type II cell communication appears *in vitro* (14, 29, 40). We propose that such a change in TGFβ receptor activity is a crucial event in the regulation of fetal lung growth and differentiation, and involves, at least in part, changes in the amount of TGFβ receptors. In addition, such a change may also include differences in the compartmentalization of TGFβ receptors within the cell, leading to the loss of specific inhibitory responses and the appearance of other responses.

Our data on changes in thymidine incorporation with TGFβ1 stimulation during gestation are consistent with this. Cell growth measured by thymidine incorporation was affected by TGFβ1 treatment differently at different gestational ages, with a sex-specific pattern. Earlier in gestation when the fibroblasts were immature and endogenous TGFβ1 binding was higher, growth was modestly reduced (male fibroblasts) or slightly stimulated (female fibroblasts). With more advanced maturation, female fibroblasts exhibited marked stimulation of growth. Male fibroblasts showed no response to low doses of TGFβ1 and were similarly stimulated at the highest doses of TGFβ1 studied. Thus, at the more mature gestation, when fibroblast-Type II cell communications stimulating surfactant synthesis are established, both female and male fibroblasts overcame the TGFβ1 growth inhibition, exhibiting a proliferative response typical of mature lung fibroblasts (39). At that time in gestation, both TGFβ1 binding (our data) and the endogenous amount of TGFβ1 production (14) are decreasing.

We do not propose that the changes in receptor binding and subtypes found in this study are sufficient alone to remove TGFβ signaling which represses lung maturation. Overall, multiple mechanisms are likely involved in the developmental change that allows positive signals to cause lung maturation. In particular, others have shown changes in the amount of TGFβ produced and/or present in the developing lung. TGFβ1 mRNA in fetal rat lung mesenchyme progressively declines with advancing gestation from Day 18 to Day 21, the developmental stage corresponding to Days 16–18 in mice (M. Post, personal communication). Torday and Kourembanas showed that primary cultures of immature fetal rat lung fibroblasts produce conditioned medium containing a TGFβ immunoreactive substance; the production declined with advancing gestational age in concert with the development in the conditioned medium of activity to stimulate Type II cell differentiation (14). Androgen pretreatment of the fetal rats eliminated the gestational decline in production of the TGFβ-like substance by fibroblasts cultured from different gestations (47). This supports our observation that *in vitro* treatment of Day 18 female fibroblasts with DHT increased specific TGFβ1 binding. Many hormones and growth factors that regulate lung maturation and surfactant production may also regulate TGFβ1 and TGFβ receptors. DHT, which delays lung maturation, stimulates TGFβ protein and receptor in several organs (14, 48). DHT *in vitro* both stimulates fetal lung cell proliferation and inhibits surfactant production (49). These effects may be mediated through effects on TGFβ signaling. Glucocorticoids stimulate lung maturation by stimulation of fibroblast-Type II cell communication leading to enhanced disaturated phosphatidylcholine synthesis (50). Dexamethasone decreases TGFβ1 mRNA and TGFβ activity in adult rat lung fibroblasts (51). Glucocorticoids also downregulate TGFβ3 mRNA in fetal lung fibroblasts (52). The downregulation of TGFβ activity by glucocorticoids has been postulated to allow growth factor–induction of epithelial differentiation and surfactant protein synthesis (53). These studies support our hypothesis that the negative regulation of Type II cell differentiation by TGFβ1 is, at least in part, mesenchyme-directed, and suggest a complex interaction of regulatory factors that control fetal lung growth and maturation. Overall, the attenuation with advancing gestation of TGFβ's mesenchyme-directed inhibition of Type II cell maturation may involve a decrease in TGFβ production in fetal lung mesenchyme, downregulation of ligand binding, changes in receptor kinetics, and changes in the intracellular signal transduction pathways. The ontogeny of the TGFβ receptors and receptor dynamics reported in this study contributes to an understanding of how the processes of growth and differentiation are coordinately regulated in the fetal lung.

In conclusion, at the time of commencement of surfactant synthesis in fetal mice, TGFβ1 inhibition of mesenchyme-directed maturation of Type II cells is overcome by several mechanisms, including a decrease in the specific binding of TGFβ1 to the fetal lung fibroblasts, a decrease in the total receptor numbers, and a switch in receptor dynamics such that there is a relative decrease in the available signaling of Type I and II receptors with advancing gestation. We believe that downregulation of TGFβ1 inhibition of maturation is an important regulatory step in lung maturation and is likely accomplished at multiple levels including changes in receptor dynamics. Further studies of the effects of various hormones and growth factors such as

glucocorticoids, dihydrotestosterone, and epidermal growth factor on the regulation of TGFβ1 binding and receptor dynamics in fetal lung fibroblasts may contribute to understanding the regulation of this complex process.

We thank Cynthia Doyle for technical assistance and gratefully acknowledge the secretarial assistance of Erdene Haltiwanger in the preparation of this manuscript.

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