Transforming Growth Factorβ Gene Expression and Action in the Seminiferous Tubule: Peritubular Cell-Sertoli Cell Interactions

Michael K. Skinner and Harold L. Moses

Departments of Pharmacology (M.K.S)
and Cell Biology
Vanderbilt University
School of Medicine
Nashville, Tennessee 37232

The potential role of transforming growth factorβ (TGFβ) as a mediator of cell-cell interactions within the seminiferous tubule was investigated through an examination of the local production and action of TGFβ. Sertoli cells and peritubular (myoid) cells were isolated and cultured under serum-free conditions. Secreted proteins from Sertoli cells and peritubular cells were found to contain a component that bound to TGFβ receptors in RRA. Reverse-phase chromatography of Sertoli cell and peritubular cell secreted proteins fractionated a protein with similar biochemical properties as TGFβ_1. This fractionated protein also contained TGFβ bioactivity in its ability to inhibit growth of an epidermal growth factor-dependent cell line. Both peritubular cells and Sertoli cells contained a 2.4 kilobase mRNA species that hybridized in a Northern blot analysis with a TGFβ_1 cDNA probe. TGFβ_1 gene expression was not detected in freshly isolated germ cells. TGFβ_1 alone was not found to influence Sertoli cell nor peritubular cell proliferation with cells isolated from a midpubertal stage of development. The effects of hormones and TGFβ on Sertoli cell differentiation and function were assessed through an examination of transferrin production by Sertoli cells. TGFβ_1 had no effect on transferrin production nor the ability of hormones to influence transferrin production. The presence of peritubular cells in a coculture with Sertoli cells also did not affect the inability of TGFβ_1 to act on Sertoli cells. Although Sertoli cell function did not appear to be influenced by TGFβ_1, peritubular cells responded to TGFβ_1 through an increase in the production of a number of radiolabeled secreted proteins. TGFβ_1 also had relatively rapid effects on peritubular cell migration and the promotion of colony formation in culture. Cocultures of Sertoli cells and peritubular cells responded to TGFβ_1 by the formation of large cell clusters with ball-like structures. Data indicate that TGFβ may have an important role in influencing the differentiation and migration of peritubular cells. Observations demonstrate the local production of TGFβ within the seminiferous tubule by Sertoli cells and peritubular cells and suggest that TGFβ may have a role as a paracrine-autocrine factor involved in the maintenance of testicular function (Molecular Endocrinology 3: 625–634, 1989)

0888-8809/89/0625–0634$02.00/0
Molecular Endocrinology

INTRODUCTION

The somatic cells of the seminiferous tubule, Sertoli cells and peritubular myoid cells, have a critical role in the maintenance and control of the process of spermatogenesis. Sertoli cells form the tubule and provide the cytoarchitectural support for the developing germinal cells. Peritubular cells surround the tubule and are separated from the Sertoli cells by a basement membrane. This complex extracellular matrix is produced cooperatively by Sertoli cells and peritubular cells (1). The environmental cell-cell interaction mediated by this complex basement membrane is important in maintaining structural integrity for the tubule and in promoting structural differentiation of the cells (2, 3). A complex array of regulatory agents are required to act on seminiferous tubule somatic cells to maintain tubule and testis function. In addition to externally derived agents such as FSH and androgens, locally derived paracrine factors are also postulated to play an important role in maintaining cellular function, growth, and differentiation (4). One example of a potentially important cellular interaction involves the production of a paracrine factor, P-Mod-S (5), by peritubular cells under androgen regulation (5, 6) that has a significant effect in modulating Sertoli cell functions involved in the control of germ cell development (7). This type of observation has led to the postulate that a number of additional locally derived paracrine factors may be important in the regulation of testicular cell function, growth, and differentiation.

A factor that has marked effects on cellular differentiation and growth is transforming growth factorβ (TGFβ) (8). TGFβ generally inhibits the growth of epithelial cell types and stimulates the growth of some mesenchymal cell types (9). TGFβ also can influence cellular differentiation (8), promote the production of extracellular matrix proteins (10), and act as a chemotactic agent for some cell types (11). TGFβ is a highly conserved protein and produced by many cell types (12). TGFβ is produced in an inactive form (13) that can be activated with acid treatment or proteolysis. TGFβ acts via unique cell surface receptors (14) that appear to be ubiquitous (15). Different forms of TGFβ have been identified, TGFβ_1 and TGFβ_2, that are highly homologous and have similar, although not identical, biological activities (16). In addition, a third gene product has been identified and designated TGFβ_3 (17), but the protein product for this TGFβ_3 gene remains to be identified. The term TGFβ will be used in the current manuscript when the production or action of a specific form of TGFβ is unknown or the actions of multiple forms are possible. TGFβ has been implicated in cell-cell interactions in a number of tissues, including the ovary (18), to regulate cell growth and differentiation. The current study was designed to investigate the possible local production of TGFβ in the seminiferous tubule and investigate the actions of TGFβ on cellular function and growth.

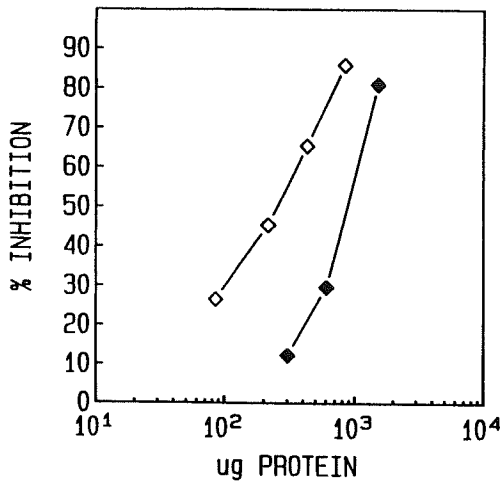

Fig. 1. TGFβ RRA on Sertoli Cell (◆) and Peritubular Cell (◇) Secreted proteins

The percent binding inhibition of ^{125}I-TGFβ_1 was determined with increasing concentrations of secreted protein. Data presented is the mean of a replicate determination and is representative of three separate experiments.

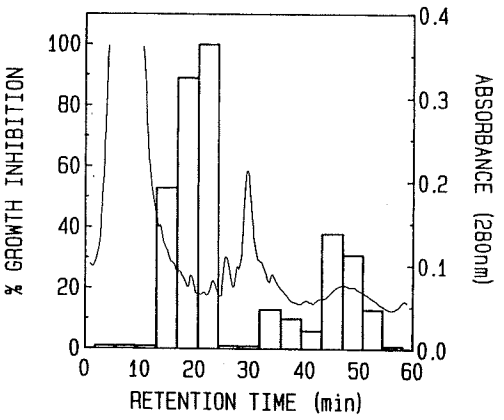

Fig. 2. Reverse Phase Chromatography of SSP

The ability of individual fractions to inhibit EGF-stimulated cell growth is expressed as percent growth inhibition (*bar graph*). Protein elution was monitored at 280 nm (histogram). The standard retention time for TGFβ_1 on this column is 22 min. Data presented is a representative experiment from three separate experiments.

RESULTS

The possible production of TGFβ was initially examined with a TGFβ RRA. Peritubular cell and Sertoli cell secreted proteins were prepared from concentrated serum-free conditioned medium and will be referred to as PSP and SSP, respectively. To activate any latent or inactive forms of TGFβ the secreted protein preparations were exposed to pH 2.0 acid conditions a minimum of 2 h before neutralization and assay. Both Sertoli cell and peritubular cell secreted proteins displaced radioiodinated TGFβ_1 from its receptor, Fig. 1. The relative amount of binding activity was greater in PSP than in SSP. These observations indicate that both PSP and SSP contain a component that can specifically bind to the TGFβ receptors. The amount of TGFβ present in SSP and PSP was estimated to be 2.5 and 5 ng/mg protein, respectively, using a TGFβ_1 standard in the RRA. To confirm the secretion of TGFβ, a preliminary study was done with a TGFβ RIA previously described (18), that is cross reactive for both TGFβ_1 and TGFβ_2. Both SSP and PSP displaced radioiodinated TGFβ_1 from the antibody used in this TGFβ RIA (unpublished observation). Therefore, peritubular cells and Sertoli cells appear to secrete a TGFβ-like substance.

To further characterize this TGFβ receptor binding activity both PSP and SSP were separated by reverse phase chromatography. Any latent or inactive forms of TGFβ were presumably activated by the pH 2.0 acid conditions of the chromatography. TGFβ biological activity was assessed by the ability of individual fractions to inhibit growth of an epidermal growth factor (EGF)-dependent cell-line. A major discrete peak of growth inhibitory activity was detected in the profiles for both SSP, Fig. 2, and PSP, Fig. 3. The retention times for both the SSP and PSP major inhibitory peaks were the same, 22 min. A minor peak of inhibitory activity was also detected at a 50-min retention time for both PSP and SSP, Figs. 2 and 3. The presence of these growth inhibitory peaks indicates that both PSP and SSP contain TGFβ biological activity. Authentic porcine TGFβ_1 was found to have a retention time between 20 and 22

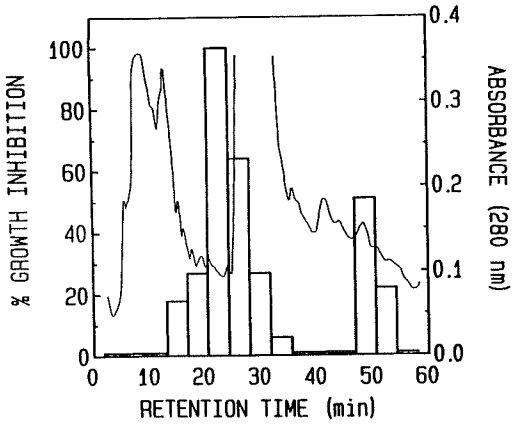

Fig. 3. Reverse Phase Chromatography of Peritubular Cell Secreted Proteins

The ability of individual fractions to inhibit EGF-stimulated cell growth is expressed as percent growth inhibition (*bar graph*). Protein elution was monitored at 280 nm (histogram). The standard retention time for TGFβ_1 on this column is 22 min. Data presented is a representative experiment from three separate experiments.

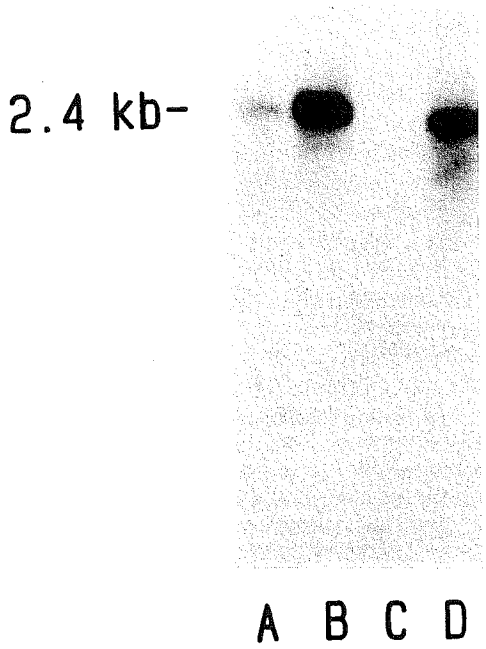

Fig. 4. Northern Blot Analysis for TGFβ_1 Gene Expression

Polyadenylated RNA was isolated from an AKR-2B cell-line, 2 μg (A); peritubular cells, 5 μg (B); germ cells, 10 μg (C); and Sertoli cells, 5 μg (D). RNA was specifically hybridized with a [^{32}P]cDNA probe for TGFβ_1. Data presented is a representative experiment from three separate experiments.

min while TGFβ_2 was more hydrophobic and had a retention time between 26 and 28 min (data not shown). SSP primarily contained TGFβ activity which eluted with a similar retention time as TGFβ_1 with negligible activity eluting at the retention time 26 to 28 min of TGFβ_2, Fig. 2. PSP contained TGFβ activity which eluted with the 20–22 min retention time of TGFβ_1, however, PSP also contained a significant amount of activity which eluted at the 26–28 min retention time of TGFβ_2, Fig. 3. Results imply that SSP contains TGFβ activity which coelutes with TGFβ_1 while PSP contains TGFβ activity which coelutes with both TGFβ_1 and TGFβ_2. These observations support the postulate that peritubular cells and Sertoli cells produce TGFβ.

Gene expression of TGFβ_1 was examined to help confirm active synthesis and secretion of TGFβ by peritubular cells and Sertoli cells. Polyadenylated RNA was obtained from freshly isolated and cultured peritubular cells and Sertoli cells. Northern blot analysis of this RNA with a TGFβ_1 cDNA probe previously described (12) is shown in Fig. 4. Hybridization was detected on a single mRNA species from both peritubular cells and Sertoli cells. The size of the mRNA species was similar in peritubular cells and Sertoli cells and comparable to a 2.4 kilobase (kb) species previously shown in a AKR-2B embryonic mouse cell-line (19), Fig. 4. In approximately half of the RNA preparations examined the Sertoli cell transcript was slightly smaller than the 2.4 kb transcripts in peritubular cells and AKR-2B cells. The reason for this smaller size is currently unknown and may be generated due to variable processing by the Sertoli cell or an artifact of the isolation procedure. The relative amount of hybridization was reduced in freshly isolated cells which implies mRNA levels increase during the culture of the cells, data not shown. No hybridization was detected with germ cell RNA. Combined results imply that the TGFβ_1 gene is expressed in peritubular cells and Sertoli cells but not germ cells.

The actions of TGFβ_1 on Sertoli cell function were assessed through examining effects on transferrin production by Sertoli cells. Sertoli cells were cultured in the absence or presence of various hormones and TGFβ_1, Fig. 5. TGFβ_1 was not found to affect basal levels of transferrin production nor the ability of different concentrations of FSH to stimulate transferrin production. The ability of a combination of FSH, insulin, retinol, and testosterone to stimulate transferrin production was also not affected by TGFβ_1, Fig. 5. Observations indicate that TGFβ_1 affects neither transferrin production by Sertoli cells nor the ability of FSH or other regulatory agents to stimulate transferrin production.

Peritubular cells produce a paracrine factor that influences Sertoli cell function (7) and the presence of peritubular cells augments the actions of hormones on Sertoli cell function (6). Therefore, the effects of TGFβ_1 on a coculture of peritubular cells and Sertoli cells were examined. Sertoli cell cultures were seeded with approximately a 25% contamination with peritubular cells and transferrin production examined. As previously described the basal level of transferrin production was increased when peritubular cells were present from 18–35 ng/μg DNA, Figs. 5 and 6. TGFβ_1 was not found to affect transferrin production in these cocultures in the absence, Fig. 6, or presence of hormones, data not shown. Therefore, TGFβ_1 does not appear to affect

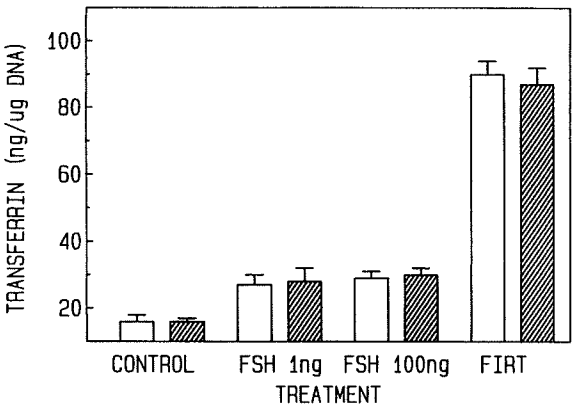

Fig. 5. Transferrin Production by Sertoli Cells Cultured in the Absence (□) or Presence (▨) of TGFβ_1 (10 ng/ml)

Sertoli cells were also cultured in the absence (control) or presence of FSH, 1 and 100 ng/ml, and a combination of FSH, insulin, retinol, and testosterone, FIRT. Data is presented as nanograms of transferrin/μg Sertoli cell DNA and is the mean ± SEM for three different experiments done in triplicate.

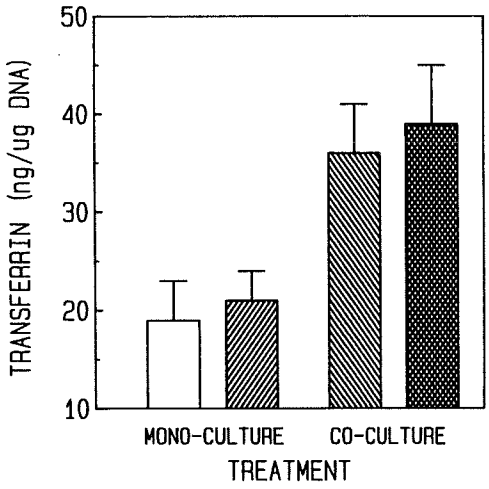

Fig. 6. Transferrin Production by Monocultures of Sertoli Cells and Cocultures of Sertoli Cells and Peritubular Cells, 3:1 Ratio, Respectively

Cells were cultured in the absence [□ and (▧)] or presence [(▨) and (××)] of TGFβ_1 (10 ng/ml). Data is presented as nanograms of transferrin per μg DNA and is the mean ± SEM for three different experiments done in triplicate.

Sertoli cell function indirectly through the peritubular cells.

The effect of TGFβ_1 on Sertoli cell and peritubular cell proliferation is shown in Fig. 7. Cells were treated starting on day 1 of culture for 4 days followed by a DNA assay to determine alterations in cell number, Fig. 7. Sertoli cell growth was not affected by TGFβ_1 nor hormones. Peritubular cell growth was also not altered by TGFβ_1 but was stimulated with calf serum. Similar results were also obtained when the incorporation of [^{3}H]thymidine into DNA was examined after an 18-h treatment with TGFβ_1 or 10% calf serum, data not shown. TGFβ alone had no effect on [^{3}H]thymidine

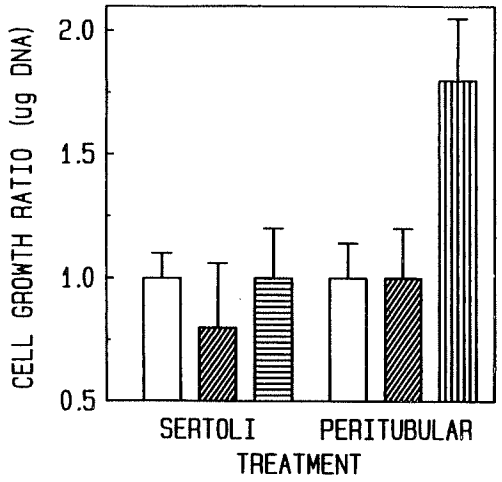

Fig. 7. Effects of Regulatory Agents on Sertoli Cell and Peritubular Cell Growth

Cells were cultured in the absence (□) or presence of 10 ng/ml TGFβ_1 (▨), combination of FSH, insulin, retinol, and testosterone (▤) or 10% calf serum (▥), for 72 h, then examined for DNA content (micrograms of DNA). Data is presented as a cell growth ratio as compared to control cultures. The mean ± SEM is presented for three different experiments done in triplicate.

incorporation into either Sertoli cell or peritubular cell DNA. The combined results indicate that TGFβ_1 alone does not influence Sertoli cell or peritubular cell growth with cells isolated at a midpubertal stage of development.

Radiolabeled secreted protein profiles of Sertoli cells and peritubular cells were examined as a qualitative measure of cellular functions. The effects of TGFβ_1 on radiolabeled secreted proteins are shown in Fig. 8. Although hormones can stimulate the production of Sertoli cell radiolabeled secreted proteins, data not shown, TGFβ_1 had no detectable effects. In contrast, TGFβ_1 promoted the apparent production of a number of radiolabeled secreted proteins by peritubular cells, Fig. 8. Radiolabeled proteins at 180, 220, and 300–400 kilodaltons, as well as a number of smaller proteins, were all increased with TGFβ_1 treatment. The radiolabeled secreted proteins applied to the polyacrylamide sodium dodecyl sulfate (SDS) gel were derived from the same number of cells, as determined with a DNA assay. These observations indicate that TGFβ_1 has major effects on the apparent production of a number of peritubular cell secreted proteins, but has no detectable effect on Sertoli cell secreted proteins.

The actions of TGFβ_1 on Sertoli cell and peritubular cell morphology in culture is shown in Fig. 9. Cells were cultured in the presence of 1% calf serum for 1 day, then treated with TGFβ_1 for an additional 1–4 days of culture. Sertoli cell morphology was not influenced by the presence of TGFβ_1, even after a 4-day treatment. However, peritubular cell morphology and migration was markedly altered by TGFβ_1 treatment. After 24 h of treatment peritubular cells started migrating toward

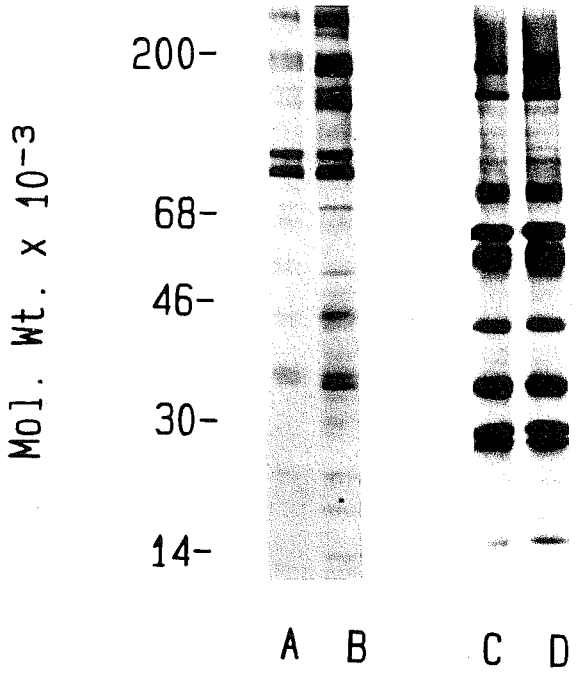

Fig. 8. Fluorogram of Radiolabeled Secreted Proteins from Peritubular Cells (A, B) and Sertoli Cells (C, D)

Cells were cultured in the absence (A, C) or presence (B, D) of TGFβ_1, 10 ng/ml. Data is a representative experiment from three different experiments.

small islands of cells and after 48 h of treatment formed small clusters of cells, Fig. 9. After 72–144 h of treatment large colonies, balls, of cells were formed that had long cellular extensions for attachment to the plastic substratum. Longer treatment with TGFβ_1 resulted in the balls or colonies of cells to increase in size and have a reduced ability to remain attached to the culture substratum. Cocultures of peritubular cells and Sertoli cells, 25:75 ratio, respectively, were also found to be affected by TGFβ_1. Cells migrated to cell colonies and had a tendency to form irregularly shaped cell clusters, Fig. 9. Longer treatment with TGFβ_1 resulted in the formation of large cell clusters that maintained attachment to the culture substratum. In contrast to the peritubular cell clusters, in the cocultures a sparse monolayer of cells was often present between the cell clusters, Fig. 9. The effects of TGFβ_1 on cell morphology and migration were relatively rapid and were initiated within 24 h and completed within 72–144 h. Although effects could be observed in the absence of serum, optimal effects required 1% calf serum. Higher concentrations of serum appeared to interfere with the morphological effects of TGFβ_1. Observations indicate that TGFβ_1 has a significant effect in promoting peritubular cell migration and the formation of unique cellular structures, balls, in culture. Unique cell clusters were also formed in cocultures of peritubular cells and Sertoli cells in response to TGFβ_1 treatment. In contrast, TGFβ_1 had no detectable effect on Sertoli cell morphology.

DISCUSSION

TGFβ has been implicated as a paracrine factor in a number of tissues for the regulation of cellular growth and differentiation (9, 16). The current study demonstrates that both Sertoli cells and peritubular cells are sites for the local production of TGFβ within the seminiferous tubule. Both cell types secrete a substance that can bind to TGFβ receptors and inhibit the ability of EGF to stimulate growth of an EGF-dependent cell line. TGFβ has previously been shown to be produced in a latent inactive form that can be activated by exposure to acid conditions (9, 13). Although low levels of receptor binding activity could be detected in nonacid treated secreted protein preparations, maximal TGFβ receptor binding activity and growth inhibitory activity required acid treatment of both PSP and SSP. Therefore, observations imply that as with most cell types a latent form of TGFβ is produced by both cell types that will require activation. Multiple forms of TGFβ are present which are different gene products of apparently functionally related proteins (16, 17). The protein products TGFβ_1 and TGFβ_2 have been characterized and appear to have some differential effects on specific cell types (16). The protein product of other TGFβ genes such as TGFβ_3 have not been identified or characterized (17). Reverse phase chromatography of secreted proteins indicated that Sertoli cells produce a TGFβ activity which coelutes primarily with TGFβ_1 while peritubular cells produce TGFβ activity which coelutes with both TGFβ_1 and TGFβ_2. Observations imply that Sertoli cells produce TGFβ_1 while peritubular cells produce both TGFβ_1 and TGFβ_2. Other forms of TGFβ whose reverse phase chromatographic properties are unknown at present may also be produced and will need to be considered as a limitation to any data interpretation. Investigations with specific antisera for TGFβ_1 and TGFβ_2 have been initiated and will help elucidate further the specific forms of TGFβ produced.

To further investigate the production of TGFβ the presence of TGFβ_1 gene expression was investigated. Sertoli cells and peritubular cells were both found to express the TGFβ_1 gene with a 2.4 kb message being identified. Therefore, Sertoli cells and peritubular cells from rat testis synthesize and secrete TGFβ_1 as a potential paracrine factor within the seminiferous tubule. Investigations of the possible expression of TGFβ_2 and TGFβ_3 have been initiated and will help elucidate what additional forms of TGFβ may be produced in the seminiferous tubule. A mixed population of germinal cells from various stages of development was not found to contain the TGFβ_1 message. Although germinal cells do not appear to express the TGFβ_1 gene, the possible actions of TGFβ on germinal cell development cannot be excluded. Examination of the possible presence of TGFβ receptors on germ cells and effects on cell function remains to be investigated. Detailed analysis with techniques such as *in situ* hybridization for TGFβ will be informative to confirm data presented in the current study.

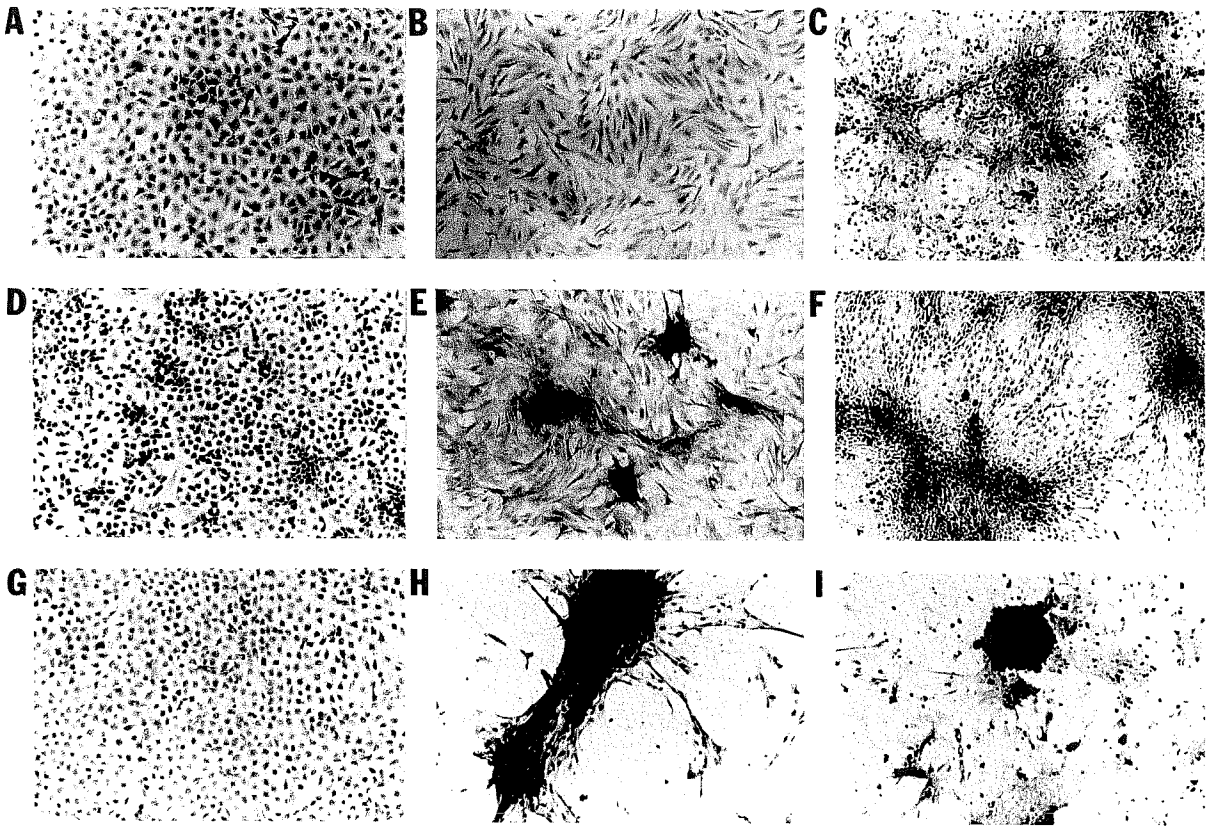

Fig. 9. Morphological Analysis of Sertoli Cells (A, D, G), Peritubular Cells (B, E, H), and Cocultures of Sertoli Cells and Peritubular Cells, 3:1 Ratio, respectively (C, F, I)

Cells were cultured in the absence (A, B, C) or presence of TGFβ_1 (10 ng/ml) for 24 h (D, E, F) or 96 h (G, H. I). Cells were fixed and stained with hematoxylin and eosin on day 3 (D, E, F) or day 5 (A, B, C, G, H, I) of culture. Data presented is at a 135× magnification and is representative of a minimum of three different experiments done on three different cell preparations.

The observation that Sertoli cells and peritubular cells produce TGFβ implies the possible involvement of TGFβ in growth regulation within the seminiferous tubule. Neither Sertoli cell nor peritubular cell growth was influenced by TGFβ_1 alone with cells isolated from a mid-pubertal stage of development. This was examined with an analysis of both cell proliferation and [^{3}H]thymidine incorporation into DNA. Therefore, TGFβ_1 does not appear to be a growth stimulator for either cell type at this stage of development. One of the primary functions of TGFβ is its ability to inhibit cell proliferation (9). The ability of TGFβ to inhibit growth stimulation of Sertoli cell and peritubular cell proliferation remains to be investigated. Sertoli cells have previously been shown to produce an EGF-like substance (20). Recent studies have demonstrated that an EGF-like substance, transforming growth factorα (TGFα), is produced by Sertoli cells and peritubular cells and subsequently can act on peritubular cells and possibly prepubertal Sertoli cells to stimulate cell proliferation (21). The ability of TGFβ to inhibit the actions of EGF/TGFα is a well established biological activity of TGFβ. It is speculated that TGFβ production in the seminiferous tubule will function in part to inhibit the actions of a locally produced EGF/TGFα substance. Sertoli cells are a terminally differentiated nonproliferating cell type in the adult. Sertoli cells lose their ability to proliferate during midpuberty (22) and this is the stage of development the animals in the current study were obtained. Whether TGFβ may inhibit the stimulatory actions of EGF/TGFα on Sertoli cell growth at an earlier stage of testis development, particularly the prepubertal stage, remains a possibility to be investigated. Peritubular cells continue to proliferate at any stage of testis development and may require a more dynamic growth regulation than Sertoli cells. TGFβ may have an active role as a growth inhibitor for peritubular cells to counter the stimulatory actions of a locally produced EGF/TGFα substance. Germ cells undergo a rapid rate of cell proliferation, but little information is currently available concerning control mechanisms. The possible role of TGFβ in germ cell growth is currently unknown. Further understanding of general growth regulation in the testis will provide insight into the role of TGFβ in growth regulation in the seminiferous tubule. The local production of TGFβ by Sertoli cells and peritubular cells, as well as the production of EGF/TGFβ substances (20, 21), suggests that TGFβ may have a role as a growth regulator in the tubule.

TGFβ also has the ability to regulate cell differentiation for a number of cell types (9, 16). Whether this effect on cell differentiation is an indirect effect of its ability to inhibit cell growth remains to be investigated. The postulate is made that the inhibition of cell growth by TGFβ may simply allow the cell to express a more differentiated state and be more responsive to hormone regulation. The effect of TGFβ on Sertoli cell function and differentiation was examined using transferrin production as a functional marker for the cell. Transferrin production by Sertoli cells (23) is under hormonal regulation (24) and provides a marker protein for Sertoli cell function and differentiation (4). TGFβ was not found to affect transferrin production by Sertoli cells nor the ability of hormones, including FSH, to influence cell function. This result is in contrast to the previous observations made on ovarian cells in which TGFβ_1 appears to enhance the ability of FSH to stimulate granulosa cell steroidogenesis (25). Whether TGFβ may influence Sertoli cell functions at an earlier stage of development, *i.e.* prepubertal, remains a possibility to be investigated. Peritubular cells produce a paracrine factor, termed P-Mod-S, under androgen regulation that can modulate Sertoli cell function (5). Therefore, regulatory agents can influence Sertoli cell function indirectly through actions on peritubular cells (4). For this reason the effect of TGFβ_1 on cocultures of Sertoli cells and peritubular cells was examined. The presence of peritubular cells had no effect on the inability of TGFβ_1 to influence transferrin production by Sertoli cells. Combined results imply that TGFβ_1 has minimal effects on the functions of Sertoli cells isolated at midpuberty or the ability of hormones to influence Sertoli cell function. Therefore, TGFβ does not appear to play a major role in the regulation of adult Sertoli cell function or differentiation. Further information, however, regarding the effects of TGFβ on the developmental regulation of Sertoli cell function and differentiation is required.

To assess the effects of TGFβ on general cellular function, radiolabeled secreted proteins were examined. Although regulatory agents such as FSH, insulin, and retinol influence the secretion of radiolabeled proteins by Sertoli cells, TGFβ_1 had no effect on Sertoli cell secretory products. Peritubular cells, however, did respond to TGFβ_1 through an increase in the apparent production of 180, 220, and 300–500 kilodalton proteins. Specific peritubular cell secreted proteins with these same molecular weights have previously been identified as collagen (1), fibronectin (26), and proteoglycans (27), respectively. Therefore, TGFβ may increase the production of extracellular matrix components by peritubular cells. Previously TGFβ has been shown to increase extracellular matrix production by several cell types (10). Synthesis and secretion of extracellular matrix components by peritubular cells are involved in the formation of the basement membrane of the seminiferous tubule. This complex extracellular matrix between peritubular cells and Sertoli cells is essential to maintain the structural integrity of the tubule and cytoarchitecture of the epithelium (2, 3). TGFβ, therefore, may have a role in the production and maintenance of this extracellular matrix through its actions on peritubular cells. Peritubular cell-Sertoli cell interactions mediated via extracellular matrix may be regulated in part through the local production of TGFβ. The observation that TGFβ can promote the production of a number of secreted proteins implies that TGFβ can influence peritubular cell differentiation and function. Regulation of peritubular cell function is postulated to be important for the maintenance of testicular function and the process of spermatogenesis (4).

TGFβ has also been shown to influence cell migration and colony formation for a number of cell types (11). Several fibroblast cell types respond to TGFβ as a chemotactic agent (9, 16). Although Sertoli cells did not alter cell morphology or migration in response to TGFβ_1, peritubular cells had a profound response to TGFβ_1. Within 24 h peritubular cells initiated migration toward cell colonies and eventually formed large clusters, balls, of cells. The ability of TGFβ_1 to influence peritubular cell migration may in part be due to potential effects on the production of extracellular matrix components which would allow the cell to migrate and form contacts with other cells more readily. Observations presented suggest that TGFβ may potentially act as a chemotactic agent for the peritubular cell, but this possibility remains to be quantified. Cocultures of peritubular cells were also found to form large cell clusters in response to TGFβ_1. Both Sertoli cells and peritubular cells appeared to be present in these cell colonies, which is speculated to be due to the ability of Sertoli cells to readily attach to peritubular cells. Peritubular cells are postulated to be a primary participant in the formation of the cell colony. The formation of ball-like cell clusters has previously been described in long-term, 14–20 days cocultures of Sertoli cells and peritubular cells (28). It is speculated that the local production of TGFβ by the cells may play a role in the formation of these ball-like cell structures previously described. The delayed response previously observed may have been due to the low levels of TGFβ present. Observations presented in the current study suggest that TGFβ may have a role in influencing the migration and possible chemotaxis of peritubular cells within the seminiferous tubule. The ability of TGFβ to influence the development of a number of tissues has previously been described (9, 16, 29). An interesting possibility to investigate will be whether TGFβ may play a role in recruiting peritubular cells from the nondifferentiated fibroblast population in the interstitium of the testis. In response to TGFβ these potentially nondifferentiated fibroblasts may in part differentiate to become peritubular cells, produce extracellular matrix and migrate to form the exterior wall of the seminiferous tubule. Further investigation of the developmental and hormonal regulation of TGFβ production in the seminiferous tubule will be required to investigate this hypothesis.

Interactions between peritubular cells and Sertoli cells provide an example of mesenchymal-epithelial cell interactions (4). Cellular interactions between mesen-

chymal cells and epithelial cells are present in most tissues and is postulated to be critical for tissue development and function (30). The ability of TGFβ to mediate important mesenchymal-epithelial cell interactions is a possibility that is suggested by data presented in the current study. Further investigation of peritubular cell-Sertoli cell interactions mediated via TGFβ will provide insight into the regulation of testicular cell growth and differentiation, as well as develop a better understanding of mesenchymal-epithelial cell interactions.

The current study demonstrates the local production of TGFβ in the seminiferous tubule by Sertoli cells and peritubular cells. $TGF\beta_1$ does not appear to act as a growth stimulator for either cell type at a midpubertal stage of development. The ability of TGFβ to act as a growth inhibitor for growth stimulators within the tubule is postulated to be a potential function of TGFβ. With cells isolated at the midpuberty stage, $TGF\beta_1$ was not found to affect Sertoli cell transferrin production nor the ability of hormones to affect transferrin production. However, the ability of TGFβ to modulate cell growth and differentiation at earlier stages of testis development remains to be examined. TGFβ was found to influence peritubular cell function and migration. The data imply that TGFβ may have a role in the maintenance of peritubular cell differentiation and possibly chemotaxis. Clearly the local production and action of TGFβ within the seminiferous tubule implies that TGFβ may be a paracrine-autocrine factor participating in cell-cell interactions required to maintain testis function and the process of spermatogenesis.

MATERIALS AND METHODS

Cell Preparation and Culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (31) with a modified procedure previously described (26). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml) (Gibco, Grand Island, NY) to remove interstitial cells, followed by a collagenase digestion (1 mg/ml, type I, Sigma, St. Louis, MO) and then a hyaluronidase digestion (1 mg/ml, Sigma). Sertoli cells were then plated in 24-well (1 ml/well) multiwell plates at approximately 5×10^5 cells per well or in 150-mm culture plates. Cells were maintained at 32 C in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco). Sertoli cultures were treated as described in *Results* at the time of plating and retreated after 48 h of culture when the medium was replenished. Unless otherwise stated, a 72-h medium collection on day 5 of culture was obtained for analysis and the cells harvested for RNA isolation or DNA assay. Sertoli cell cultures were treated as outlined in *Results* with test substances or with FSH (100 ng/ml), insulin (5 μg/ml), retinol (0.35 μM), and testosterone (1 μM). Data presented were obtained with 10 ng/ml $TGF\beta_1$ (R and D Systems, Minneapolis, MN), but were also confirmed with a concentration of 2.5 ng/ml. The contamination of the Sertoli cell preparations with peritubular cells was determined to be less than 1% as previously described (26) with the use of fibronectin production and desmin cytoskeletal elements as unique functional markers for the peritubular cells.

Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (7). Peritubular cells were plated in medium containing 10% calf serum and grown to confluence. Cells were then subcultured and plated at 25% confluence. After 3–4 days of culture, subcultured cells were confluent and cells were either obtained for RNA isolation or washed for 24 h with serum-free medium. The cells were then cultured for up to 2 weeks in serum-free medium with 48-h medium collections. The peritubular cell preparations were found to be a highly purified cell population with the use of cytochemistry for alkaline phosphatase activity and desmin cytoskeletal elements.

Germ cells were prepared from the testis of adult 60-day-old rats. After removal of the tunica, seminiferous tubules were gently teased apart and chopped. Tubules were incubated 30 min in serum-free medium. The cells released into the medium were harvested and residual tubule fragments gravity sedimented. This crude cell preparation contained predominantly germ cells at various developmental stages as determined morphologically.

Freshly collected peritubular cell and Sertoli cell serum-free conditioned medium were made 25 μM phenylmethylsulfonyl fluoride and 0.1 mM benzamidine and then centrifuged at 1000 × *g* for 15 min at 4 C to remove cell debris. When required, medium was frozen and stored at −20 C. Conditioned medium was concentrated 100-fold by ultrafiltration with an Amicon system using a membrane with 3000 molecular weight exclusion limit. Cells used for morphological analysis were fixed with formalin and stained with hematoxylin and eosin.

Electrophoresis and Fluorography

Sertoli cell and peritubular cell cultures were maintained for 48 h starting on day 5 of serum-free culture in glycine and methionine-free media containing 5 μCi/ml [^{3}H]glycine and [^{35}S] methionine. The radiolabeled media were collected, centrifuged, and used for analysis. Proteins were electrophoretically analyzed on SDS 7.5-15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (32) buffer system. The gels were fluorographed with diphenyloxazole in acetic acid as previously described (33).

Transferrin RIA

Transferrin production by Sertoli cells was assayed by a RIA described previously (24). An aliquot of the culture media was incubated with rabbit anti-rat transferrin antibody (Cooper Biomedical, Melvern, PA) and iodinated transferrin for 1 h at 37 C followed by 1 h incubation with goat anti-rabbit immunoglobulin G antibody (Sigma). Complexed antibody was then precipitated with polyethylene glycol (Sigma), pelleted by centrifugation, and radioactivity in the pellets determined. All data was normalized per micrograms of Sertoli cell DNA at the time of media collection and expressed as nanograms of transferrin per micrograms of DNA.

DNA and Protein Assays

DNA was measured fluorometrically with ethidium bromide (34), as previously described (7). At the end of the culture period, the media was removed, ethidium bromide buffer (EBB) (20 mM sodium chloride, 5 mM EDTA, 10 mM Tris, pH 7.5; Sigma) was added to the wells, and the cells were sonicated. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide, 100 U/ml heparin in EBB) and diluted 1:2 with EBB buffer and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantitate DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 2.5 μg DNA. Total protein concentration was measured according to the method of Bradford (35).

TGFβ RRA

TGFβ$_1$ from porcine platelets (36) was obtained from R and D Systems (Minneapolis, MN) and used in a RRA previously described (9, 14). TGFβ was radioiodinated with a modified chloramine-T procedure (37), and binding assays were carried out using dishes at a density of 1×10^5 to 2×10^5 AKR-2B cells per well. After 24 h, the cells were washed with binding buffer (Dulbecco's PBS, pH 7.4, containing 0.1% BSA and 5 mM $MgCl_2$). Then, 1 ml binding buffer containing 0.2 ng ^{125}I-labeled TGFβ (15,000 cpm) with various levels of competitors was added to each well. Nonspecific binding was determined in the presence of 1 μg 50% pure unlabeled TGFβ. After 2 h of incubation at room temperature the cells were washed three times with binding buffer, and the bound radioactivity was extracted with PBS containing 0.5% Triton X-100.

TGFβ Growth Assay

Inhibition of EGF stimulated growth of an EGF-dependent cell line was used as an assay for TGFβ bioactivity. Growth of an EGF-dependent cell line, MK cells, was performed as previously described (38). Samples were incubated in the presence of EGF on MK cells cultured at 37 C for 72 h. Cell number was then determined to assess EGF growth stimulation. EGF was supplied by the Vanderbilt University, Reproductive Biology Research Center, Tissue Culture Core Laboratory, directed by Dr. G. Carpenter.

Cell Growth Assays

Sertoli cells and peritubular cells were plated at 25% confluence in 24-multiwell culture plates and incubated for 24 h in 1 ml culture medium containing 1% calf serum, followed by 48 h culture in medium containing 0.1% calf serum. Then, the cells were treated for 18 h with various concentrations of growth regulators. The medium was replaced with 0.5 ml culture medium containing 1 μCi [^{3}H]thymidine, and the cells were incubated for 4 h. The amount of [^{3}H]thymidine incorporated into DNA was then determined, as previously described (18). Alternatively cells were cultured for 72 h in the presence of growth regulators followed by a DNA assay to determine alterations in cell number.

RNA Isolation and Northern Analysis

Polyadenylated RNA was obtained from Sertoli cells and peritubular cells. Cells were extracted with 5 M guanidine isothiocynate, 10 mM EDTA, 50 mM Tris, pH 7.5, 8% (vol/vol) β-mecaptoethanol then precipitated with 1 M LiCl 18 h at −20 C. The resulting pellet was reconstituted in 4 ml 1% SDS, 1 mM EDTA, 0.1 M NaCl, 10 mM Tris-HCl, and homogenized if necessary. The sample was then digested with 50 μg/ml proteinase K for 30 min at 37 C, then adjusted to 0.4 M NaCl, and subsequently incubated with 0.1 g oligodeoxythymide affinity gel 4–18 h at room temperature with slight agitation. Poly(A) RNA was eluted and precipitated with ethanol 18 h at −20 C. Poly(A) RNA was separated electrophoretically on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and analyzed with a Northern blot procedure previously described (39). A ^{32}P-radiolabeled human TGFβ$_1$ cDNA probe was used in the hybridization at 42 C overnight, then washed in three 30-min incubations of 0.2% (wt/vol) SDS and 0.15 M NaCl, 16 mM sodium citrate as previously described (19).

Reverse-Phase HPLC

Reverse-phase chromatography was performed as previously described (7) on an analytical Vydac-C4 column. Unless otherwise stated, the column was equilibrated in 0.1% (vol/vol) trifluoroacetic acid, 0.5% (vol/vol) ethylene glycol, 1 mM triethylamine, pH 2.0. Acidified samples were loaded and eluted at 0.5 ml/min with a 60-min linear gradient from 25–60% acetonitrile. Fractions were collected (2 min) and dried in a speed-vac apparatus (Savant, Hicksville, NY). Dried samples were then reconstituted for analysis and when necessary stored at −20 C.

Acknowledgments

We thank Susan Schiltz, Lisa Halburnt, Jana Wolfshohl, and Byron Glenn for technical assistance; Mary Couey and Loretta Cheairs for assistance in preparation of the manuscript; Dr. Cathy Anthony for the morphological analysis; and Dr. Jorma Keski-Oja and Dr. R. M. Lyon for the TGFβ RRAs. We also acknowledge the Vanderbilt University Reproductive Biology Research Center, Cell Culture Core Laboratory.

Received December 9, 1988. Revision received January 13, 1989. Accepted January 18, 1989.

Address requests for reprints to: Dr. Michael K. Skinner, Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232.

This study was supported by NCI Grant CA-42572 (to H.L.M.) by Mellon Foundation and NIH Grant HD-20583 (to M.K.S.) who is a PEW Scholar.

REFERENCES

1. Skinner MK, Tung PS, Fritz IB 1985 Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. J Cell Biol 100:1941–1947
2. Dym M, Fawcett DW 1970 The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol Reprod 3:308–326
3. Hadley MA, Byers SW, Suarez-Quian CA, Kleinman HK, Dym M 1985 Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation and germ cell development *in vitro*. J Cell Biol 101-2:1511–1522
4. Skinner MK 1987 Cell-cell interactions in the testis. Ann NY Acad Sci 513:158–171
5. Skinner MK, Fritz IB 1985 Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell function. Proc Natl Acad Sci USA 82:114–118
6. Skinner MK, Fritz IB 1985 Androgen stimulation of Sertoli cell function is enhanced by peritubular cells. Mol Cell Endocrinol 40:115–122
7. Skinner MK, Fetterolf PM, Anthony C 1988 Purification of a paracrine factor, P-Mod-S, produced by testicular peritubular cells that modulates Sertoli cell function. J Biol Chem 263:2884–2890
8. Keski-Oja J, Leof EB, Lyons RM, Coffey RJ Jr, Moses HL 1987 Transforming growth factors and control of neoplastic cell growth. J Cell Biochem 33:95–107
9. Moses HL, Tucker RF, Leof EB, Coffey RJ Jr, Halper J, Shipley GD 1985 Type-β transforming growth factor is a growth stimulator and a growth inhibitor. In: Feramisco J, Ozanne B, Stiles C (eds) Cancer Cells. Cold Spring Harbor Press, Cold Spring Harbor, NY, vol 3:65–71
10. Ignotz R, Massague J 1986 Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345
11. Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH 1987 Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor-β. J Exp Med 165(1):251–256
12. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV 1985 Human transforming growth factor-α complementary DNA

sequence and expression in normal and transformed cells tumor cell lines. Nature 316:701–705
13. Lawrence DA, Pircher R, Kryce-Martinerie C, Jullien P 1984 Normal embryo fibroblasts release transforming growth factors in a latent form. J Cell Physiol 121:184–188
14. Tucker RF, Branum EL, Shipley GD, Ryan RJ, Moses HL 1984 Specific binding to cultured cells of ^{125}I-labeled type-β transforming growth factor from human platelets. Proc Natl Acad Sci USA 81:6757–6761
15. Cheifetz S, Like B, Massague J 1986 Cellular distribution of type I and type II receptors for transforming growth factor-α. J Biol Chem 261:9972–9978
16. Sporn MB, Roberts AB, Wakefield LM, Assoian RK 1986 Transforming growth factor-α: biological function and chemical structure. Science 233:532–534
17. Jakowlew SB, Dillard PJ, Kondaiah P, Sporn MB, Roberts AB 1988 Complementary deoxyribonucleic acid cloning of a novel transforming growth factor-β messenger ribonucleic acid from chick embryo chondrocytes. Mol Endocrinol 2:747–755
18. Skinner MK, Keski-Oja J, Osteen KG, Moses HL 1987 Ovarian thecal cells produce transforming growth factor-β which can regulate granulosa cell growth. Endocrinology 121:786–792
19. Coffey RJ, Sipes NJ, Bascom CC, Graves-Deal R, Pennington CY, Weissman BE, Moses HL 1988 Growth modulation of mouse keratinocytes by transforming growth factors. Cancer Res 48:1596
20. Holmes SD, Spotts G, Smith RG 1986 Rat Sertoli cells secrete a growth factor that blocks epidermal growth factor (EGF) binding to its receptor. J Biol Chem 261:4076–4082
21. Skinner MK, Takacs K, Coffey RJ 1989 Transforming growth factor-alpha gene expression and action in the seminiferous tubule: peritubular cell-Sertoli cell interactions. Endocrinology 124:845–854
22. Clermont Y, Perey B 1957 Quantitative study of the cell population of the seminiferous tubules in immature rates. Am J Anat 100:241–267
23. Skinner MK, Griswold MD 1980 Sertoli cells synthesize and secrete transferrin-like protein. J Biol Chem 255(20):9523–9525
24. Skinner MK, Griswold MD 1982 Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biol Reprod 27:211–221
25. Adashi EY, Resnick CE 1986 Antagonistic interactions of transforming growth factors in the regulation of granulosa cell differentiation. Endocrinology 119:1879–1881
26. Tung PS, Skinner MK, Fritz IB 1984 Fibronectin synthesis is a marker for peritubular cell contaminants in Sertoli cell enriched cultures. Biol Reprod 30:199–211
27. Skinner MK, Fritz IB 1985b Structural characterization of proteoglycans produced by testicular peritubular cells and Sertoli cells. J Biol Chem 260:11874–11883
28. Tung PS, Fritz IB 1980 Interactions of Sertoli cells with myoid cells *in vitro*. Biol Reprod 23:207–217
29. Rosa F, Roberts AB, Danielpour D, Dart LL, Sporn MB, Dawid IB 1988 Mesoderm induction in amphibians: the role of TGF-β_2-like factors. Science 239:783–785
30. Cunha G, Chung LWK, Shannon JM, Taguchi O, Fujii H 1983 Hormone-induced morphogenesis and growth: role of mesenchymal-epithelial interactions. Recent Prog Horm Res 39:559–598
31. Dorrington JH, Roller NF, Fritz IB 1975 Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. Mol Cell Endocrinol 3:57–70
32. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
33. Skinner MK, Griswold MD 1983 Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem J 209:281–284
34. Karsten U, Wollenberger A 1977 Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates. Anal Biochem 77:464–470
35. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
36. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB 1983 Transforming growth factor-α in human platelets: identification of a major storage site, purification, and characterization. J Biol Chem 258:7155–7160
37. Frolik CA, Wakefield LM, Smith DM, Sport MB 1984 Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney cells. J Cell Biol 259:10995–11000
38. Carpenter G, Zendegui J 1985 A biological assay for epidermal growth factor/urogastrone and related polypeptides. Anal Biochem 153:279–282
39. Thomas PS 1980 Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201–5205