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Cellular localization of fibronectin gene expression in the seminiferous tubule

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Summary

The cellular location of fibronectin expression within the seminiferous tubule was investigated in order to better understand testicular cell functions and cell–cell interactions. Peritubular cells were shown to actively synthesize and secrete fibronectin in culture by the detection of a radiolabeled 220 kDa secreted protein that is immunologically similar to fibronectin and by the quantitation of fibronectin in peritubular cell conditioned medium with a fibronectin enzyme-linked immunosorbent assay. Sertoli cells did not produce detectable levels of fibronectin when assayed by either of these procedures. A 6.5 kb fibronectin messenger RNA was detected in freshly isolated or cultured peritubular cells, but no fibronectin gene expression was detected in Sertoli cells or developing germinal cells. Combined results imply that the peritubular cells are the only apparent site of fibronectin expression within the seminiferous tubule. During the development of the testis the levels of fibronectin expression increased to a maximum at early puberty (15-day-old rats) and then slowly declined. The results demonstrate that fibronectin can be utilized as a unique functional and biochemical marker for peritubular cells when compared to other cell types in the seminiferous tubule. Production of fibronectin by peritubular cells provides an example of the ability of peritubular cells and Sertoli cells to cooperate in the production of individual components of the basement membrane of the seminiferous tubule. This cellular interaction is an example of a mesenchymal/stromal–epithelial interaction which is postulated to be important for the physiology of many tissues.

Introduction

The cell types which make up the seminiferous tubule include Sertoli cells, peritubular (myoid) cells and germinal cells. Sertoli cells are the epithelial cell type, both morphologically and cytochemically, which form the tubules, provide the physical support to developing germinal cells and play a critical role in the maintenance of the process of spermatogenesis. Testicular peritubular cells are a mesenchymal (i.e. stromal) cell type which surround the tubules and are separated from the basal surface of Sertoli cells by a complex extracellular matrix (Dym and Fawcett, 1970). The basement membrane between peritubular cells

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and Sertoli cells helps provide structural integrity to the tubule and is produced cooperatively by Sertoli cells and peritubular cells (Skinner et al., 1985a). Components of this basement membrane that have been identified include collagen type I, collagen type IV, laminin (Skinner et al., 1985a), unique proteoglycans (Skinner and Fritz, 1985a) and fibronectin (Skinner et al., 1985a). Fibronectin is a multifunctional protein with both collagen and heparin binding sites (Hynes, 1981). It has been shown that fibronectin can influence the growth, differentiation and morphology of a number of cell types (Spiegelman and Ginty, 1983). A large number of investigations have indicated that fibronectin has an important physiological role in the extracellular matrices of most mammalian cell types (Hynes, 1981).

An initial report using seminiferous tubule tissue indicated that fibronectin was produced primarily by testicular peritubular cells (Tung et al., 1984). In contrast, recently reported data from cytochemical procedures have suggested that Sertoli cells also provide a possible additional site of synthesis (Borland et al., 1986). The current study was designed to more definitively determine the cellular localization of fibronectin expression in the seminiferous tubule using a biochemical and molecular approach. Information obtained from this investigation is important to enable a better understanding of the cellular functions of individual cell types within the seminiferous tubule and to determine the potential use of fibronectin as a biochemical marker of cellular function. In addition, determination of the cellular localization of fibronectin expression provides insight into the ability of mesenchymal cells and epithelial cells to cooperate in the production and formation of a basement membrane.

Materials and methods

Cell preparation and culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (Dorrington et al., 1975) with a modified procedure previously described (Tung et al., 1984). Decapsulated testis fragments were digested first with trypsin (2.5 mg/ml) (Gibco, Grand Island, NY, U.S.A.) to remove Leydig cells, followed by a collegenase digestion (1 mg/ml type 2, Sigma, St. Louis, MO, U.S.A.) and then a hyaluronidase digestion (1 mg/ml, Sigma). When required Sertoli cells were plated in 24-well (1 ml per well) Linbro (Gibco) plates at approximately 5×10^5 cells per 2 cm^2. Cells were maintained at 32° C in a 5% CO_2 atmosphere in Ham's F12 medium (Gibco). Sertoli cell cultures were treated as described in the Results section 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. Sertoli cell cultures were treated as outlined in the Results section with (NIADDK-oFSH-16, National Pituitary Agency) FSH (100 ng/ml), insulin (5 μg/ml), retinol (0.35 μM) and testosterone (1 μM). Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (Skinner and Fritz, 1985b). Peritubular cells were cultured in the presence of 10% calf serum and grown to confluence in 2–5 days. After the peritubular cells were confluent they were maintained in serum-free cell culture conditions described above. A cytochemical analysis of the purity of the cell preparations indicates that Sertoli cell preparations are greater than 99% pure. The peritubular cell population contains approximately 50% highly differentiated myoid cells and 30% less differentiated myoid cells with the remainder of the cell population being an undifferentiated fibroblast (Anthony and Skinner, 1989). The peritubular cell population contained no detectable Sertoli cells or endothelial cells. Germinal cells were isolated from adult (> 60-day-old) rat testis as previously described (Bellve et al., 1977) and utilized immediately for RNA isolation.

Gel electrophoresis and fluorography

Sertoli cell and peritubular cell cultures were maintained for 48 h starting on day 5 of culture with glycine and methionine-free media containing 5 μCi/ml [^{3}H]glycine and 5 μCi/ml [^{35}S]-methionine. The media was collected, centrifuged, and the radiolabeled proteins were electrophoretically analyzed on sodium dodecyl sulfate 5–15% polyacrylamide gradient slab gels under reducing

conditions (Laemmli, 1970). The gels were fluorographed with diphenyloxazole in acetic acid as previously described (Skinner and Griswold, 1983).

Fibronectin enzyme-linked immunosorbent assay

Non-equilibrium, competition enzyme-linked immunosorbent assays (ELISA) were performed as previously described (Skinner et al., 1985) for fibronectin (Dorrington and Skinner, 1986). MicroELISA plates (Dynatech Lab., Alexandra, VA, U.S.A.) were coated overnight with human fibronectin at 4°C. Samples and standards were incubated overnight at 4°C in the presence a 1 : 20 000 final dilution of goat anti-rat fibronectin that is cross-reactive with human fibronectin (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.). After a 30 min incubation of the antigen-antibody solution in the coated plate the bound antibody was detected with an alkaline phosphatase-conjugated second antibody as previously described (Dorrington and Skinner, 1986). This assay had a sensitivity of 2 ng fibronectin with a detection limit of 150 ng fibronectin.

Cytochemistry

Fibronectin histochemistry was performed using an immunoperoxidase detection procedure. Cells were fixed with cold methanol then incubated in 0.3% hydrogen peroxide in methanol. Fixed cells were then rinsed with phosphate-buffered saline, 0.15 M NaCl, 50 mM phosphate, pH 7.0 (PBS) followed by 1% horse serum then incubated for 1 h with goat anti-rat fibronectin (1 : 100 final dilution) (Calbiochem-Behring Corp.). Cells were then rinsed with PBS and horse serum followed by an incubation with a biotinylated second antibody and then avidin-conjugated to peroxidase (Vector Labs, Burlingame, CA, U.S.A.). Peroxidase activity was detected with diaminobenzidine and the cells rinsed with PBS. Cells were counterstained with Gill's hematoxylin then dehydrated and mounted for microscopy.

Northern blot analysis

RNA was isolated from freshly isolated cells, cell cultures (generally from 150 mm culture plates) or whole tissue by a guanidine isothiocyanate and LiCl procedure (Cathala et al., 1983). 10 μg of total RNA was loaded in each lane and electrophoretically separated on a denaturing 1.2% agarose gel then transferred to a nylon membrane (MSI). The blots were hybridized with nick-translated SP64-plasmid containing the fibronectin cDNA (Schwarzbauer et al., 1983) (kindly provided by Dr. R. Hynes, MIT, Boston, MA, U.S.A.). Hybridization was performed at 42°C overnight then the blot washed in three 30 min incubations of 0.2% (w/v) sodium dodecyl sulfate (SDS) and 0.15 M NaCl, 16 mM sodium citrate prior to autoradiography. For analysis of fibronectin mRNA from rats of different ages, the total RNA from each testis was resuspended in a constant volume (0.5 ml). Therefore, the hybridization to equal volumes of RNA represented the relative amount of mRNA per testis for each sample (Morales et al., 1987). Relative differences in hybridization were determined by scanning densitometry of autoradiograms.

Results

Using a histochemical procedure, fibronectin was detected on both peritubular cells and Sertoli cells in culture (Fig. 1). The intensity of staining was greater on peritubular cells and was independent of the duration of culture. Sertoli cell cultures contained less intense staining that decreased with extended culture periods. The cytochemical analysis confirms previous observations of the presence of fibronectin on both peritubular cells and Sertoli cells (Borland et al., 1986). However, determination of the site of production of fibronectin will require biochemical procedures.

The synthesis of fibronectin was examined by the addition of radiolabeled amino acids to Sertoli cell and peritubular cell cultures. The electrophoretic profiles of the actively synthesized and secreted proteins of the two cell types are unique (Fig. 2). These data indicate that the functions of the two cell types are distinct and that the cultured cell populations have negligible levels of contamination with each other. A 220 kDa radiolabeled protein secreted by peritubular cells has previously been shown to be fibronectin (Tung et al., 1984). This radiolabeled fibronectin is not detected in Sertoli cell radiolabeled secreted proteins (Fig. 2). Studies have also indicated that

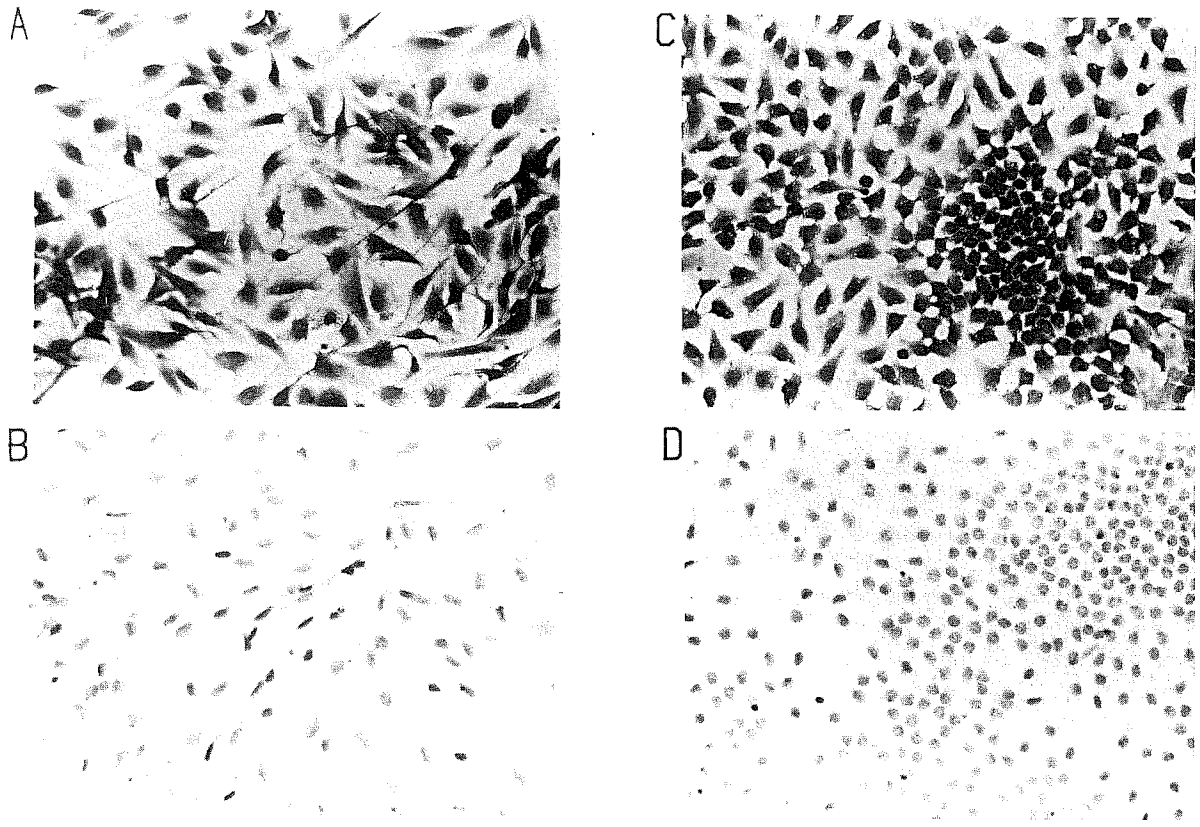

Fig. 1. Fibronectin immunocytochemical analysis of serum-free cultures of peritubular cells (*A*) and Sertoli cells (*C*). Controls with non-immune antiserum are also shown for peritubular cells (*B*) and Sertoli cells (*D*). 200× magnification.

immunoprecipitation from radiolabeled secreted proteins is only detectable in peritubular cell secreted proteins (Tung et al., 1984).

To provide a more quantitative measure of the amount of fibronectin in Sertoli cell and peritubular cell secreted proteins an ELISA assay was utilized. Non-detectable levels of fibronectin were present in Sertoli cell secreted proteins from cells cultured in the absence or presence of a variety of regulatory agents (Fig. 3). In contrast, secreted proteins from cultured peritubular cells contained a significant amount of fibronectin. Fibronectin production by peritubular cells appeared to be non-responsive to the regulatory agents analyzed (Fig. 3). Since fibronectin becomes insoluble and deposits onto the surface of the cell by interactions with the extracellular matrix, the amount of soluble fibronectin detected in the secreted protein preparation provides a minimum estimate of the amount actually produced by peritubular cells.

To determine more directly the sites of synthesis of fibronectin the cellular localization of fibronectin gene expression was determined. RNA was isolated from peritubular cells, Sertoli cells and germ cells and analyzed by Northern blot analysis with a complimentary DNA (cDNA) probe to fibronectin (Schwarzbauer et al., 1983). A 6.5 kb fibronectin mRNA was only detected in RNA from peritubular cells while Sertoli cells and germinal cells did not contain a fibronectin transcript (Fig. 4). Similar results were also obtained with 10 μg polyadenylated RNA (data not shown). Fibronectin expression was also detected in liver RNA preparations (Fig. 4). Results were similar with freshly isolated cells or cultured cells. Analysis of the blots for a constitutively produced pro-

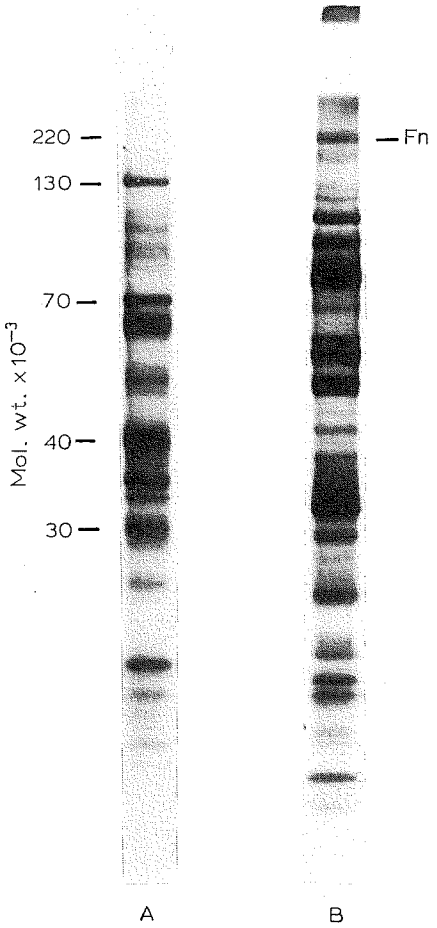

Fig. 2. Electrophoretic profile of Sertoli cells (A) and peritubular cell (B) radiolabeled secreted proteins. Radiolabeled [^{35}S]-cystine and [^{35}S]methionine secreted proteins were separated electrophoretically on 5–15% polyacrylamide gradient gels then fluorographed. Location of fibronectin is designated (Fn).

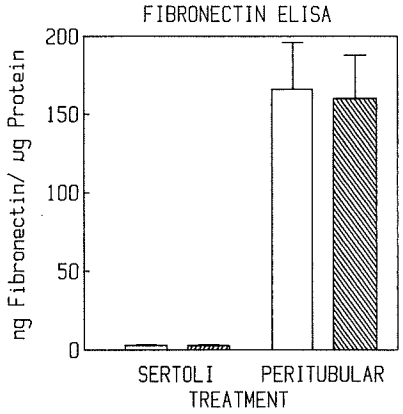

Fig. 3. Fibronectin ELISA on Sertoli cell and peritubular cell conditioned medium. Cells were cultured in the absence (open bars) of presence (hatched bars) of regulatory agents (FSH, insulin, retinol and testosterone) and a 72 h serum-free medium collection on day 5 of culture analyzed. Fibronectin data is represented as ng fibronectin/μg total protein and presented as the mean $\pm$ SEM from three experiments done in triplicate. Levels shown for Sertoli cells are below the detection limit of the assay.

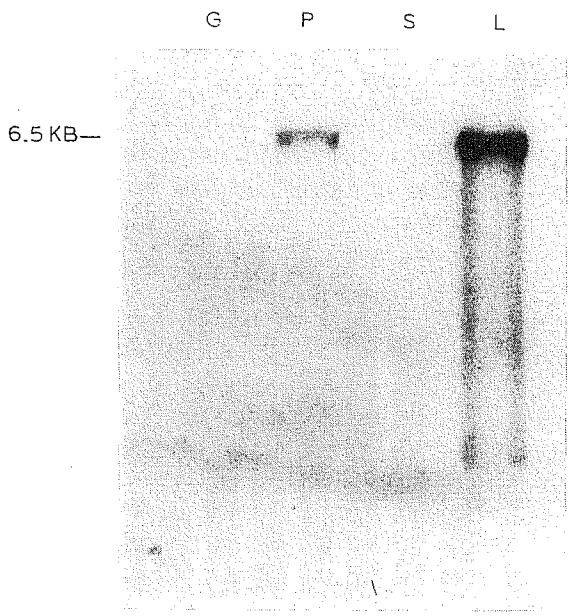

Fig. 4. Northern blot analysis for fibronectin gene expression in germinal cells (G), peritubular cells (P), Sertoli cells (S), and liver (L). Total RNA was isolated and 10 μg analyzed by Northern analysis described in Materials and Methods. RNA was isolated from freshly isolated tissue except for Sertoli cells and peritubular cells which were isolated from cultured cells.

tein cyclophilin (Skinner et al., 1989) indicated that the integrity of the RNA was good and similar amounts of RNA were analyzed (data not shown).

The developmental pattern of fibronectin mRNA expression was examined using RNA from the testis of rats of several different ages. Total RNA was obtained from testes from rats in each age group and was examined by a Northern blot for the presence of fibronectin mRNA (Fig. 5). Testes from rats of all age groups (5- and 10-day-old prepubertal; 15- to 35-day-old, and adult) expressed detectable levels of fibronectin mRNA.

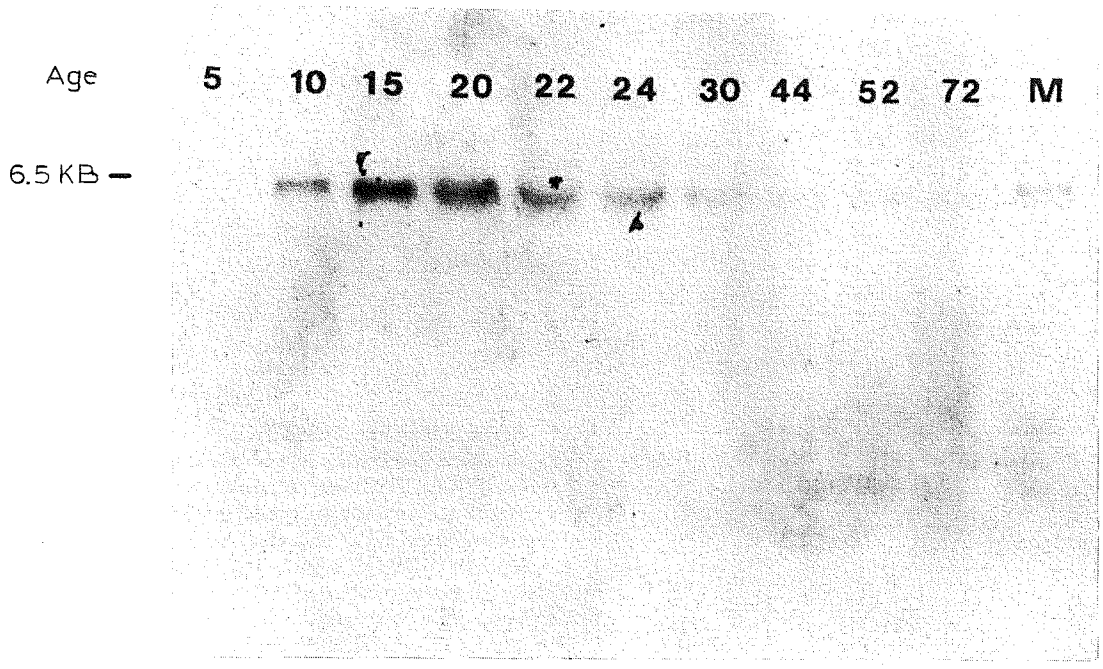

Fig. 5. Northern blot analysis for fibronectin gene expression in testis isolated at different stages of development. Total RNA was isolated from whole testis from 5-, 10-, 15-, 20-, 22-, 24-, 30-, 52-, 72-day-old animals and equivalent amounts of RNA per testis analyzed. RNA from isolated peritubular (myoid) cells (M) was also analyzed.

Densitometric scanning of the bands showed that levels of fibronectin mRNA per testis increased and peaked at 15 days of age then declined (Fig. 5).

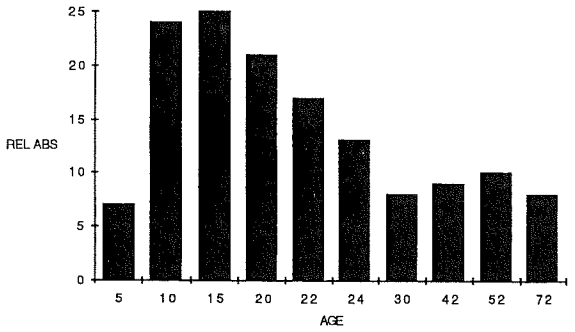

Fig. 6. Quantitation of fibronectin gene expression in whole testis isolated at different stages of development. The Northern blot shown in Fig. 4 was scanned with a densitometer and the relative absorbances plotted versus the age of the rat when the testis was isolated.

Discussion

Fibronectin was found to be actively synthesized and secreted by seminiferous peritubular cells through the detection of a radiolabeled secreted protein and the presence of fibronectin in conditioned medium by ELISA. Fibronectin gene expression was also demonstrated in peritubular cells by Northern analysis of RNA isolated from the cells. Using these same procedures Sertoli cells were not found to have detectable levels of fibronectin gene expression or synthesis. In addition, a mixed population of germinal cells from various stages of development were also not found to express fibronectin. The observation that germinal cells do not produce fibronectin implies that fibronectin is not a haploid expressed gene. The observation that Sertoli cells and germinal cells do not express the fibronectin gene is limited by the sensitivities of the methods utilized and low levels of expression may be observed by more sensitive procedures such as the use of the polymerase

chain reaction method. However, expression in Sertoli cells or germ cells if present would be negligible in comparison to the expression in peritubular cells. Examination of fibronectin production by a wide variety of cell types has indicated that mesenchymal cell types generally synthesize fibronectin while a number of epithelial cell types do not produce fibronectin (Yamada, 1983). This observation is supported by data presented in the current study in that the mesenchymal peritubular cell type expresses fibronectin while the epithelial-like Sertoli cell type does not produce fibronectin. Although the current study suggests that fibronectin production may be predominate by mesenchymal cell types, the Sertoli cell is a unique epithelial cell in that it is a terminally differentiated non-growing cell type in adult tissue. Other epithelial cell types which actively grow and migrate, such as the granulosa cell in the ovary (Skinner et al., 1985b), have been shown to produce fibronectin. Therefore, it is likely that the physiological needs of a tissue will have as much of a role in dictating the types of gene expression required as does the origin of the cell type. Combined results indicate that the peritubular cells appear to be the primary site of fibronectin production in the seminiferous tubule; however, fibronectin can deposit onto the surface of either peritubular cells or Sertoli cells.

The peritubular cell population utilized has been recently characterized cytochemically (Anthony and Skinner, 1989) confirming the presence of different cell populations (Palombi et al., 1988). Observations imply that the majority of the cell population is peritubular myoid cells at various stages of differentiation with a minor contaminate of an undifferentiated fibroblast (Anthony and Skinner, 1989). Preliminary analysis with an in situ hybridization procedure previously described (Morales, 1987) on the peritubular cell population indicated that all the cells in the culture were positive for fibronectin gene expression (unpublished observation). Therefore, data indicate that all the peritubular cells, including the more highly differentiated myoid cells, express fibronectin.

Demonstration that fibronectin expression is only detected in peritubular cells indicates that fibronectin can be used as a unique functional marker for peritubular cells when compared to the other cell types of the seminiferous tubule. This observation confirms a previous report that fibronectin may be used as a marker of peritubular cell contamination in Sertoli cell cultures (Tung et al., 1984). Peritubular cells have an important functional role in the seminiferous tubule to provide structural integrity to the tubule, in part through the production of an extracellular matrix (Dym and Fawcett, 1970). Recently, peritubular cells have also been shown to produce a paracrine factor, P-Mod-S, under androgen regulation (Skinner and Fritz, 1985b) that has a dramatic effect in the regulation of Sertoli cell function and differentiation (Skinner et al., 1988). Therefore, peritubular cells have an integral role in the maintenance and control of testicular function. The development of unique functional markers such as fibronectin helps provide the tools required to understand the functions of this important testicular cell type.

Analysis of the levels of fibronectin gene expression during testis development indicated that levels peaked at early puberty and then gradually declined to a steady-state level. This increased level of fibronectin expression at day 15 correlates with the development of a mature seminiferous tubule and the onset of meiosis. Interstitial tissue will also likely express fibronectin, therefore, the developmental study cannot be only attributed to the differentiation of peritubular cells. Data obtained from this developmental study were normalized per testis rather than RNA to reduce the variable associated with RNA contributed by germinal cells increasing during puberty. The current observations support the proposal that a large number of testicular somatic cell functions are fully developed at the onset of meiosis during puberty.

Previously, Sertoli cells and peritubular cells have been shown to cooperate in the production and formation of an extracellular matrix (i.e. basement membrane) (Skinner et al., 1985a). Sertoli cells produce collagen type IV and laminin while peritubular cells produce collagen type I and IV (Skinner et al., 1985a), and as the current study demonstrates, fibronectin. Each cell type also produces unique proteoglycans (Skinner and Fritz, 1985). Therefore, each of these cell types contributes unique components to the extracellular

matrix that exists between themselves. In essentially every tissue where an epithelial cell is in contact with a mesenchymal cell a complex extracellular matrix exists, often a basement membrane. The combined observations made on Sertoli cells, which is an epithelial-like cell, and peritubular cells, which is a mesenchymal-like (i.e. stromal) cell, imply that both cell types may cooperate in the production of the extracellular matrix. Mesenchymal–epithelial cell interactions are postulated to have an important role in the development of most tissues (Cunha et al., 1983). The ability of peritubular cells and Sertoli cells to cooperate in the production and formation of an extracellular matrix provides an example of a mesenchymal–epithelial cell interaction. This type of interaction will likely be important for the development and functions of most tissues.

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