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Research Article

Regulation of Blood-Testis Barrier (BTB) Proteins in Sertoli-Germ Cell Nanotube Formation in EF-Treated Spermatogenesis

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Abstract

Objective: The aim of this study was to analyze visual data analysis of nanotubes formation and actin expression between Sertoli germ cells, gene silencing with the use of FAK siRNA and EF application, confirmation with mRNA levels, cell viability test and immunofluorescent staining associated ezrin, Fascin 1, FAK and N-cadherin. Expressions of blood-testicular barrier (BTB) proteins were evaluated.

Materials and Methods: As the experimental group of the research; control group (CG), in which intercellular nanotubes and cargo proteins were followed under normal culture conditions; Sertoli and germ cells co-culture; co-culture of testosterone (T) group, Sertoli and germ cells in which intercellular nanotubes and cargo proteins are tracked; The group in which actin organization and intercellular nanotubes and cargo proteins are monitored, and the group in which the focal adhesion kinase is suppressed with siRNA (FAK RNAi) and the co-culture of Sertoli and germ cells, the electromagnetic field applied group (EF), in which intercellular nanotubes and cargo proteins are followed under normal culture conditions, were used.

Results: In the control groups, nanotubes formations started at the 6th hour during the culture and increased at the 40th hour, while the number of nanotubes formation and disappearance was 52 in the Control group; 58 in the EF group; 44, 12 in the FAK RNAi group and 5 in the EF+ FAK RNAi group. It was shown that actin associated nanotubes formations were significantly decreased in FAK RNAi and EF+ FAK RNAi groups compared to control. Stable nanotubes formation rate but low disappearance rate was detected in the EF applied group. It was observed that there was a decrease in ezrin and Fascin 1 expressions in nanotubes formation regions, except for control and testosterone groups, and there was no significant difference in N-cadherin expression levels. It was determined that FAK, Ezrin and Fascin 1 cargo passage were significantly retained in the cytoplasm in the FAK RNAi groups.

Conclusions: With the results we obtained; It has been shown that the FAK molecule has an important role in the germ cell development process *in vitro*. It has been shown that in Sertoli-germ cell co-culture in which FAK gene is silenced and FAK RNAi and EF applied together, vesicle contents cannot be released by endocytosis and these molecules affect nanotubes formation due to decreasing the ratios of FAK, ezrin and Fascin 1 proteins. Based on our results, a research pattern and culture model were proposed for the detection of intercellular signaling due to the passage of regulatory proteins and nanotubes formation.

Keywords: Focal Adhesion Kinase (FAK); Nanotubes; Spermatogenesis; Sertoli-Sertoli and Sertoli-germ

Introduction

The Sertoli cell plays a crucial part in the formation of the male genitalia during both the embryonic and the spermatogenic stages of development [1]. Because of the Sertoli cell is responsible for producing sperm [2]. The number of Sertoli cells that are found in the testes has a direct bearing on the size of the testes as well as the amount of sperm that is produced by those testes. When a person reaches the age of puberty, postnatal Sertoli cells will stop differentiating and multiplying, at which point they will begin to provide sustenance to germ cells. This process will continue until the age of death. Because of this, the quantity of Sertoli cells has an immediate and direct impact on an adult male's fertility.

Endocrine hormones like Follicle-stimulating hormone (FSH), estrogen, and thyroxine, as well as other paracrine growth factors like Insulin-like growth factors I (IGF-I) and Insulin-like growth factors II (IGF-II), Fibroblast growth factors (FGF), somatostatin C, activin, Transforming growth factor (TGF), and Interleukin (IL)-1, are involved in the regulation of cellular proliferation and differentiation (IL) 1, The importance of the exponential growth of the population cannot be emphasized [2–6]. Through the use of mice testicular gene deletion, testicular specific overexpression, and *in vitro* culture of spermatogonial stem cells [7,8], it was demonstrated that GDNF is required for the continued existence of spermatogonial stem cells (SSCs) as well as their capacity to proliferate. Studies have shown that GDNF and Follicle-stimulating hormone (FSH) can promote the proliferation of neonatal rat testicular Sertoli cells in a synergistic manner [9]. On the other hand, studies have shown that GDNF alone can promote the embryonic development of mouse Sertoli cell proliferation [10]. In addition to this, research has demonstrated that GDNF and FSH can stimulate the proliferation of newborn rat testicular Sertoli cells in a synergistic manner [9,10].

Nanotubes are primarily responsible for their development, and actin-derived cytoplasmic membrane protrusions are the primary culprits in this process. Nanotubes permit the passage of cellular cargo as well as signals. In a number of separate studies, it was discovered that nanotubes are the medium via which cancer cells transport retroviruses [11,12], apoptotic signals [13], and calcium signals [14,15]. In contrast to corrugated junctions, it is feasible for it to contact distantly localized cells via a single cell extension in order to generate a tubular cytoplasmic connection. This is because it is able to touch distantly localized cells in order to develop tubular cytoplasmic connection. In addition to this, they are able to keep communication going between cells that are already in close proximity to one another, even if the cells in question are moving in opposite directions [16]. Even though the first observation was made in PC12 cells, non-cancerous cells such as myeloid, dendritic, natural killers and T cells [14,15,17] as well as mesenchymal stromal cells [18] have been extensively studied in the scientific literature. This is the case even though the first observation was made in PC12 cells. Despite the fact that the initial observation was made in PC12 cells, this is the conclusion that may be drawn. The primary human renal epithelial cells used in the study [19] were found to include nanotubes within their cellular architecture. This was discovered in a recent study. Initial research into the ectoplasmic specializations of the blood-testis barrier uncovered the presence of nanotubes between Sertoli cells as a result of the expression of Fascin 1 [20] and ezrin [21]. This was discovered through the expression of Fascin 1. It is difficult to comprehend the function of focal adhesion kinase (FAK) in the microtubular dynamics of the testis and the tubulin-based cytoskeleton all through the seminiferous epithelial cycle in spermatogenesis [22]. The goal of this study is to investigate the regulation of the regulatory proteins of the blood-testicular barrier (BTB) necessary for the achievement of good spermatogenesis in cell communication according to nanotubes formations and disappearance numbers between cells. This is being done so that the researcher can better understand how to achieve good spermatogenesis. These results were obtained from reverse transcriptase polymerase chain reaction (RT-PCR) analyses, in which the FAK gene was suppressed by 80% after being transfected with siRNA pairs using RNAi technology. RT-PCR is a technique that uses polymerase chain reactions to amplify and transcribe genetic material.

Materials and Methods

Materials

The research's experimental groups are as follows:

- **Control group (CG):** Co-culture of Sertoli and germ cells at densities of 0.5 x 106 and 0.05 x 106 cells/ml with nothing added, the group in which intercellular nanotubes and cargo proteins are observed under standard culture conditions.
- **Testosterone group (T):** The group that tracks intercellular nanotubes and cargo proteins.
- **Group in which siRNA inhibited focal adhesion kinase (FAK RNAi):** Co-culture of Sertoli and germ cells at densities of 0.5 x106 and 0.05 x106 cells/ml, in which FAK will be inhibited using RNAi technology; the group that will monitor

actin organization, intercellular nanotubes, and cargo proteins.

- **Electromagnetic field group (EF):** Co-culture of Sertoli and germ cells at densities of 0.5 x 106 and 0.05 x 106 cells/ml with an applied electromagnetic field, the group in which intercellular nanotubes and cargo proteins are observed under normal culture conditions.
- **FAK siRNA inhibits the application of an electromagnetic field (EF+ FAK RNAi):** Co-culture of Sertoli and germ cells at densities of 0.5 x106 and 0.05 x106 cells/ml, in which the FAK to be generated using RNAi technology and the electromagnetic field were applied; group in which intercellular nanotubes and cargo proteins are monitored under normal culture conditions.
- **Sertoli-germ cell culture experiment setup**: The main isolation of Sertoli cells was observed in a culture dish for only seven days. Five baby rat testicles yielded around 2 million Sertoli cells (10 testicles). The addition of testosterone, siRNA transfection (FAK RNAi), and EF+ FAK RNAi steps on day 3 were removed from the control group by sowing germ cells between 1:5 Sertoli cells. The live follow-up was continued for 24 hours, and on the fourth day, data analyses were conducted. In two replicates, 100,000 Sertoli cells per well of 500,000 germ cells in a primary isolation were cultivated in 6-well culture dishes from each group. Nanotubes and blood-testicular barrier regulator proteins are observed in groups with 0.5 x 106 and 0.05 x 106 cell seeding densities, respectively. Five groups utilized around 3 million Sertoli cells and 15 million germ cells. According to common knowledge, a single group can be subjected to three distinct studies with two repetitions in 6-well culture dishes. Simultaneously, cell lysate was collected for PCR studies, and coverslips were utilized for immunofluorescence labeling. This method took a total of five days, and the outcomes of the experiment were followed in tandem with at least three statistical analyses.
- **Fluorescent intensity analyses and imaging of living cells:** Fluorescence intensity measurements of nanotubes and cargo proteins, as well as live recording and viewing of their intercellular transfer, are given by the JuLi FL fluorescent live cell imaging system.
- **Double-label immunofluorescence labelling:** In order to accomplish double-label immunofluorescent labeling, the cultured cells were fixed with a thin, circular coverslip (cover slip) inserted in the culture dishes containing Sertoli cells and germ cells.
- **Reverse transcriptase utilizing polymerase chain reaction (RT-PCR):** Following Sertoli cell and germ cell RNA separation, total RNA was back-transcribed to cDNA using M-MLV back-transcriptase, and cDNAs were reacted with Ezrin, Fascin 1, FAK, and S16 primers.
- **Immunoblot (IB) analyses:** After co-culturing Sertoli cells and germ cells (0.5 x 106), lysates were collected in Eppendorf tubes for each experiment and prepared in sample buffer solution for loading 15 g of protein onto the SDS-PAGE system top gel to visualize FAK responsiveness after loading, electrophoresis, and transfer. Utilizing the FUJI film imaging equipment and a fluorescent luminous chemical on a PVDF membrane, the experiment was conducted.
- **Cytotoxicity tests on cells:** The X-Celligence system (ACEA Biosciences) performed the proliferation, adhesion, and dissemination of cells, real-time cell analysis in all groups with micro-electrodes in a gold-plated 16-well microplate and computed and graphed the cell incidence.
- **Endocytosis testing:** On the day of preparation, 500 g of the amine reagent pHrodoTM Green STP (molecular probes, catalog # 35369) was dissolved in 75 l DMSO to create an 8.9 mM stock solution.
- **Statistical analysis:** Depending on the consistency of the results in each experimental group, the differences between the individual sample means were evaluated using the student's t test, and the differences between the groups were studied using an ANOVA and the necessary Post Hoc tests. It was judged significant when *p0.05.

The results of *in vitro* studies

Morphological studies

- **Quantity and duration of nanotubes development in SC-GC in the control group:** In the control group, particularly for the regulation of all germ cells (GC) with Sertoli cell (SC) dynamics and the execution of migration, the serial, rapid, and regular continuity of nanotubes formations served as a benchmark. Based on this reference, it was established that the nanotubes-mediated insertion of germ cells between SC was substantially faster than when nothing was applied at the 4^{th} and 40^{th} hours.

- In the testosterone group, the number and duration of SC-GC nanotubes development. The pace and structure of nanotubes forms in the testosterone group were comparable to those of the control group, and the density of germ cells per SC was measured. The creation of the SC-GC nanotubes found between clustered GC and SC is depicted. The rate of nanotubes development dropped at a similar pace in the testosterone group based on the number of nanotubes created and lost (Figure 1).
- During the 0–48-hour culture period, the number of nanotubes between SC-GC rose at a similar rate in the control and testosterone groups (Figure 2).
- Parallel to the control and testosterone groups, the number of nanotubes remained consistent and unaffected during the culture (Figure 3).
- Nevertheless, it was found that the dynamics of nanotubes development persisted with a much reduced acceleration in the electromagnetic field (EF) treated group compared to the control and testosterone groups (Figure 4).
- **The number and duration of SC-GC nanotubes development in the FAK RNAi group:** Although the frequency of nanotubes between Sertoli-germ cells was low in groups treated with FAK RNAi, germ cell clusters similarly gathered green cargo vesicles when the transit of siRNA pairs was examined with the siGLO indicator (Figure 5). The FAK RNAi group was shown to have slower germ cell migration.
- **Number and duration of EF+ FAK RNAi SH-GH nanotubes formation:** When the EF effect was applied to cells in conjunction with FAK siRNA, it was discovered that nanotubes forms were greatly reduced and germ cells, with the exception of Sertoli cells with an aggregation propensity, were detected. In the EF+ FAK RNAi group, nanotubes formation was drastically reduced, and the formation rate was irreversibly impaired (Figure 6).
- In groups in which the FAK gene of Sertoli-germ cell culture was silenced, the gene silencing efficiency rate was found to be 80%. In cells tagged with the protein-level phosphorylated version of p-FAK Tyr397, gene silencing was established (Figure 7).
- **Examining the strength of actin expression:** Intergroup variations in actin architecture, nanotubes formation, and cargo transport were observed in a co-culture of 0.5×10^6 SC-GC cells. While it was demonstrated that the nanotubes structures remained incomplete prior to formation, particularly in the FAK RNAi groups, it was found that they were long and ready to be used as bridges that can transfer their contents in the control and Testosterone groups, and that they remained short and stable in the EF group (Figure 8).
- **The location of ezrin and N-cadherin expressions intercellularly:** In the SC-GC culture with a low concentration of tesotsterone compared to the control, tesotsterone group, the expression of actin binding protein in cell-cell interactions decreased, and nanotubes formations were interrupted in the FAK RNAi and EF+ FAK RNAi groups during intercellular cargo transfer (Figure 9).
- **Endocytosis testing:** Protein emission acquired by labeling the cargo proteins transported into the cell by endocytosis revealed Fascin 1 (Figure 10a), FAK (Figure 10b), and ezrin (Figure 10c) proteins in the control and testosterone groups at 0 minutes, 15 minutes, 30 minutes, and 60 minutes. While its cytoplasmic distribution was shown to be high, its distribution was low in the EF, FAK RNAi, and EF+ FAK RNAi groups.
- **Evaluation of the morphology of germ cell development in co-culture with Sertoli cells:** In the control and testosterone groups, spermatocytes with big nuclei were observed to form, whereas in the FAK RNAi and EF+ FAK RNAi groups, spermatocytes retained their spherical sparmatid shape. In contrast, the EF group displayed less large-nucleated germ cell shape and spherical spermatid-like germ cells than the control and testosterone groups. Statistical analysis and classification of spermatocyte-like and round spermatid-like germ cells revealed the quantity of germ cells (Figure 11).

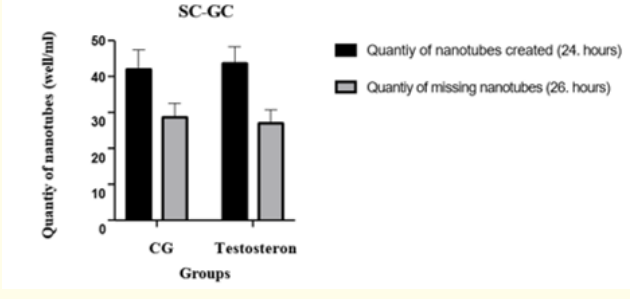

Figure 1: Analysis of the formed and lost nanotubes structures in the control and testosterone groups between 24. and 26. Hours.

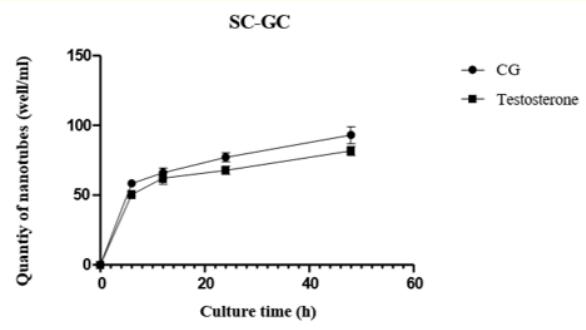

Figure 2: The number of SC-GC-specific nanotubes forms between the control and testosterone groups.

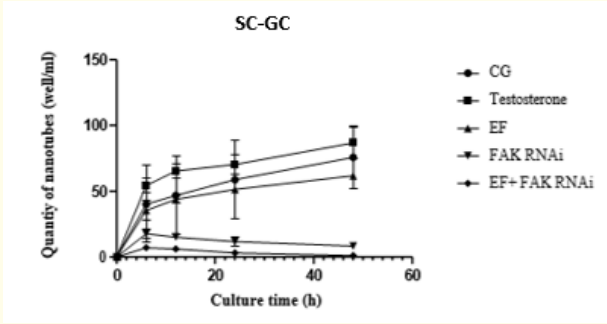

Figure 5: The number of nanotubes formations between SC-GC in all groups.

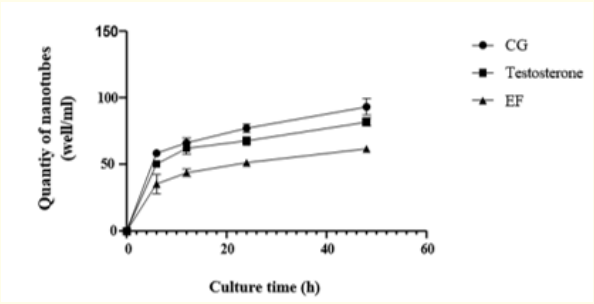

Figure 3: Count of SC-GC nanotubes forms unique to the control, testosterone, and EF groups.

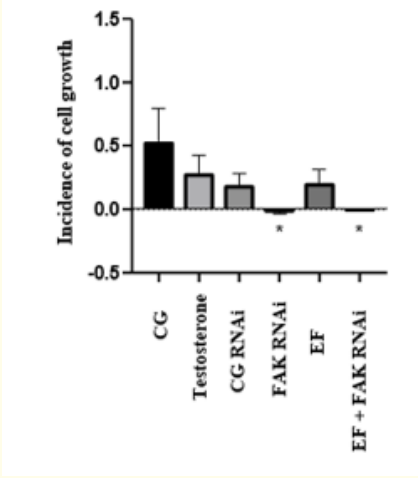

Figure 6: Evaluation of the incidence of germ cell proliferation by group in SC-GC co-culture.

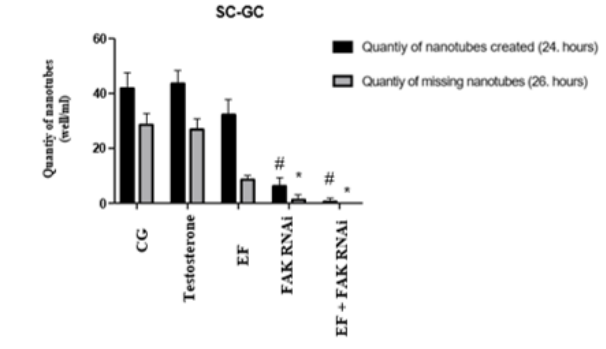

Figure 4: Analysis of produced and lost nanotubes structures in all groups between 24 and 26 hours.*, # $p < 0.05$.

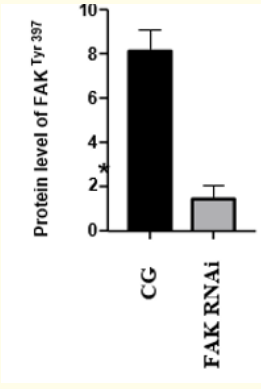

Figure 7: Detection of p-FAKTyr-397 protein levels in the FAK gene silencing group by immunoblotting following RNAi transfection.

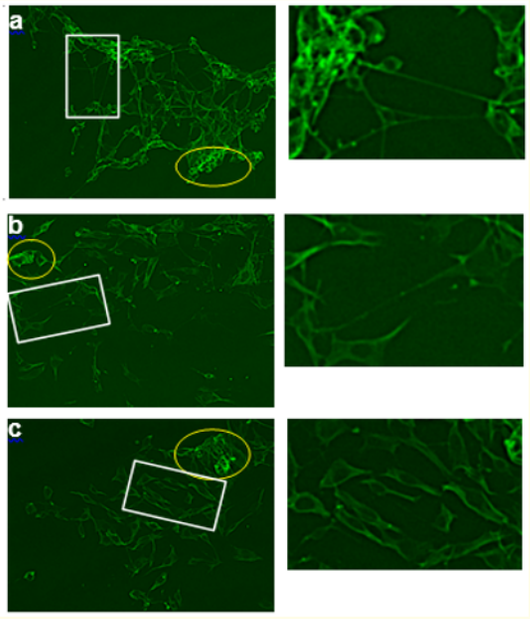

Figure 8: Nanotubes creation and actin expression. Observation groups selected for cargo transit: (a) Control, (b) EF, and (c) FAK RNAi. (The micrographs on the right were taken at a magnification 10 times that of the designated location).

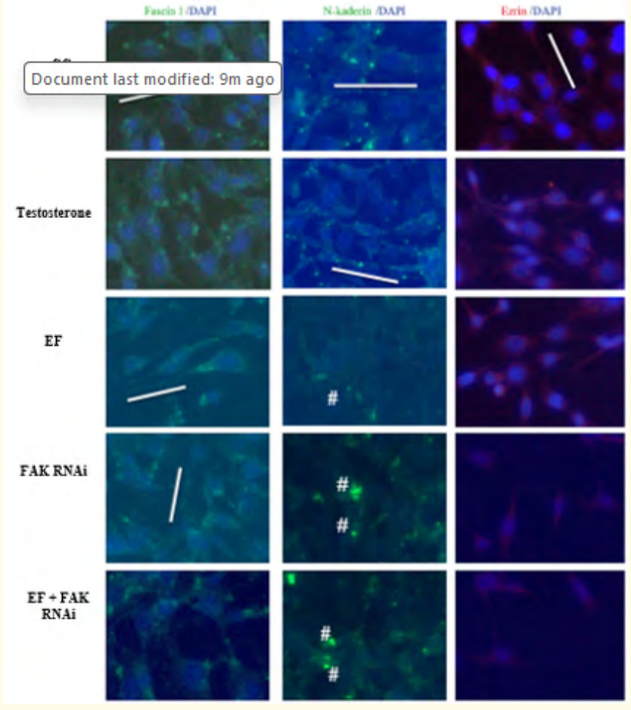

Figure 9: Fascin 1, N-cadherin, and Ezrin proteins are expressed within cargo channels and intercellular nanotubes. Cell density 0.5 x 106 (1:4). Fascin 1 and N-cadherin (green), ezrin (red), and the nuclear envelope (blue). # indicates clustered germ cell areas, with nanotubes between cells.

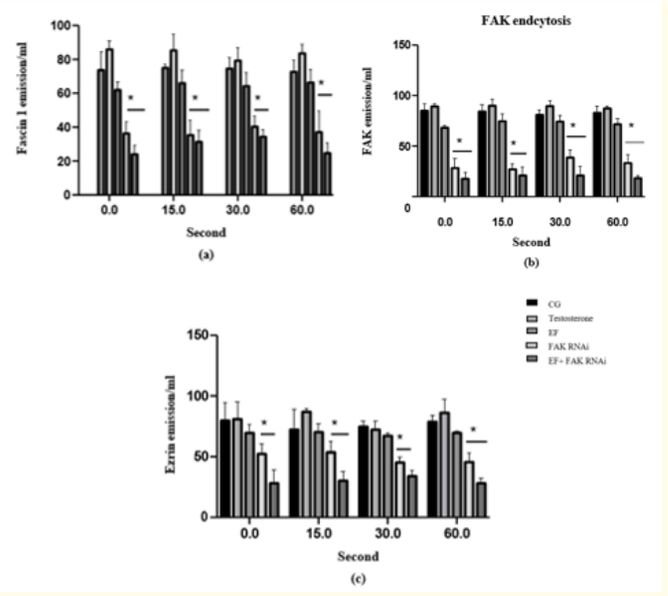

Figure 10: Endocytosis testing (a) Fascin 1, (b) FAK, (c) Ezrin.

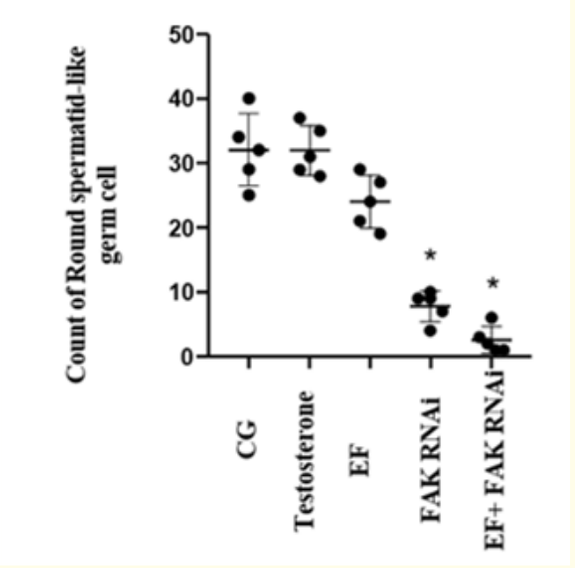

Figure 11: Quantitative comparison of germ cells between groups. The statistical ratio of round spermatid count in the graph was determined by separately counting 100 locations for each group (*; $p < 0.05$).

Discussion

The results of our study showed that the rate of SC-GC nanotubing decreased significantly in the absence of FAK, under the influence of FAK genes, and under the influence of EF. The rate of cell-cell ezrin and Fascin 1 communication also decreased because there were no nanotubes structures. In this process, these nanotubes

structures help Sertoli cells keep their germ cells alive. It was seen that when ezrin and Fascin 1 expressions went down, so did the germ cell uptake potential. Based on this information, it has been shown that the FAK molecule is important for the development of germ cells in a lab dish. It has been shown that when the FAK gene is turned off and FAK RNAi and EF are both used on Sertoli-germ cell co-cultures, the contents of vesicles cannot be released by endocytosis. This is because these molecules change the ratios of FAK, ezrin, and Fascin 1 proteins, which prevents nanotubes from forming. The traffic between cells depends on these nanotubes, and it shows that the inducer FAK gene plays an important role in the development of Sertoli-Sertoli and Sertoli-germ cells. It has been shown that when ezrin and Fascin 1 proteins don't work, FAK proteins that turn on target BTB regulator proteins mess up where they are in the cell. Because of these things, how the formation of FAK-associated F-actin affects the formation of nanotubes Sertoli-germ cells may be an important way for the *in vitro* development process to protect itself.

Ezrin and Fascin 1 proteins, which are known to have positive effects on *in vitro* development potential in the spermatogenesis process and which we have shown to have these effects in our studies, cannot have these effects on nanotubes formation and F-actin regulation in cells where FAK genes have been turned off.

It has been found that an electric field can move actin filaments because of how they are made. Different effects happen depending on what kind of surface is used. It has been found that ions move around the actin filaments and along them [24]. In this case, changes in the electrical charge of actin filaments, which can be caused by magnetic or electrical fields, will also change how the ions in the area move and cause metabolic changes in cells. By affecting each other, the way ions move through ion channels and along actin filaments controls how cells are put together inside and between cells [25].

In the testosterone-treated cells in which the FAK gene was silenced along with EF, the protein density in the cytoplasm, which was lowered due to involvement/entrapment in the vesicle content, was shown to have grown dramatically. In addition, it has been demonstrated that N-cadherin expression is lowered in cells in which the FAK gene is silenced, but stays stable when simply EF is applied. However, EF treatment had no influence on irregular actin and nanotubes development in the group in which the FAK gene was silenced, and it caused a significant drop in the formation and disappearance rate of natural tunnels. All of these data demonstrate that EF acts independently of the FAK protein and cannot do this activity without it. It demonstrates that EF alone can be a crucial regulator in the spermatogenesis pathway. In the normal process, the stabilization of the nanotubes and the continuity of F-actin expression are maintained under the influence of FAK. The decrease in ezrin, Fascin 1, and N-cadherin expressions in the absence of FAK suggests that FAK regulates the signaling pathway by providing feedback to ezrin, Fascin 1, and N-cadherin proteins. makes you think [20,22]. In addition, it demonstrates that the enhanced germ cell agglutination in FAK gene silencing cells cannot be regulated in the presence of EF, and that EF inhibits the synthesis of actin and nanotubes during spermatogenesis via the FAK mechanism. In the process of nanotubes formation, which can occur *in vitro* during the spermatogenesis process and is related to orientation, it can be concluded that EF reflects the equivalent development process with control and testosterone based on the principle of working together while the FAK gene is present. Due to the combined activities of these actin bundling proteins, the actin-based cytoskeleton at the respective places, namely the "old" and "new" BTB, undergoes modifications. As recently shown [26,27], reorganization of actin microfilaments at the "old" BTB also enhances endocytic vesicle-mediated trafficking events, allowing "old" integral membrane proteins to be swiftly endocytosed, transcytosed, and recycled to the "new" BTB. The combination of these alterations destabilizes the "old" BTB, allowing preleptotene spermatocytes to pass the barrier. At the "new" BTB, newly recycled integral membrane proteins and their related peripheral proteins are employed to construct the barrier behind the transiting preleptotene spermatocytes. As the "new" BTB is constructed behind these germ cells, the breakdown of the "old" BTB above the preleptotene spermatocytes in transit does not result in a rupture of the immunological barrier. On the other hand, it has been demonstrated that the FAK gene plays a pivotal role, that EF and the damage to this signaling pathway cannot be repaired independently, and that actin stabilization cannot be performed without FAK. p-FAK-Tyr407 is known to support BTB integrity, such as the assembly of the "new" BTB, whereas p-FAK-Tyr397 promotes BTB restructuring, such as the disruption of the "old" BTB, according to research [28,29]. Together with other protein kinases [30-32], these two forms of FAK serve as molecular switches to induce intrinsic activities of the relevant actin regulatory proteins via phosphorylation.

In accordance with all these findings, it has been shown that the action of EF on the PI3K/c-Src/FAK signaling pathway during

spermatogenesis does not damage the mechanism of external actin and nanotubes production, but rather "stabilizes" it. It has also been demonstrated that the EF effect has no appreciable influence on the protein expressions involved in the regulation of BTB *in vitro*, and it is recommended that the EF effect can be employed as an active application approach in the *in vitro* actin mechanism. It has been demonstrated that interruption of the FAK-mediated signaling pathway during *in vitro* spermatogenesis is not caused by EF and cannot enhance the production rate and direction of nanotubes. Our results highlighted the significance of Sertoli-germ cell signaling and nanotubes-mediated protein traffic in the *in vitro* spermatogenesis development process and demonstrated that EF application, which is known to affect polarity-dependent actin, and *in vitro* signaling traffic affect the rate of nanotubes formation. This is significant because it sheds light on the potential for actin, ezrin, and Fascin 1 to act via the mechanism and advances *in vitro* germ cell survival and development.

Conclusion

This study introduces a novel cell-specific model to explore molecular mechanisms of Sertoli cell–germ cell interactions, specifically addressing the understudied role of intercellular nanotubes and cargo transfer in spermatogenesis. Using electromagnetic fields combined with siRNA-mediated silencing of key cytoskeletal and regulatory proteins (Ezrin, Fascin 1, N-cadherin), we reveal new insights into cellular communication during spermatogenic processes. Experiments were performed with [X] biological replicates per condition, and sample sizes were determined based on [rationale, e.g., power analysis or precedent in related studies]. Although this *in vitro* model allows detailed mechanistic studies under controlled conditions, its generalizability to *in vivo* spermatogenesis is limited by the absence of complex tissue architecture and systemic influences. Nonetheless, this platform provides a valuable tool for advancing our understanding of spermatogenic regulation and supports further *in vivo* research.

Ethical Approval

Twenty-weeks-old male wild-type rat pups were obtained from Istanbul University Animal Research Unit with approval number: 35980450 and rats were housed under temperature and light-controlled conditions with ad libitum access to food.

Authors' Contributions

None

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