Biotechnological evaluation of extracellular matrix proteins expressed by cultured testicular cells

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Sertoli cells and peritubular myoid cells play an important role in germ cell maturation and contribute in the process of spermatogenesis. The production of extracellular matrix components (ECM) by these cells is thought to mediate testicular development. We have selected primary culture of Sertoli cells, secondary culture of peritubular myoid cells and a transformed mouse cell line (MSC-1 cells) to evaluate production of selected ECM proteins. Previously we have reported biosynthetic cooperation between Sertoli cells and peritubular myoid cells with respect to production of ECM proteins and in particular, the production of fibronectin by peritubular myoid cells in vitro was enhanced when cultured in association with Sertoli cells. Here we evaluated primary culture of Sertoli cells, secondary culture of peritubular myoid cells, and the transformed MSC-1 cells in terms of expression of laminin and fibronectin by using various biotechniques. Laminin, but not fibronectin was detectable by immunocytochemistry in primary culture of Sertoli cells when cultured on glass coverslips. Secondary culture of peritubular myoid cells produced both laminin and fibronectin. In MSC-1 cells, both laminin and fibronectin were detected. Levels of soluble laminin and fibronectin secreted by the three types of cells were determined by an ELISA. The dot-blot analysis using specific antibodies for laminin and fibronectin was undertaken to demonstrate the expression of these ECM proteins. Primary Sertoli cells in culture secreted laminin, but did not secrete detectable amounts of fibronectin. Peritubular myoid cells and MSC-1 cells released high amounts of both laminin and fibronectin into the medium. To further confirm the difference in fibronectin expression by these cells we have conducted western blot analysis. A specific band of fibronectin at 220 kDa was observed in peritubular myoid cells and MSC-1 cells. Again, Sertoli cells in primary culture did not express a significant level of fibronectin. Furthermore, Sertoli cells demonstrated a much higher electrical resistance than the secondary culture of peritubular myoid cells or transformed MSC-1 cells when cultured on Matrigel coated Millipore-HA filters in bicameral chambers. Through biotechnological evaluation and screening we demonstrated the differences between these cultured testicular cells, which may be used to better understand the biology of the seminiferous tubule, and capture nuances of the *in vivo* cellular microenvironment.

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Introduction

The extracellular matrix (ECM) components, including the basement membrane, play a key role in regulating cytoskeletal structure as well as facilitating cell-cell interactions, embryonic development and morphogenesis [1-3]. There is evidence that certain biologically active components of the ECM, including fibronectin and laminin are present within the seminiferous tubule basement membrane [4]. In rodent testis, the basement membrane surrounding the seminiferous tubules possesses the backbone of laminin chain synthesized by Sertoli cells and in the adjacent connective tissue fibronectin is synthesized by peritubular myoid cells. These ECM molecules are thought to be involved in the process of testicular development.

The basement membrane of the rat testis surrounding the seminiferous tubules is produced via cooperation between the Sertoli cells and peritubular myoid cells [4-7]. In three

week old rats a network of ECM components populate the peritubular zone and the post-natal development of the peritubular zone is synchronous with the tubular changes. At around two weeks of gestation, laminin together with collagen Type IV and heparan sulfate proteoglycan have been shown to be involved in the organization of testicular cords in the undifferentiated gonad, and fibronectin was present even earlier [8, 9]. In adult rat testis, laminin, collagen Type IV and heparan sulfate proteoglycan have been localized in the basement membrane close to the outer boundary of the Sertoli cells [4]. On the other hand, fibronectin was present only in the basement membrane layer below the peritubular myoid cells. From *in vitro* studies [5], it was reported that laminin and collagen Type IV were localized within Sertoli cells, whereas fibronectin, collagen Types I and IV were localized within the peritubular myoid cells cultured on glass cover-slips for five days.

Sertoli cells are unique epithelial cells that are non-growing terminally differentiated cell types in adult testis. Other epithelial cell types, which actively grow and migrate, such as the adult mouse Sertoli cell line, MSC-1 cells have been included in this study to compare with primary rat Sertoli cell cultures. It is known that MSC-1 cells do not express inhibin-α or FSH receptors [10]. This is an important difference from primary cultures of Sertoli cells. Testicular tumors composed of MSC-1 cells were developed in transgenic mice carrying a fusion gene composed of human Mullerian inhibiting substance (MIS) transcriptional regulatory sequences linked to the SV40 T-antigen gene [10]. MIS is a glycoprotein hormone expressed by Sertoli cells that induces the regression of Mullerian ducts during development of the male reproductive tract. The MSC-1 cells have been claimed to be composed of a single cell type [11]. Though these cells have histological resemblance with Sertoli cells, the researchers [10] reported morphologically heterogeneous populations of cells consisting of large, epitheloid-shaped cells displaying extensive cytoplasmic processes as well as smaller spindle-shaped cells.

To determine the extent of similarities and differences between Sertoli cells and MSC-1 cells, we examined the expression of laminin and fibronectin in these cell types. Study of these bioactive secreted proteins in primary, secondary and transformed cell cultures is necessary to further characterize their use in cell culture research. We used the testicular tumor cell type from nongerminal cells within the seminiferous tubule as referred to as MSC-1 cells because these transformed cells have many similarities with primary Sertoli cells cultures (10, 11). As adult Sertoli cells are postmitotic (do not propagate *in vitro*) and are only a few percent of the cells in the testis, the use of such an transformed cell line (MSC-1) would be helpful in studying the features of normal primary culture of Sertoli cells. On the other hand, it has been demonstrated *in vivo* that loss of peritubular myoid cells results impaired spermatogenesis (12). Peritubular myoid cells are used for the obvious reason that they express fibronectin protein *in vitro* and thus serve as a candidate for positive control. Additionally, in the connective tissue adjacent to the peritubular myoid cells fibronectin is synthesized *in vivo* by peritubular myoid cells (13). Because of this strategic anatomical location of peritubular myoid cells in the rodent testis surrounding the seminiferous tubules and neighboring Sertoli cells, we have included these cells in our study.

We have applied biotechnological assays to measure cell permeability barrier or functional polarity of the cultured cells in a two-compartment bicameral culture system. We measured electrical resistance between the upper and lower chambers of the two-compartmental culture system, which has proven useful to study functional and morphological polarity of Sertoli cells [14-17]. Sertoli cells form the blood testis barrier through tight junctions and divide the seminiferous epithelium into basal and

adluminal compartments. This compartmentalization is necessary for germ cell division and differentiation. Therefore, the formation of the unique testicular environment with the involvement and contributions of these cells warrant further evaluation of the primary, secondary and transformed cell lines. This culture approach of studying cell fate involving laminin and fibronectin would lead to our understanding and emerging interest in biology of different tissues, and thus advance the field of biotechnology.

Materials and methods

Animals

All rats aged 19-21 days were purchased from Harlan Sprague-Dawley (Indianapolis, IN). For each Sertoli cell and peritubular myoid cell isolation, ten male rats (19- to 21-day-old CD albino pups) were used. All animals were housed in a room with controlled lighting (12 h of light and 12 h of darkness daily) and temperature (20-22^{o}C).

Sertoli Cell Isolation and Culture

Sertoli cells were isolated by a modification of procedures outlined previously [18], and described elsewhere [19, 20]. Briefly, testes were aseptically removed, decapsulated, and minced into 0.5 mm segments. The chopped tubules were suspended in a Ca^{2+}- and Mg^{2+}-free Hank's balance salt solution (HBSS), and incubated at 33°C in a shaker water bath with 0.25% trypsin and 700 μl DNAse (1 mg/ml) for 30 min, followed by a washing through a sterilized stainless steel grid (0.6 mm^2) with 10 ml HBSS plus trypsin inhibitor (2 mg/ml). The tubules were further digested in 20 ml HBSS containing collagenase (1 mg/ml), hyaluronidase (1 mg/ml) and 100 μl DNAse (1 mg/ml) for 30 min, and the supernatant was saved for peritubular myoid cell isolation. The resultant cell aggregates were washed with 1% BSA in HBSS at least two times by low speed centrifugation (3 min at 60 X g). The enriched population of Sertoli cell fractions were cultured in Dulbecco's modified Eagle's medium:Ham's F-12 medium in a 1:1 ratio with 4 mmol/L of added glutamine and 15 mmol/L HEPES (DMEM/F-12) supplemented with 5% fetal bovine serum (FBS) and antibiotics (final concentrations: gentamicin sulfate, 1 μg/ml; penicillin, 2U/ml; streptomycin sulfate, 2 μg/ml; amphotericin B, 5 ng/ml). Sertoli cells were plated onto 22x22 mm glass cover-slips in a 35-mm diameter culture dish (Falcon Plastics, Lincoln Park, NJ) at approximtely 1 X 10^6 cells per dish. Cells were incubated at 33^{o}C in a humidified incubator in an atmosphere of 95% air and 5% CO_2. After 24 hour, media from all dishes were removed and discarded. Subsequently, culture media were changed every 2 days and the cultures were maintained for a period of 6 days.

On Day 3 of culture, contaminating spermatogenic cells were lysed with a hypotonic solution of 20 mM Tris-HCl (pH 7.4) for 5 min as described previously [21]. To check for possible Leydig cell and peritubular cell contamination in Sertoli cell preparations, Sertoli cell cultures were occasionally stained histochemically for 3-β hydroxysteroid dehydrogenase [22] and alkaline phosphatase [23], respectively. Sertoli cell cultures were virtually negative for Leydig cell contamination and there was consistently less than 1% peritubular myoid cells.

Culture of Peritubular Myoid Cells

Peritubular myoid cells were isolated from the collagenase/hyaluronidase supernatant according to procedures described previously [18, 24]. These cells were cultured in DMEM/F-12 containing 10% FBS, subcultured on day 4, and plated at a cell density of approximately 2 X 10^5 cells per dish. In this study, we used secondary culture of peritubular myoid cells as a positive control for fibronectin expression.

Culture of MSC-1 Cells

The mouse Sertoli cell line was established from 13-week-old mouse testis [10]. We obtained these cells from the laboratory of Dr. M. D. Griswold, Washington State University. MSC-1 cells were cultured in 5% FBS in DMEM with 3.7

g/L NaHCO3, 4.5 g/L D-glucose, 10 ml/L antibiotic antimycotic, 100 mg/L gentamycin and 5 ml/L insulin-transferrin-selenium liquid media supplement, pH 7.3, at 37°C in 5% CO_2 and 95% air. Approximtely 1 X 10^6 cells per dish were plated in 35-mm diameter culture dishes and maintained for at least four days before use.

Cell Viability Study

Cell viability was routinely determined by trypan blue exclusion. Greater than 95% of the cultured cells were viable; a majority of the dead cells had round shapes that were removed while changing media.

Immunocytochemical Assay of Laminin (LN) and Fibronectin (FN)

Affinity purified rabbit anti-mouse LN (Cat # 9393) and rabbit anti-human FN (Cat # F3648), both were obtained from the Sigma Chemical Co (St. Louis, MO). Rabbit IgG used for negative controls, and Vectastain ABC (Avidin-biotin peroxidase complex) Elite Rabbit IgG kit were obtained from Vector Laboratories (Burlingame, CA). The manufacturer's instructions were followed to determine the immunolocalization of LN and FN on cover-slip cultures fixed in 3% paraformaldehyde in PBS for 10 min [25].

Enzyme-linked Immunosorbent Assay (ELISA)

Competition ELISA were performed following the procedure as described elsewhere [5, 26]. Rat FN and rabbit anti-human FN, mouse LN and rabbit anti-mouse LN were purchased from Sigma Chemical Co. (St. Louis, MO). HRP-conjugated goat anti-rabbit IgG (1:1000) and o-phenylenediamine (OPD) were received from Organon Teknika Corporation (Durham, NC). Color development in the plates was monitored at 490 nm with a Dynatech MR5000 plate reader.

Protein Assay

Conditioned medium from the cell culture dishes were removed and stored at – 20°C for further assay. Protein concentration was quantified as described briefly. Protein from 2 ml medium was precipitated in 2% Sodium deoxycholate-trichloroacetic acid. The precipitate was re-suspended in phosphate buffered saline and total protein was determined using a BCA protein assay kit (Pierce Chemical Company, Rockford, IL).

Dot blot Analysis

Equal amounts of protein in aliquots of Sertoli cells, peritubular myoid cells and MSC-1 cell conditioned medium were spotted on nitrocellulose paper strips. After drying, the strips were probed with either rabbit anti-human FN or rabbit anti-mouse LN at a dilution of 1:100.

Western blot Analysis

Equal amounts (10 µg) of protein samples were loaded in each lane, and separated by 7.5 % SDS-PAGE. Proteins were transferred at 200 V to Hybond-ECL nitrocellulose membranes (Pharmacia Biotech, Buckinghamshire, UK) and blocked for 1 hour at 4°C in 1% Blotto non-fat dry milk (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were incubated with 0.5 µg/ml rabbit polyclonal FN for 1 hr at room temperature and subsequently in 1 µg/ml peroxidase conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) at room temperature. ECL western blotting detection analysis (Amersham Biosciences, Buckinghamshire, UK) was used to visualize fibronectin expression on X-ray film (Eastman Kodak Co., Rochester, NY). We purchased fibronectin from human plasma that was used as positive control (Sigma Life Science, St. Louis, MO).

Electrical Resistance in Bicameral Culture Chambers

Freshly isolated Sertoli cells, MSC-1 cells as obtained above (2-3 x 10^6 cells/0.64 cm^2/well) were plated on Matrigel coated (Collaborative Research Inc., Bedford, MA) filters in bicameral chambers (Millicell-HA; Millipore Corporation, Bedford, MA) and cultured for 7 days. After culture for 3, 5 or 7 days, the transepithelial electrical resistance was measured by Millicell-ERS meter (Millipore Corporation, Bedford, MA)

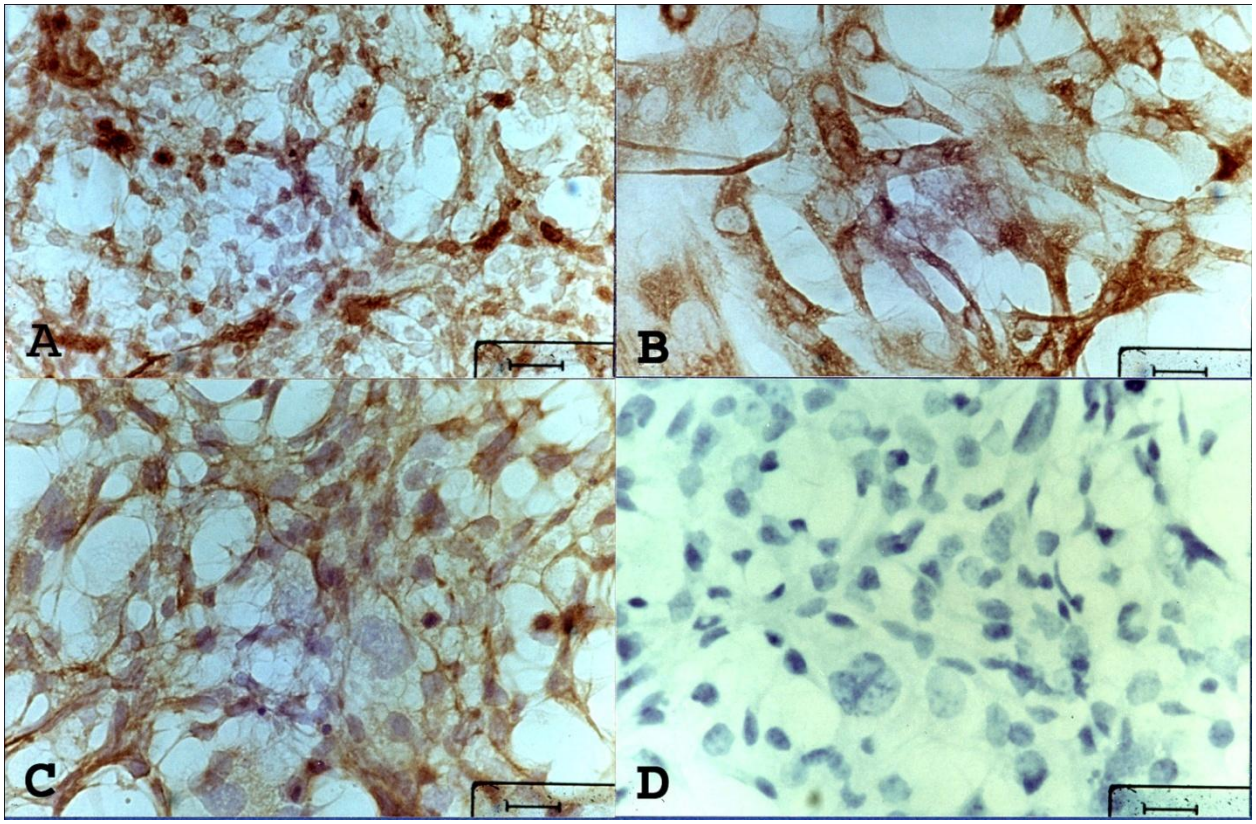

Figure 1: Immunocytochemical Assay of laminin (LN). A polyclonal rabbit anti-mouse LN antibody was used at 1:30 dilution. Laminin was detected in cultured Sertoli cells (A). Peritubular myoid cells had mostly perinuclear staining (B). MSC-1 cells demonstrated staining pattern on the surface of the cells with anti-LN (C). The rabbit IgG negative control did not show any immunoreactivity of LN in MSC-1 cells (D). Figures are representative fields of three separate experiments in duplicate samples. Bar = 10 µm

according to the instructions in the instruction manual. The net value of electrical resistance was computed by subtracting the background reading measured on Matrigel-coated, cell-free control chambers from the value of cell plated chambers. Finally the values were multiplied by the effective area of the filters in the apical chamber (0.64 cm^2).

Statistical Analysis

Electrical resistance data in duplicate from three separate experiments were presented as ohms per cm^2. Mean and standard error mean were calculated. Difference between the groups was analyzed by one way analysis of variance. The group that registered significant difference ($P < 0.05$) were analyzed by multiple comparison tests using Prism 3.02 software (Graphpad Inc., San Diego, CA).

Results

Sertoli cells exhibited a positive immuno-cytochemical reaction to anti-LN antibody (Figure 1A). Peritubular myoid cells had mostly perinuclear staining pattern with occasional nuclear deposit (Figure 1B). Cultured MSC-1 cells showed staining pattern on the surface of the cells with anti-LN antibody (Figure 1C). The rabbit IgG negative control did not show any expression of LN in MSC-1 cells (Figure 1D) and in other cell types used in this study.

The rabbit IgG negative control did not show any immunoreactivity in Sertoli cells (Figure 2A). Sertoli cells did not stain with anti-FN antisera (Figure 2B). Close examination of the figure showed occasional FN-staining on the contaminating peritubular myoid cells (Figure 2B). Peritubular myoid cells showed intense

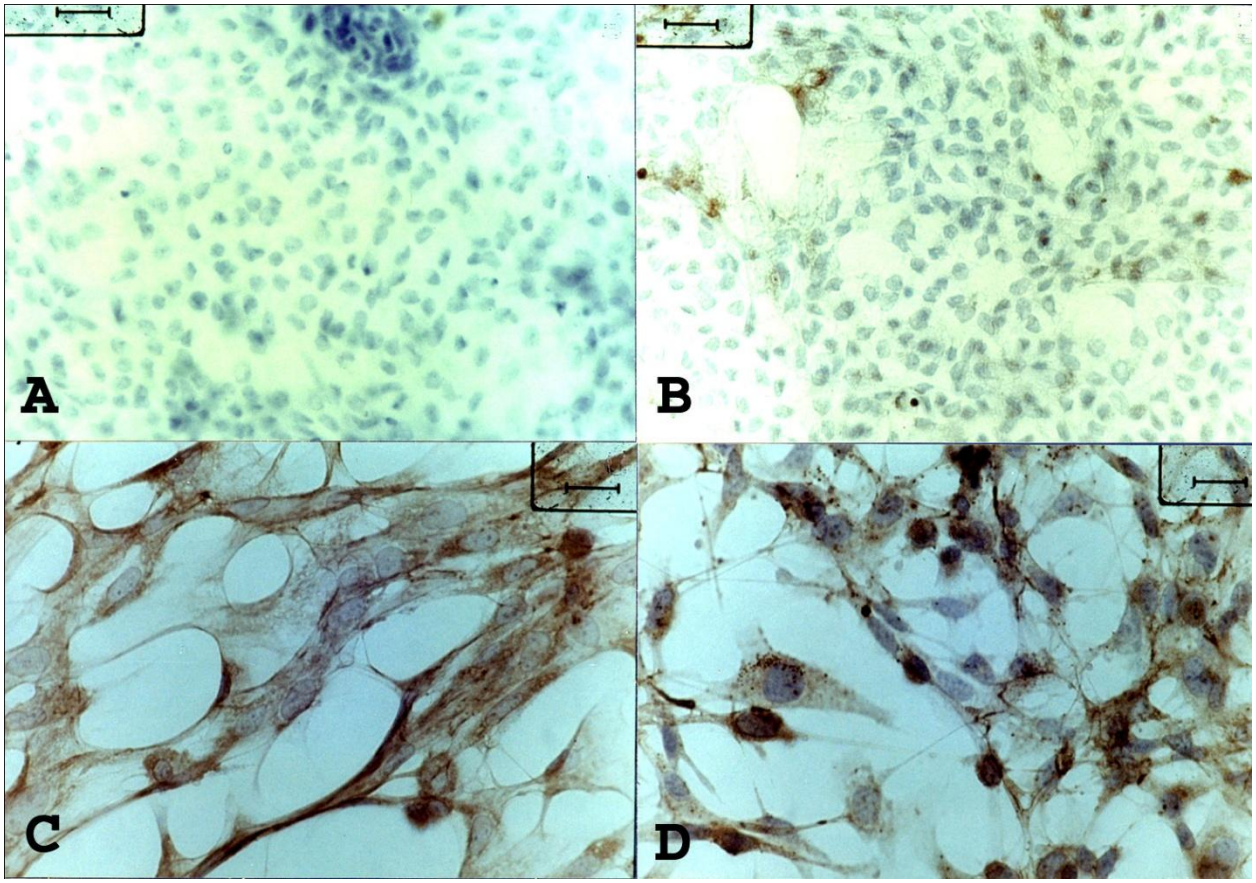

Figure 2: Immunocytochemical Assay of fibronectin (FN). A polyclonal rabbit anti-human FN antibody was used at 1:400 dilution. The rabbit IgG negative control did not show any immunoreactivity in Sertoli cells (A). Sertoli cells did not stain with anti-FN; however, some contaminating myoid cells showed FN localization (B). Peritubular myoid cells showed positive staining with anti-FN antibody (C). The MSC-1 cells also showed positive FN staining (D). Figures are representative fields of three separate experiments in duplicate samples. Bar = 10 µm

staining with anti-FN antibody (Figure 2C). The MSC-1 cells showed positive FN staining (Figure 2D).

Results from the ELISA for FN and LN on Sertoli cell, peritubular myoid cells and MSC-1 cell conditioned medium are presented in Figure 3. Sertoli cells demonstrated a very negligible level of FN, but a very high level of LN. Peritubular myoid cells had a very high level of FN and an intermediate level of LN. In MSC-1 cells, higher levels of both FN and LN were detected.

The immunoreactivity of FN and LN were determined by dot-blot immunoassay using equal amounts of proteins from the conditioned medium of Sertoli cells, peritubular myoid cells and MSC-1 cells (Figure 4). Sertoli cells did not produce detectable level of FN whereas FN was expressed both in peritubular myoid cells and MSC-1 cells. Laminin was detected in all of the three cell types. To further verify the difference of FN expression in these cells we have conducted western blot analysis. A specific band of FN at 220 kDa was observed with human plasma FN positive control (Lane 1**,** Figure 5), in peritubular myoid cells (Lane 2) and MSC-1 cells (Lane 4). Again, Sertoli cells did not express a significant level of FN (Lane 3, Figure 5).

The electrical resistance data are presented in Table 1. Sertoli cells values were consistently higher throughout the tested period of time. On

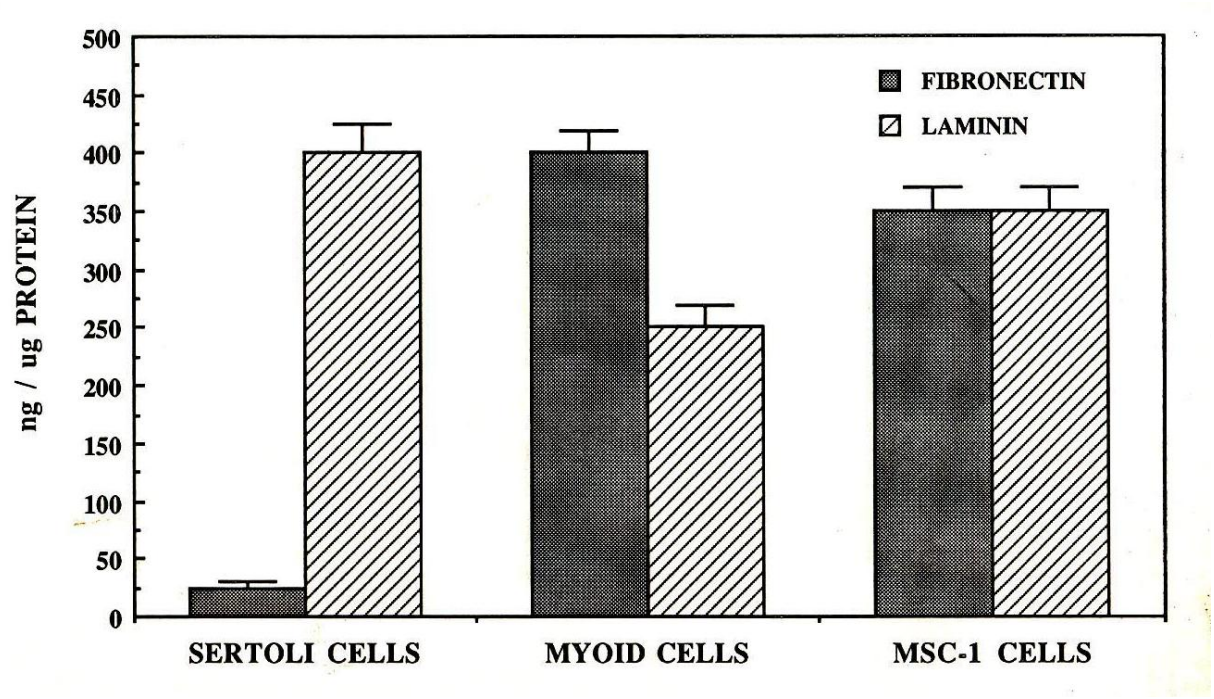

Figure 3: Enzyme-linked Immunosorbent Assay (ELISA). Fibronectin and laminin ELISA were performed on Sertoli cell, peritubular myoid cell and MSC-1 cell conditioned medium. Cells were cultured first for three days in the presence of 5% FBS, and then for next 72 hour in serum-free medium. On day six, media was collected for assay. Data represent ng/μg cell protein and are presented as the mean ± SEM from three separate experiments done in duplicate samples.

day 5 and day 7 Sertoli cells demonstrated significantly increased electrical resistance as compared to the other cell lines.

Discussion

These data clearly show that Sertoli cells from three weeks old rats produce laminin, but negligible amount of fibronectin. Peritubular myoid cells and MSC-1 cells produce both laminin and fibronectin *in vitro*. Hence, immature rat Sertoli cells and MSC-1 cells demonstrate a clear difference in terms of fibronectin production. The difference could result from a number of reasons. MSC-1 cells maintained at 37°C proliferate in culture while Sertoli cells grow *in vitro* in close association with each other have a lower cell proliferation potential. Also when we examined microscopically, the shape of a larger population of MSC-1 cells do not resemble Sertoli cells and that is in agreement with Peschon et al. as previously described [10]. This cell line contained a morphologically heterogeneous cell population consisting of large, epitheloid shaped cells and smaller spindle shaped cells. However, McGuinness et al. [11]

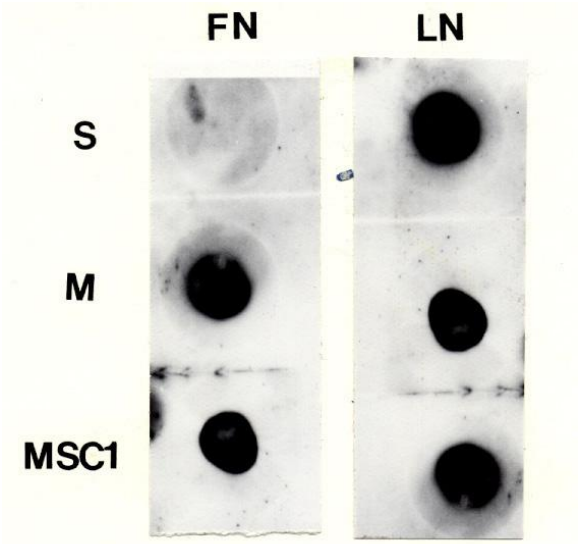

Figure 4: Dot-blot Analysis of fibronectin (FN) and laminin (LN). Equal amounts of total proteins from the conditioned medium of Sertoli cells (S), peritubular myoid cells (M) and MSC-1 (MSC1) cells were spotted on nitrocellulose paper strips. After drying, the strips were probed with either rabbit anti-human FN or rabbit anti-mouse LN at a dilution of 1:100.

Table 1. Comparison of electrical resistance (ohms per cm^2) in Sertoli cells, Peritubular cells and MSC-1 cells maintained in bicameral culture chambers

Culture type	Day 3	Day 5	Day 7
Sertoli cells	42.4 ±1.6	70.1 ± 5.8*	120.5 ± 4.9*
Peritubular cells	10.9 ± 0.6	12.8 ± 0.4	Not detected
MSC-1 cells	27.9 ± 3.1	31.4 ± 2.5	50.4 ± 4.7

* denotes significantly different (P < 0.05) from Day 3 of Sertoli cell cultures (Day 3 versus Day 5 or Day 7).

believed that MSC-1 cells are ultrastucturally similar to cultured Sertoli cells, but both differed in a number of ways from adult Sertoli cells in vivo.

To determine the extent of differences between MSC-1 cells and Sertoli cells, we have observed that MSC-1 cell line overexpressed fibronectin whereas primary cultures of Sertoli cell did not express a detectable level of fibronectin. This may be due to the fact that MSC-1 cells are the testicular tumor cell type from nongerminal cells within the seminiferous tubule. According to McGuinness et al. [11] MSC-1 cells express biochemical markers associated with Sertoli cells including mRNA for androgen binding protein, sulfated glycoprotein-1 and sulfated glycoprotein-2 and transferring, but not the receptor for FSH. However, the level of expression in all the above parameters was less in MSC-1 cells than in cultured Sertoli cells. Again, MSC-1 cells expressed messages associated with cell replication that are not expressed in Sertoli cells [11].

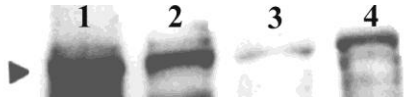

Figure 5: Western blot Analysis **of fibronectin (FN).** Western blot analysis with 10 µg of total soluble proteins from human plasma FN (Lane 1) was used as positive control. A specific band of FN at 220 kDa was observed in peritubular myoid cells (Lane 2) and MSC-1 cells (Lane 4). Sertoli cells (Lane 3) did not express a significant level of FN. The experiment was repeated three times.

Another major difference is the proliferative behavior of MSC-1 cells (the ability of MSC-1 cells to divide), which is not available in adult Sertoli cells. Sertoli cells stop dividing after three weeks of birth [27]. In this study, the MSC-1 cells express fibronectin, a marker normally associated with the peritubular myoid cells. All peritubular cells, including the more highly differentiated myoid cells, express fibronectin [28]. This observation confirms a previous report that fibronectin may be used as a marker of peritubular myoid cell contamination in Sertoli cell cultures [18]. However, FN expression in MSC-1 cells and peritubular myoid cells does support the possibility of a highly dynamic and modified function of secondary cultures or transformed cells, which are different from a stable primary Sertoli cell cultures. For example, another Sertoli cell line, TM-4 cells, have previously been isolated from prepubertal mice, and only limited Sertoli cell functions are expressed in TM-4 cells [29]. The cell line may not represent a true alternative for studying Sertoli cells in primary culture. We are quite aware of the difficulty in comparing primary cultures of Sertoli cells with Sertoli cell lines or even with Sertoli cells *in vivo* [30]. However, the ability of primary cultured cells or cell lines to respond under various experimental conditions does provide insight into our understanding of potential roles of these cells *in vivo*. In the bicameral culture system we have observed that the Sertoli cells produced consistently and significantly higher electrical resistance than the levels achieved by the other cell lines. This clearly demonstrated that the primary culture of Sertoli cells is able to form the monolayer of an effective epithelial cell permeability barrier, which other cell lines were not able to form. More such studies comparing similarities and differences between these cultured cells should help us to understand the

various roles played by these cells in biotechnology research.

During the initial culture period the requirement of serum in promoting cell proliferation seemed to be evident, and the deposition of fibronectin required the presence of fetal bovine serum. Fibronectin has been implicated in the spreading and growth of cells in culture [31-33]. Fibronectin could also be attributed due to deposition of serum FN and the mitogenic factor present in serum. However, a difference in the distribution of cellular fibronectin in primary cultured epithelial cells and transformed cell lines is possible due to varied responses to cytoskeletal organization and cell proliferation. Together, this distribution provides capacity for enormous functional complexity. The ability of FN to bind other growth factors present in the microenvironment (through the presence of serum or ECM) may also modulate cell behavior including cell proliferation and migration [34]. The ECM components produced by these cells (primary, secondary or transformed tumor cells) may also sequester other non-growth factor proteins and thereby modulate signaling pathways [35]. Characterization of these cells under controlled environment and comparing them in terms of production of ECM proteins may provide an emergent area of interest in biotechnology research. Recent advances [36] on the generation of *in vivo* like ECM construct for supporting 3D cell cultures are underway. However, a successful integration of biology still remains challenging, but it is needed to be addressed.

Acknowledgement

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