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Embryonic microenvironment suppresses YY1 and YY1-related genes in prostate cancer stem cells

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A B S T R A C T

Yin yang 1 (YY1), a transcription factor, plays crucial roles in cell fate specification, differentiation, and pluripotency during embryonic development. It is also involved in tumorigenesis, drug resistance, metastasis, and relapse caused by cancer stem cells (CSCs), particularly in prostate cancer (PCa). Targeting YY1 could potentially eliminate prostate CSCs (PCSCs) and provide novel therapeutic approaches. PCa tissues often exhibit elevated YY1 expression levels, especially in high-grade cases. Notably, high-grade PCa tissues from 58 PCa patients and $CD133^{high}/CD44^{high}$ PCSCs isolated from DU145 PCa cell line by FACS both showed significantly increased YY1 expression as observed through immunofluorescence staining, respectively. To investigate the embryonic microenvironment impact on YY1 expression in CSC populations, firstly PCSCs were microinjected into the inner cell mass of blastocysts and then PCSCs were co-cultured with blastocysts. Next Generation Sequencing was used to analyze alterations in YY1 and related gene expressions. Interestingly, exposure to the embryonic microenvironment significantly reduced the expressions of YY1, YY2, and other relevant genes in PCSCs. These findings emphasize the tumor-suppressing effects of the embryonic environment by downregulating YY1 and YY1-related genes in PCSCs, thus providing promising strategies for PCa therapy. Through elucidating the mechanisms involved in embryonic reprogramming and its effects on YY1 expression, this research offers opportunities for

Abbreviations: CSCs, cancer stem cells; HESCs, Human embryonic stem cells; ICM, inner cell mass; ICSI, intracytoplasmic sperm injection; PCa, prostate cancer; PCSCs, prostate CSCs; TF, transcription factor; YY1, Yin yang 1.

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further investigation into focused therapies directed against PCSCs, therefore enhancing the outcomes of PCa therapy. As a result, PCa tumors may benefit from YY1 and associated genes as a novel therapeutic target.

1. Introduction

In terms of all cancer types, prostate cancer (PCa) ranks as the second most common in men and the fourth most common overall. While the survival rate for PCa patients diagnosed at localized and early stages is 80–85 %, this rate drops to 28 % for patients with metastases and late-stage tumors. According to the American Cancer Society in 2023, the likelihood of recurrence in treated patients within 5 years is 20–30 % [1]. Despite various conventional treatment methods such as chemotherapy, radiotherapy, and hormonal therapy, the main cause of this recurrence is attributed to cancer stem cells (CSCs), which represent a small subset of cells within heterogeneous solid tumors that possess the ability to self-renew and differentiate. CSCs drive tumor initiation, maintenance, migration, recurrence, plasticity, and drug resistance leading to cancer progress independently of treatment [2]. CSCs can originate from genetic alterations in normal stem cells, the process of dedifferentiation in specialized cancer cells, or the fusion of cells. The CSC hypothesis, which elucidates the hierarchical arrangement of malignancies, posits that these cells are accountable for the diversity within tumors. CSCs are considered the initiation point of tumorigenesis, making targeting CSCs in cancer therapy a promising approach for effective therapies [3,4]. They are identified using biomarkers such as CD44, CD133, and ALDH1 [5]. Therapeutic methods involve focusing on biomarkers, blocking pathways promoting renewal, implementing treatments encouraging cell differentiation, and utilizing techniques that harness the immune system. Current research focuses on understanding the molecular mechanisms, biomarkers, and clinical treatment trials of CSCs, with future directions emphasizing combination therapies, personalized medicine, and strategies involving the tumor microenvironment. The cancer disease has a diverse etiology, and its molecular mechanisms are not yet fully understood. It is of great importance to have a better understanding of CSC molecular signaling mechanisms and to identify and target molecules that serve as initiators or inhibitors of cancer for targeted PCa therapy.

Yin yang 1 (YY1), a transcription factor (TF) expressed during various stages of embryonic development, is closely associated with tumorigenesis, drug resistance, metastasis, and relapse caused by CSCs in adult tissues. It also plays important functions in regulating cell fate specification, differentiation, and pluripotency [6]. YY1 is a significant therapeutic target for identifying prostate CSCs (PCSCs) and metastasis, as it is typically overexpressed in PCa tissues compared to normal tissues [7]. As a dual gene regulator, YY1 acts as an activator and an transcription inhibitor. It is involved in regulating normal stem cells, such as organogenesis and tissue regeneration, and the regulation of CSCs, including metastasis, therapy resistance, and recurrence [8]. YY1 is upregulated in various types of cancer, including bladder cancer [9], breast cancer [10], cervix cancer [11], colon cancer [12], gastric cancer [13], glioma [14], Hodgkin lymphoma [15], leukemia [16], liver cancer [17], lung cancer [18], melanoma [19], osteosarcoma [20], ovary cancer [21], pancreas cancer [22], prostate cancer [3], renal cancer [23], thyroid cancer [24], testicular seminoma [25]. Manipulating YY1 in cancer may provide a promising targeted approach for new treatment strategies.

In prostate cancer, the role of YY1 is two-sided and depends on the tumorigenic potential of the cells. For DU145 PCa cells, which have moderate tumorigenicity and lack sensitivity to androgen receptors, YY1 exerts several tumor-supporting effects. These effects include the suppression of tumor factor X-linked inhibitor of apoptosis-associated factor-1 [26], tumor suppressive miR-146a [27], and heterogeneous nuclear ribonucleoprotein M [3], all of which contribute to tumor growth, migration, and invasion. On the other hand, in LnCap PCa cells, which are low tumorigenic but sensitive to androgen-receptor, YY1 plays a hindering role by repressing MMP2, MMP3, and MMP9, which are involved in invasion and migration [28]. PC3 PCa cells, known for their highly tumorigenic and metastatic behaviors, exhibited an elevated nuclear expression pattern of YY1, which disrupted the Fas signaling pathway, leading to apoptosis [29]. In a study by Arum Park et al., the expression of YY1 was inhibited in DU145, LnCAP, and PC3 cells through treatment with NP-001, a YY1 inhibitor [30]. Given the dual roles of YY1 in prostate cancer, our objective was to examine the expression patterns of YY1 in DU145 cells within the context of embryonic reprogramming.

The "embryo" possesses significant reprogramming capabilities, and the disordered environment during early embryonic development gradually adopts a stable arrangement by regulating diverse genetic, epigenetic, molecular, transcriptional, and signaling networks [31]. The nonpermissive nature of the embryonic environment for tumor growth is widely recognized since it has a distinctive capability to reprogram and counteract tumorigenicity[32]. Human embryonic stem cells (hESCs), obtained from the inner cell mass of blastocysts, are a kind of stem cell that can develop into any cell type in the human body[33]. During this phase, there exists a resemblance in the behavioral patterns between ESCs and the embryonic environment, as well as tumor cells. These shared characteristics include cell invasion, cell migration, activation of signaling pathways, maintenance of pluripotency, and regulation of gene expression crucial for self-renewal and differentiation, among others. The characteristics of cancer progression and embryonic development share striking similarities, particularly when examined through the lens of CSC biology. Both processes involve cells that exhibit high plasticity, self-renewal capacity, and the ability to differentiate into multiple cell types. In embryonic development, stem cells differentiate in a controlled manner to form the various tissues and organs of the body. Similarly, CSCs within tumors can differentiate into diverse cell types that constitute the heterogeneous cell populations of a cancer [34]. The Wnt/β-catenin pathway regulates cell fate decisions, proliferation, and differentiation in both contexts. Similarly, the Notch pathway influences cell differentiation during embryogenesis and helps maintain the undifferentiated state and self-renewal capabilities of CSCs. The Hedgehog pathway, essential for pattern formation and organogenesis in embryos, also plays a significant role in the self-renewal and proliferation of CSCs [35]. These shared pathways underscore the parallels between normal developmental processes and cancer progression, providing insights into potential therapeutic targets. In embryonic development and cancer progression, the microenvironment, or "niche," plays a critical role in regulating stem cell behavior. During embryogenesis, the niche provides essential signals that ensure proper development and differentiation of ESCs [36]. Similarly, CSCs reside in a specialized niche within the tumor microenvironment, which supplies cues to maintain their stem-like properties, promote survival, and enhance resistance to therapies. This parallel highlight the importance of the microenvironment in supporting stem cell functions in both normal development and cancer. Identifying shared characteristics between cancer progression and the shift to the fetal stage suggests that the embryonic environment may play a significant role in reprogramming cancer cells [37,38]. The co-culture systems evaluate the reprogramming activity between two distinct cellular components. With its strong anticancer effects and ability to stop tumor growth in prostate cancer, it provided a new and successful way to use hESCs in cancer treatment [39]. The therapeutic potential of reprogramming cancer cells can be explored through the interactions between tumor cells and an embryonic environment. Significant parallels were discovered while examining embryonic development and the biology of cancer (stem) cells.

However, what occurs is that while certain cells follow their regular path to create healthy human tissues, other cells, known as CSCs, also initiate the development of cancer. Can cancer cells be redirected towards a benign state by exposing them to the micro-environments that embryonic cells are exposed to? The literature still needs to provide clear solutions to these questions. Treating and examining cancer cells using embryonic cells, secretions, and/or microenvironments produced by these cells is necessary to address these inquiries.

Deepening our understanding of the intersections between CSC biology and embryonic development provides novel perspectives on treatment strategies. In this regard, targeting YY1, which plays key roles in the "cancer-stem cell-embryonic development" axis, may offer new tools for prostate cancer treatment. Based on this fundamental concept, we hypothesized that embryonic reprogramming influences the expression of YY1, which is linked to the prognosis of PCa. This study aimed to evaluate the YY1 expression levels in PCSCs and examine how the embryonic microenvironment influences changes in YY1 expression, as well as the expression of genes associated with YY1 in CSC populations capable of metastasis.

Our findings have indicated that PCSCs, believed to contribute to the poor prognosis of PCa, exhibit higher levels of YY1 expression compared to the bulk population containing non-CSCs. To investigate the potential for reprogramming the embryonic microenvironment, two distinct approaches were utilized for $CD133^{+}/CD44^{+}$ PCSCs: (i) microinjection of PCSCs into the inner cell mass (ICM) of the blastocyst using the intracytoplasmic sperm injection (ICSI) method and (ii) co-culture of PCSCs with the blastocyst. Transcriptome analysis of PCSCs using Next Generation Sequencing revealed changes in the expression of YY1 and YY1-related genes, suggesting a down-regulation effect.

2. Materials and methods

2.1. Cell culture

The human PCa cell line DU145 and the human normal prostate epithelial cell line RWPE-1 were provided by The American Type Culture Collection (ATCC, USA). DU145 cells were cultured in Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12; Biological Industries Ltd., Israel) containing 10 % heat-inactivated Fetal Bovine Serum (FBS; Gibco, Invitrogen Life Technologies, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, USA) in 25 cm^2 polystyrene flasks (Corning Life Sciences, UK). RWPE-1 cells were grown in keratinocyte serum-free medium containing L-glutamine, 5 ng/ml of human recombinant epidermal growth factor, 0.05 mg/ml of bovine pituitary extract (Life Technologies, UK), and 1 % penicillin-streptomycin. Cells were incubated and harvested when 80 % confluency was reached with 0.05 % trypsin (Sigma-Aldrich, USA). The culture medium was used to inactivate the trypsin enzyme before the cells were collected, centrifuged at 500 g for five minutes, and resuspended in the culture medium.

2.2. Cancer stem cell isolation by fluorescence-activated cell sorting (FACS)

Flasks with 80 % cell confluency were harvested by non-enzymatic cell dissociation solution (Sigma-Aldrich, USA) and resuspended in Dulbecco's phosphate-buffered saline (DPBS, Invitrogen, USA). 1×10^{6} cells were treated with CD133 and CD44 antibodies diluted to 1:100 in FACS wash solution (0.5 % bovine serum albumin, 2 mM NaN_3, and 5 mM ethylenediaminetetraacetic acid; Miltenyi Biotec, UK) for 15 min at 4°C. An isotype and concentration-matched phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-labeled control antibody were used, and the tested samples were PE-labeled CD133/1 and FITC-labeled CD44. After 3–5 min, the cells were resuspended after being cleaned with the FACS wash solution. The cells were sorted into $CD133^{+}/CD44^{+}$ population (sorting cells: CSCs) and non-sorting counterparts (bulk population: non-CSCs) by FACSAria flow cytometer (BD Biosciences, USA). Sorting cells were then grown in a serum-free medium to form spheroids and marked for further experimental usage.

2.3. Morphological analysis of PCSCs by spheroid formation assay

PCSCs' capacity to form spheroids was assessed in 3D non-adherent culture conditions. 10^4 cells/well in a 6-well plate pre-coated with a thin layer of 3 % agar (w/v) (Difco Laboratories, Inc.; BD Diagnostic Systems, Detroit MI, USA) in DMEM-F12 containing 10 % FBS were grown as a monolayer and incubated at 37°C. Fresh medium was added to the culture media every two to three days to eliminate cellular waste and poorly formed spheroids. The multicellular tumor spheroids were photographed on day 7 under a microscope (Olympus BX-51; Olympus, Germany) when the spheroid formation occurred to validate the presence of CSCs, as these cells can form spheroids in three-dimensional culture systems.

2.4. Histopathological analysis

The low- and high-grade prostate cancer tissue samples were obtained and fixed in a formalin solution for about 3 days. Subsequently, the tissues were embedded in paraffin blocks and sectioned into slices with a thickness of 5 μm. The FFPE slices underwent H&E staining for histological analysis and were visualized under an Olympus BX-51 microscope (Olympus, Japan).

2.5. Immunohistochemistry

YY1 staining in low- and high-grade tumor tissues obtained from patients diagnosed with PCa was performed using a rabbit anti-YY1 antibody. Tissues fixed with 4 % paraformaldehyde (PFA), embedded in paraffin blocks, and sectioned (5 μm). Sections were deparaffinized with xylene, passed through decreasing alcohol series, and washed with water and PBS. Antigen retrieval was made in citrate buffer (pH:6) for 30 minutes and washed with PBS. The tissues were drawn with DakoPen, and endogenous peroxidase activity was inhibited in a 3 % H_2O_2 solution for 15 min. Sections were left in block solution for 30 min to inhibit non-specific binding. Primary antibody (sc-7341, Santa Cruz, 1/100 diluted) was added and kept in a humid environment at +4°C overnight. Following washing, the sections were incubated with a biotin-labeled secondary antibody for 30 min, streptavidin peroxidase enzyme solution for 30 min, and washed with PBS at room temperature. DAB was added for 10 min and removed with distilled water. Counterstaining was made with Mayer's Hematoxylin, mounted with a mounting medium, and analyzed under an Olympus BX-51 microscope. ImageJ software was used to evaluate the YY1 staining intensity in tumor tissues. The first step was color deconvolution, and then the YY1 staining intensity was measured in the deconvoluted DAB image. For each case, 10 areas were randomly selected using a standard-sized circle, and the region of interest (ROI) was measured in each area.

2.6. Immunofluorescence staining

RWPE-1 normal prostate epithelial cells, $CD133^{+high}/CD44^{+high}$ prostate CSCs, and non-CSCs were fixed in 4 % PFA for 15 min, treated with 0.1 % Triton X-100 for 10 min at room temperature for cell permeabilization, and blocked with 5 % bovine serum albumin for 1 h. Cells were incubated with YY1 antibody (sc-7341, Santa Cruz, 1/100 diluted) and Ki-67 antibody (ab16667, Abcam, 1/100 diluted), and tumor spheroids were incubated with CD133 antibody (ab19898, Abcam, 1/100 diluted) overnight at 4°C, treated with FITC-conjugated secondary antibody for 1 h at room temperature and counterstained with DAPI. Fluorescence staining was visualized by a fluorescence microscope (Olympus-BX51, Olympus Corporation, Japan). ImageJ software was used to assess the mean fluorescence intensity within a cell. Three images were taken for each group, and at least 30 cells were analyzed.

2.7. Oocyte collection and blastocyst obtention

All experimental methods have received approval from the Ege University Ethics Committee for Animal Experiments (2015–024) and adhere to the norms. CD1 mice were purchased from Kobay Experimental Animals Laboratory (Kobay DHL A.Ş., Ankara, Turkey). 5 IU pregnant mare serum gonadotropin (PMSG) was administered intraperitoneally to induce superovulation in female CD1 mice housed in a 14-hour light and 10-hour dark cycle. This step was followed by the application of 5 IU hCG in Pregnyl solution (Organon, USA), and each hCG-treated female mouse was housed in individual cages with a CD1 breeding male mouse. Whether or not mating occurred was evaluated according to the presence of vaginal plugs the next morning. Female mice with vaginal plugs were separated for embryo isolation. The females were euthanized by cervical dislocation after anesthetization of 10 mg/kg xylazine (Bayer, Germany) and 60 mg/kg ketamine hydrochloride (Parke-Davis, USA) combined intramuscularly approximately 46 h after mating applied according to the Anesthesia for Laboratory Animals Guidelines of University of Oregon. The ampulla part of the oviduct, containing the embryos at the two-cell stage, was flushed in 30 mm Petri dishes (Nunc, USA) with a thin injector tip, allowing the embryos to pass into the external medium heated to 37^0 C (Sage, USA). The embryos were transferred into an embryo culture medium and incubated (Sage, USA) to reach the early blastocyst stage for microinjection.

2.8. PCSCs injection into ICM of blastocyst by intracytoplasmic sperm injection method

The embryos at the blastocyst stage were transferred to the external medium (Sage, USA) at 37°C, then transferred to the blastocyst culture medium (G2 plus, Vitrolife, Sweden), and incubated in 37 C°, 5 % CO_2, 95 % humidity conditions (New Brunswick, USA). One hour later, 5 μl of high-viscosity medium (PVP, Vitrolife, Sweden) was added to the external medium (G-Mops Plus, Vitrolife, Sweden) on the micromanipulation petri dish (60×60 mm Nunc, Thermo Scientific, USA). The drops were covered with 3 ml of gassed mineral oil (Ovoil, Vitrolife, Sweden). After equilibration at 37°C, an embryo was transferred to each drop, and the PCSC suspension was added to an empty medium drop. The micromanipulation of 100 PCSCs was applied to each embryo by inoculating from 3 o′clock with the help of the holding pipette with an inner diameter of 10 μm (Swemed, Sweden) into the inner cell mass located at 9 o′clock. Cell inoculation was performed into the inner cell mass of >80 embryos. After micromanipulation, the embryos were washed and incubated for 2 days in a blastocyst culture medium (G2 plus, Vitrolife, Sweden) at 37 C°, 5 % CO_2, and 95 % humidity incubator (New Brunswick, USA). Embryos were checked for viability at 24 and 48 h for a high viability rate (70–80 %) and then placed in RNAlater at +4°C.

2.9. Co-culture of blastocysts and PCSCs

60 embryos and 100,000 PCSCs per well were co-cultured in an embryo culture medium and incubated at 37 °C, 5 % CO_2, and 95 % humidity incubator. After the embryos reached the blastocyst stage, the incubation was continued for 2 more days. The embryos and PCSCs were collected separately and placed in RNAlater at +4°C.

2.10. Transcriptome analysis by Next generation sequencing (NGS)

For total RNA isolation, the miRNeasy RNA isolation kit (Qiagen, ABD) was used according to the manufacturer's instructions. RNA was quantified with Qubit RNA-High Sensitivity (HS) Kit (Life Technologies, ABD), and cDNA was synthesized with SMARTer Stranded Total RNA-Seq Kit-Pico Input Mammalian (ClonTech Laboratories, ABD). The Kapa Library Quantification Kit (KapaBiosystems, ABD) was used for library quantification. The pre-diluted 6 DNA Standard, which represents a series of dilutions of the 452 bp dsDNA fragment, together with appropriately diluted library samples and primers based on Illumina® P5 and P7 flow cell oligo sequences, were amplified using qPCR with the KAPA SYBR® FAST QPCR Master Mix. A standard curve is created by plotting the average Cq value for each DNA Standard against the logarithm of its concentration in picomolar (pM). The average Cq values for diluted library samples were converted to concentration using the standard curve. Subsequently, a dimension adjustment was computed to account for the disparities between the length of the DNA Standard and the average length of fragments in the libraries. Ultimately, the concentration of each library's diluted or working solution is determined. The outputs were analyzed using the NGS system. PCR assays were performed for the analyzed genes to validate the transcriptome data.

After the microinjection of PCSCs into the blastocyst, gene expression changes in the blastocyst were excluded from the whole genome data. Changes occurring only in PCSCs after microinjection were compared with untreated PCSCs isolated by FACS. In the coculture experimental group, gene expressions in PCSCs collected after coculture were compared with untreated PCSCs isolated by FACS.

2.11. Bioinformatics

An average of 32,710,045 reads were obtained for each sample during the sequencing process. The FASTQC (Babraham Institute, UK) program was used to determine the quality of the obtained readings. The adapters detected in the readings were cleaned using the Trimmomatic (Aachen University, Germany) program. The reads were then mapped to the human reference genome (version hg38). The STAR (Cold Spring Harbor Laboratory, USA) program matched the readings with a two-stage matching mode and standard parameters. In the first step, reads were mapped to the human reference genome using Ensembl gene expressions (version 83). New isoforms and exons formed in this coupling stage were identified and re-paired by adding them to the Ensembl definitions in the second stage. For differential comparison of miRNA expressions, reads were matched to the same human reference using the Kallisto (California Institute of Technology, USA) program. The values, normalized to the total number of reads, were calculated by summing the isoforms of each gene. The calculated expressions were then converted to a single gene expression value for the genes by using the Transcripts Per Million method.

2.12. Database

STRING (https://www.string-db.org/) database predicted the protein-protein interaction network of Homo sapiens YY1- and YY2-related genes.

2.13. Statistical analysis

Data were analyzed in GraphPad Prism (GraphPad Software Inc.). The normality of data distributions was verified with Shapiro–Wilk tests. Differences between group means were analyzed using one, two, or three-way mixed model analysis of variation (ANOVA) with Tukey's multiple comparisons adjustment, followed by pairwise post hoc comparisons using a two-tailed student's *t*-test. In addition, a student's t-test was performed for ROIs digital counts. In all experiments, differences were considered significant when a p-value < 0.05 was obtained.

3. Results

3.1. YY1 expression is higher in high-grade prostate cancer tissues

Histopathological analysis of low- and high-grade tissue samples from prostate cancer patients was performed using H&E staining. Low-grade prostate adenocarcinoma is evident in non-neoplastic glands, as

shown in Fig. 1A and 1B; however, back-to-back neoplastic glands with no stroma between them were detected in high-grade prostate cancer.

Formalin-fixed paraffin-embedded tissues of patients with confirmed pathological diagnoses of prostate cancer with varying grades were analyzed for YY1 protein expression levels using immunohistochemical staining. Both low-grade (Fig. 1C) and high-grade (Fig. 1D) tumors exhibited positive YY1 staining. Furthermore, high-grade tumors showed significantly higher staining intensity ($p<0.0001$), suggesting that as the tumor progresses, YY1 expression increases (Fig. 1E).

Clinical data, including Gleason scores from prostate biopsies, prostate-specific antigen levels before biopsies, body mass index, prostate volume, and age, were collected from 58 PCa patients and analyzed concerning YY1 levels. Surprisingly, no significant correlation was found between YY1 levels and PCa patients (Table 1).

Table 1
Presentation of clinical data collected from 58 prostate cancer patients. The h-score of the yy1 protein was computed. The patients' Biopsy Gleason Score, PSA Before Biopsy, BMI, and Prostate Volume were examined and accounted for during statistical analysis. Pearson Correlation and p-values were computed. PSA: Prostate Specific Antigen. BMI: Body Mass Index.

	H-Score	
Correlations	**Pearson Correlation**	**p-value**
Biopsy Gleason Score	0.15	0.26
PSA Before Biopsy	0.04	0.76
BMI	-0.17	0.19
Prostate Volume (ml)	0.10	0.47
Age	-0.01	0.92

3.2. Prostate cancer stem cells expressed higher YY1 levels than non-cancer stem cells and normal prostate cells

We aimed to assess the differential expression patterns of YY1 in normal prostate epithelial cells (RWPE-1) and prostate cancer cells (both DU145 CSCs and DU145 non-CSCs). In the case of tumors, a relatively small subpopulation of CSCs is believed to be the primary cause of aggressiveness, metastasis, and recurrence. Along these lines, we isolated the P1 population from the entire DU145 cancer population using the FACS method based on side (SSC-A) and forward (FSC-A) scatter parameters to determine whether YY1 expression is elevated in PCSCs. From the selected P1 population, the cell-specific surface antigens CD133 conjugated to PE-A and CD44 conjugated to FITC-A were used to gate the P2 population, presenting the PCSCs, accounting for only 1.2 % of the whole population. The remaining portion was classified as DU145 non-CSCs (Fig. 2A) and given that they mimic in vivo physiological conditions and better mimic cell interactions, 3D cell culture systems are considered excellent models. Agarose was utilized to coat the cell culture plate and facilitate the formation of spheroids. Agar-coated plates prevent cells from adhering to the surface, allowing for spheroid formation. Notable changes were observed on day 7, as regular spheroidal structures were observed. Before day 7, the absence of strong cell connections resulted in poorly defined boundaries or compact spheroidal structures. On day 7, it was possible to distinguish a thin outer layer of loosely packed cells from an inner tightly packed spheroid core, with a diameter ratio of approximately 200 μm (Fig. 2B). To establish that PCSCs are responsible for the production of tumor spheroids, tumor spheroids were immunostained with CD133 (Fig. 2C). The level of CD133 expression in tumor spheroids has been determined to be significantly quite high. Ki-67 is a protein frequently used as a cell proliferation marker, and its higher level of expression is associated with an aggressive phenotype. Therefore, Ki-67 staining was used to assess proliferation in $CD133^{+}/CD44^{+}$ PCSCs and DU145 non-CSCs (Fig. 2D), with higher expression in PCSCs ($p<0.0001$) (Fig. 2E) indicating faster proliferation. Immunofluorescence staining was performed to determine the expression levels of YY1 in RWPE-1, DU145 CSCs, and DU-145 non-CSCs, and the intensity of YY1 was analyzed using ImageJ. As seen in Fig. 2F, immunofluorescence staining of YY1 protein in DU145 CSCs showed significantly higher staining intensity than the normal prostate RWPE-1 cells and DU145 non-CSCs ($p<0.0001$). In contrast, RWPE-1 displayed much lower YY1 staining intensity than DU145 cells (Fig. 2F and 2G).

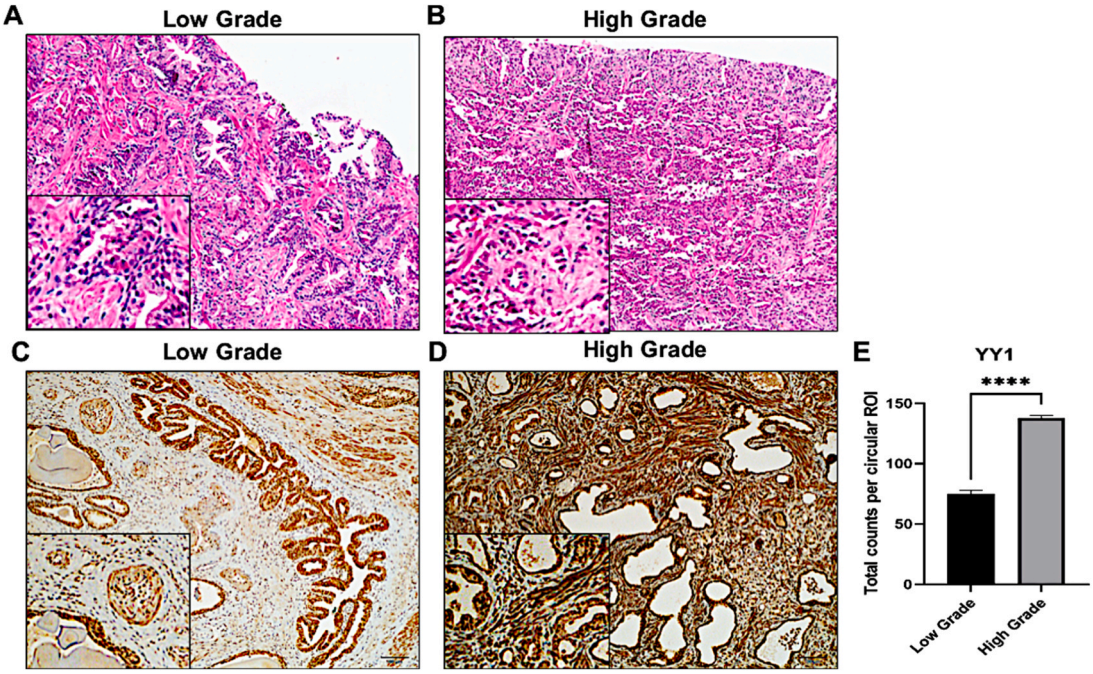

Fig. 1. H&E staining of low-(A) and high-grade (B) prostate cancer tissue samples. Immunohistochemical staining for YY1 protein expression in low-grade (C) and high-grade (D) tumors obtained from patients diagnosed with prostate cancer (Scale bar: 100 μm). (E) Bar graphs of total counts per circular ROI in tumor tissues ($p<0.0001$). Both tumors expressed YY1, while high-grade tumors showed higher staining intensity.

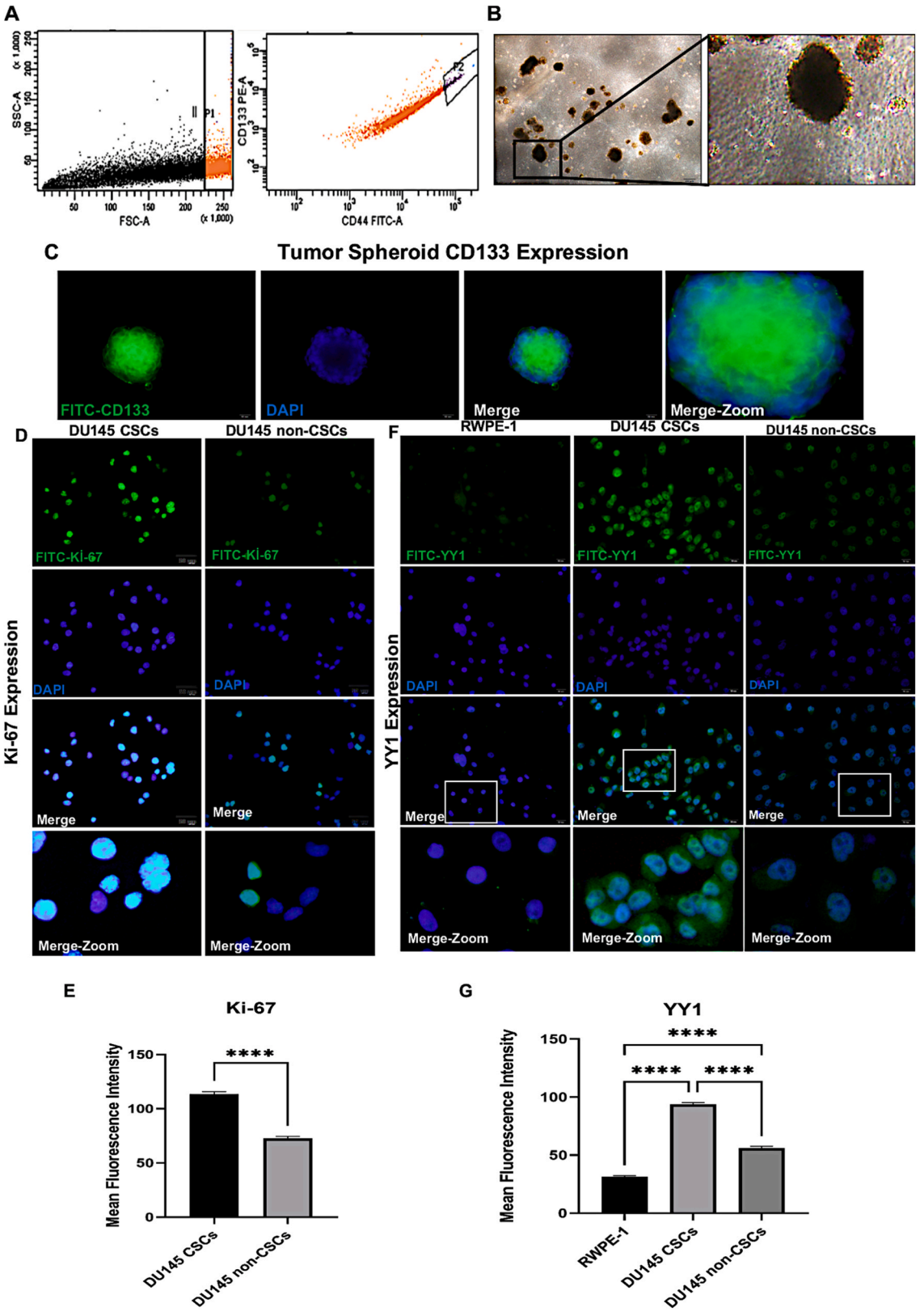

Fig. 2. YY1 is highly expressed in CD133^{+}/CD44^{+} PCSCs. The isolation of CD133^{+}/CD44^{+} PCSCs was performed using the FACS method (A). The P1 population was isolated from the total cell population with SSC and FSC parameters. The P2 population (PCSCs) was isolated from the P1 according to the CD133 PE-A and CD44 FITC-A cell surface biomarkers. Spheroid formation of CD133^{+}/CD44^{+} PCSCs photographed on day 7 (Scale bar: 200 μm) (B). CD133 expression in tumor spheroid (Scale bar: 20 μm) (C). Ki-67 expression in PCSCs and DU145 non-CSCs (D). Bar graphs of the mean fluorescence intensity of Ki-67 (E)(****$p<0.0001$). YY1 expression is presented in RWPE-1, PCSCs, and non-CSCs (Scale bar: 20 μm) (F). Bar graphs of the mean fluorescence intensity of YY1(****$p<0.0001$) (Scale bar: 20 μm) (G).

3.3. The embryonic microenvironment regulated the expressions of YY1, YY2, and YY1/YY2-related genes in PCSCs

Embryos at the two-cell stage were collected from CD1 mice. They were then cultured and incubated until the early blastocyst stage (Fig. 3A, 3B). Two approaches were used to analyze the modulation of the YY1 gene in PCSCs by inducing embryonic microenvironments. Firstly, human PCSCs were microinjected into CD1 mice blastocysts using the ICSI method (Fig. 3D). Secondly, human PCSCs were co-cultured with mice blastocysts in vitro (Fig. 3C). The evaluation of the whole transcriptome analysis of PCSCs following these two approaches showed the downregulation of YY1 expression induced by the embryonic microenvironment (Table 2, Fig. 4). The YY1 and YY2 genes showed 14.063- and 17.946-fold changes (log2), respectively using the microinjection method, and 2.443- and 2.183-fold changes (log2), respectively, using the co-culture method. The obtained results were confirmed with PCR. These results indicate that both YY1 and YY2 were downregulated following exposure to the embryonic microenvironment. Results from the microinjection method demonstrated significant downregulation in the expression of *Hdac1, Hdac2, Ep300, Ezh1, Ezh2, Sap30, Hcfc1, Ruvbl2, Ppargc1a, Rybp, Sirt1, Ddx5, Mbd2, Rnf2, Eed, Suz12*, and *Prc1* genes, with more than a 2-fold change. However, no significant upregulation was observed in the Tp53, SNAI1, and Sfmbt2 genes. On the other hand, the co-culture method showed significant downregulation in the expression of *Hdac2, Ezh1, Hcfc1, Tp53, Rybp, and SNAI1* genes, with more than a 2-fold change. Only *Ruvbl2* displayed a significant upregulation with a 2.076-fold change.

3.4. Protein-protein interaction (PPI) network of YY1 and YY2

The protein-protein interaction networks of *Homo sapiens* YY1 (Fig. 5A) and YY2 (Fig. 5B) were analyzed using the STRING database. According to STRING's findings, YY1 has interactions with MYOD1, PPARGC1A, MBD2, SAP30, UBE3A, HCFC1, HDAC1, HDAC2, SIRT1, SNAI1, DOX5, EZH2, TP53, EP300, RYBP, ACTR5, RUVBL2, AURKA, RPA1, and RPAP3. Meanwhile, YY2 is associated with ABCG2, MBTD1, EZH2, EZH1, SUZ12, PHC2, RNF2, EED, RING1, and L3MBTL2. YY1 exhibits co-expression with HDAC1, HDAC2, SAP30, and HCFC1.

4. Discussion

YY1, a transcription factor, plays a significant role in various biological functions such as apoptosis, cancer, development, and differentiation. Overexpression of YY1 has been found to induce different clinical behaviors in various types of tumors, including prostate cancer. Moreover, it has been observed that the expression levels of YY1 are significantly higher in metastatic tumors compared to the primary tumors in different forms of human cancer. This finding supports the possibility of YY1's involvement in cancer development [19,40–42]. Despite significant advancements in diagnostic and therapeutic techniques over the years, effectively targeting PCSCs responsible for tumorigenesis, drug resistance, relapse, and metastasis continues to be a major challenge in PCa treatment [43]. It has been reported that YY1 expression plays a role in various stages of the embryonic process and increases in PCa. Additionally, its expression levels tend to increase as the tumor progresses to more advanced stages [44]. Our study showed that the expression of YY1 exhibited an elevation in advanced PCa tumors and PCSCs. The increase was demonstrated using H&E staining, IHC staining, and immunofluorescence staining. However, it should be noted that we could not do Western Blot analysis, which was a limitation of our study. The upregulation of YY1 expression can be suppressed by exposing it to the embryonic microenvironment.

The development of new therapeutic strategies involving targeting YY1 is envisaged in treating PCa. The molecular echoes of the embryonic microenvironment have the potential to regulate cells that tend to deviate from normal development [45]. Previous studies have shown that cancer cells with malignant characteristics can transform into a non-tumorigenic phenotype [46–48]. Based on these findings, we suggested that PCSCs can potentially be reprogrammed by the embryonic microenvironment. The potential of embryonic microenvironment reprogramming was considered in the presented study, and changes in YY1 and related gene expressions in PCSCs were investigated. Multiple signaling pathways are actively utilized during the embryonic stage to control cell differentiation, proliferation, migration, and invasion.

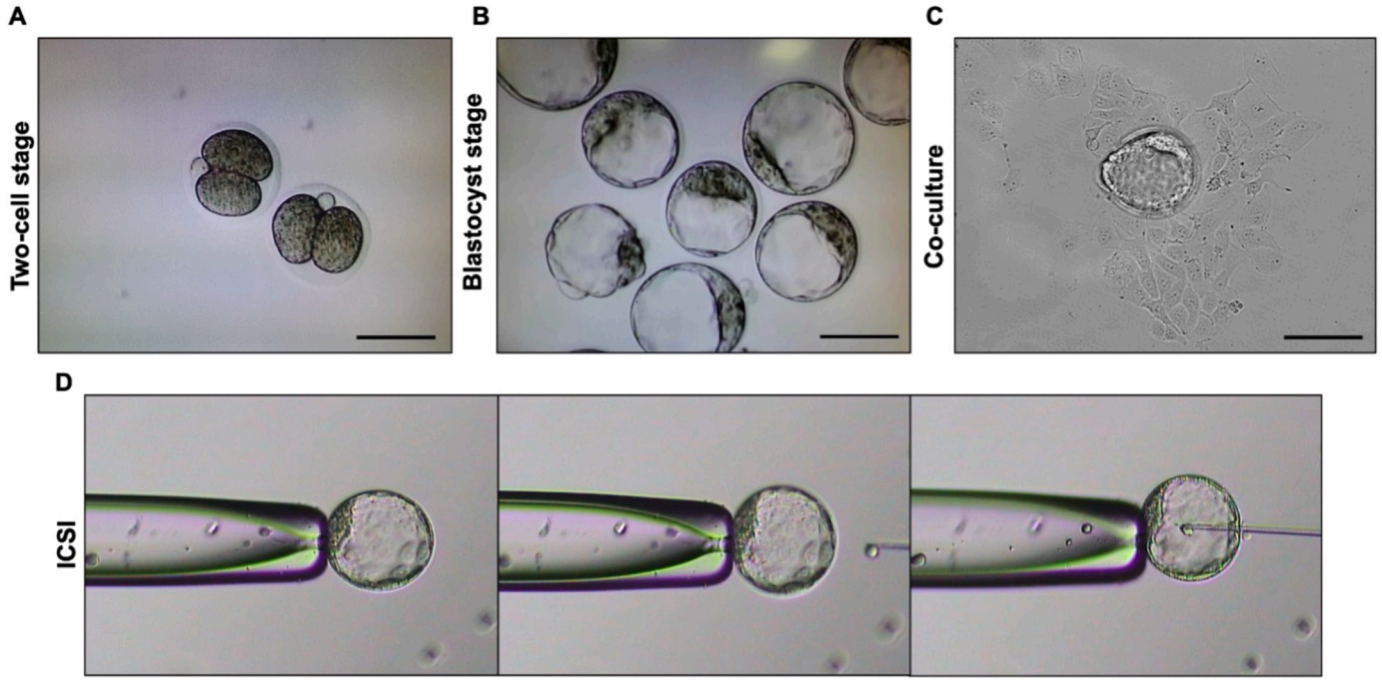

Fig. 3. Obtention of CD1 mouse blastocyst model. Embryos obtained from CD1 mice were cultured and visualized until the two-cell stage (A) and blastocyst stage (B). The blastocysts were co-cultured with $CD133^{+}/CD44^{+}$ PCSCs (C). The blastocysts were microinjected with $CD133^{+}/CD44^{+}$ PCSCs using the ICSI method (D).

Table 2
Differentiated expression of YY1, YY2, and YY1/YY2-related genes in DU145 PCSCs microinjected into a blastocyst (left) or cocultured with embryonic conditioned medium (right). Data were considered statistically significant for $p<0.05$. NA: not applicable.

Gene	Fold Change (Log2)	Regulation	p-value	Gene	Fold Change (Log2)	Regulation	p-value
Yy1	-14.063	Down	8.914e-75	Yy1	-2.443	Down	0.0152
Yy2	-17.946	Down	5.714e-21	Yy2	-2.183	Down	0.0148
Hdac1	-16.922	Down	3.871e-12	Hdac1	-0.450	Down	NA
Hdac2	-17.152	Down	2.9610e-12	Hdac2	-0.572	Down	0.0062
Ep300	-18.950	Down	2.7312e-15	Ep300	-5.484	Down	0.2642
Ezh1	-16.168	Down	1.2124e-11	Ezh1	-1.678	Down	6.7406e-10
Ezh2	-17.136	Down	4.5631e-10	Ezh2	-4.004	Down	0.4450
Sap30	-17.912	Down	1.3602e-27	Sap30	0.765	Up	0.0814
Hcfc1	-23.104	Down	4.5042e-44	Hcfc1	-10.992	Down	4.0211e-07
Ruvbl2	-14.680	Down	5.2066e-100	Ruvbl2	2.076	Up	1.6626e-25
TP53	0.534	Up	0.3992	TP53	-0.696	Down	5.6642e-07
Ppargc1a	-26.070	Down	4.9258e-09	Ppargc1a	-9.210	Down	0.0744
Rybp	-15.250	Down	8.8981e-42	Rybp	-4.049	Down	0.0032
Sirt1	-21.179	Down	3.3986e-26	Sirt1	-0.203	Down	0.3148
Ddx5	-16.157	Down	8.5251e-110	Ddx5	-1.310	Down	NA
SNAl1	2.752	Up	0.2246	SNAl1	-4.175	Down	1.4886e-08
Myod1	NA	-	NA	Myod1	NA	-	NA
Mbd2	-16.653	Down	6.8660e-12	Mbd2	NA	-	NA
Sfmbt2	1.420	Up	0.3912	Sfmbt2	0.734	Up	0.3781
Rnf2	-22.600	Down	6.4845e-33	Rnf2	-9.210	Down	0.0744
Eed	-21.054	Down	3.8472e-27	Eed	NA	-	NA
Suz12	-18.087	Down	5.3120e-11	Suz12	-3.873	Down	0.4596
Prc1	-13.366	Down	2.6373e-10	Prc1	NA	-	NA

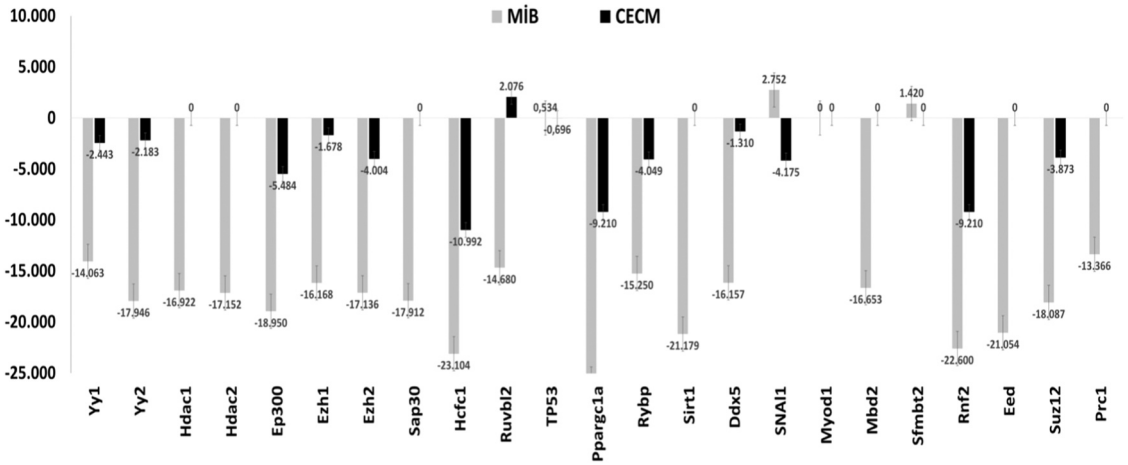

Fig. 4. Bar graph illustrating the differentiated expression of YY1, YY2, and YY1/YY2-related genes in DU145 PCSCs micro-injected into a blastocyst (MİB) and co-cultured with embryonic conditioned medium (CECM).

Tumor cells during this stage exhibit characteristics similar to those of embryonic stem cells and the embryonic environment, including cell invasion, cell migration, signaling pathways, pluripotency, and gene expression, which are crucial for self-renewal and differentiation. The similarities in signaling between cancer and fetal transition processes suggest that the embryonic environment may contribute to the reprogramming of cancer cells [37,38].

According to our research, we observed downregulation of YY1 expression in PCSCs, which was more pronounced in high-grade PCa tissues, as well as in PCSCs exposed to the embryonic microenvironment through ICSI or co-culture methods. Considering the notion that protein-protein interactions regulate the activity of YY1, we conducted a whole genome analysis with PCSCs to explore the altered gene expressions associated with YY1. It has long been established that active genes are associated with hyperacetylated histones [49]. This finding supported our discovery of downregulation in genes Hdac1 (histone deacetylase1), Hdac2 (histone deacetylase2), and Ep300 (histone acetyltransferase p300) in PCSCs upon exposure to the embryonic microenvironment. Histone modifications and chromatin remodeling play a significant role, as HDAC1 and HDAC2 are critical for removing acetyl groups from histones, leading to chromatin condensation and transcriptional repression [50]. YY1 interacts with these enzymes to facilitate repressive chromatin states. Downregulation of HDAC1 and HDAC2 may reduce YY1's chromatin remodeling activity, affecting its ability to silence target genes [51]. Additionally, EP300, a histone acetyltransferase, adds acetyl groups to histones, promoting an open chromatin structure and active transcription [50]. YY1 can function as both an activator and repressor and its interaction with EP300 might be context-dependent. Downregulation of EP300 could impair YY1's ability to activate gene transcription, shifting its role predominantly towards repression. In our study, we found that YY1, associated with Sap30, a component of the histone deacetylase complex, was significantly downregulated by the embryonic microenvironment, as reported by Huang et al. [52].

The decrease in histone acetylation, especially when using the microinjection technique, may contribute to the repression of YY1 expression by reducing chromatin accessibility. Additionally, increased histone methylation patterns can lead to a condensed chromatin state. The evolutionarily conserved proteins that comprise the mammalian

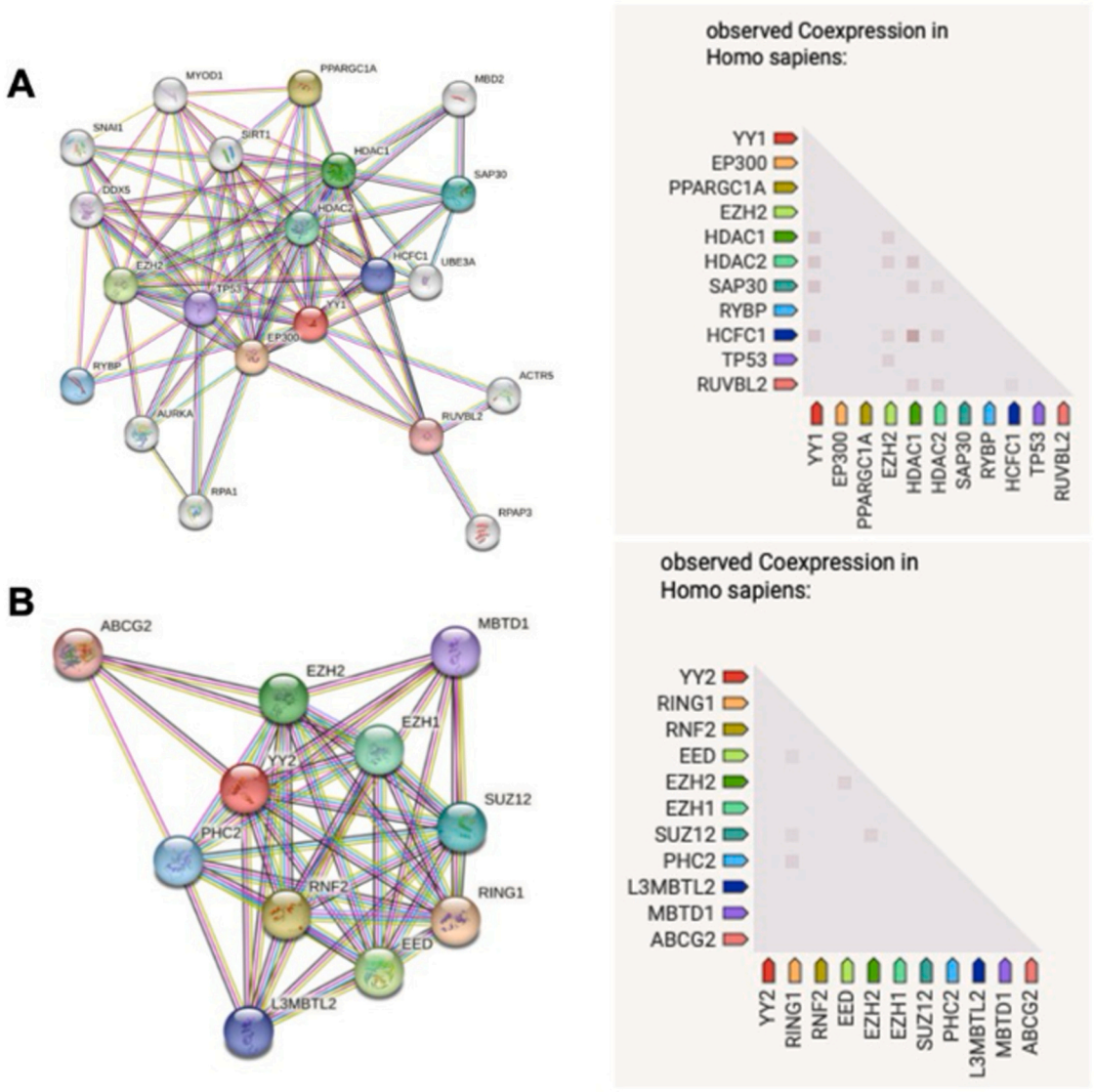

Fig. 5. Protein-protein interaction (PPI) network of (human) YY1 (A) and YY2 (B) proteins by STRING database.

PRC1 and PRC2 complexes work together to establish a repressed state. In mammals, the primary subunits of the PRC2 complex, triggering trimethylation of histone H3 at lysine 27, include SUZ12, EED, EZH2, and RBAP48/46 [53–55]. Our findings demonstrated a significant downregulation of the Ezh2, Eed, and Suz12 genes upon microinjection of PCSCs into the blastocyst ICM. This indicates that the embryonic microenvironment impacts the methylation patterns of H3K27 and subsequently alters the chromatin state in CSCs. YY1 recruits PRC2 to genomic loci to maintain repression [56]. Downregulation of these genes may disrupt YY1's function, leading to gene derepression. It is worth noting that these epigenetic silencing markers, particularly Ezh2, are highly expressed in various tumors, including high-grade PCa tumors [54]. Additionally, Geert et al. conducted a study indicating that Ezh2 overexpression is correlated with PSA recurrence in PCa and proposed Ezh2 as a potential target for PCa immunotherapy [57]. Another study revealed the interaction between YY1 and EZH2 at the miR-146a promoter binding site, limiting the tumor suppressor action of miR-146a in PCa cells [58].

Based on our transcriptome findings, we also observed a significant decrease in Hcfc1 expression. The association between YY1 and HCFC1, which is likely through transcription start site-binding sites, along with the chromatin-modifying activities exhibited by HCFC1, aligns with its role in cell-cycle development. This correlation may explain the concurrent decline in the expression of both genes [59]. Transcriptional co-regulators and chromatin modifiers like SAP30 and HCFC1 are crucial for YY1's function. SAP30, a component of the Sin3A co-repressor complex, is recruited by YY1 to repress gene transcription, and reduced expression of SAP30 could weaken YY1-mediated repression [60]. HCFC1 interacts with YY1 at transcription start sites and is involved in chromatin modification [61]. Downregulation of HCFC1 may impair YY1's ability to regulate the cell cycle and transcription, affecting cell proliferation and tumor progression. Furthermore, we discovered that RuvBL2, a constituent of the INO80 chromatin remodeling complex, is downregulated. Evidence suggests that the YY1-RuvBL1-RuvBL2 complex participates in processes beyond transcription, such as homologous recombination, wherein YY1 and RuvBL2 collaborate as ATPases [62,63]. Downregulation of RUVBL2 might hinder these interactions, affecting YY1's role in maintaining genomic stability. Similarly, RYBP, which interacts with YY1 and is involved in both apoptosis and transcriptional regulation, could disrupt YY1's ability to regulate apoptosis and other cellular processes critical for preventing tumorigenesis when downregulated [64]. The Rybp gene, which is found to be downregulated in our study, is responsible for encoding the protein Ring 1 and YY1 binding. It interacts with proteins

involved in apoptosis regulation, exerting pro- or anti-apoptotic effects in both the cytoplasm and nucleus of different cell types. Understanding the molecular mechanisms by which RYBP's effects can be "positive" or "negative" in various microenvironments is crucial. This is because RYBP can function as both a tumor suppressor gene and an oncogene in different types of cancers and even within the same cancer type. This phenomenon supports the concept that malignancies, including cancer stem cells, exhibit heterogeneity [65,66]. The ubiquitination-related pattern is also a key component for PCa prognosis [67], and this pattern is also worth investigating in the future stages of our experimental setup.

The embryonic microenvironment encompasses numerous bioactive molecules with paracrine activity, as well as extracellular vesicles that are released by embryonic stem cells. This microenvironment includes exosomes, small and large non-coding RNAs, microvesicles, membrane particles, peptides, and small proteins known as cytokines [68]. Both short and long non-coding RNAs play essential roles in tumor development as suppressors or inducers, making them the focus of various studies. For instance, a tumor suppressor called miR-186 has shown the ability to inhibit the expressions of YY1 and CDK6, effectively reducing cell proliferation in vitro for PC-3, LnCAP, and DU145, as well as inhibiting tumor growth in vivo [69]. A study focused on cistrome analysis of YY1 in advanced prostate cancer revealed that YY1 regulates the expression of various genes involved in tumorigenesis, including BRD2/4 and PFKP [70]. Furthermore, in PC3 cells, YY1 negatively modulates the expression of the tumor suppressor XAF1, resulting in its downregulation. This, in turn, hinders cell proliferation and triggers apoptosis [71].

It has been noted that YY2, another zinc-finger transcription factor and a member of the YY family, is significantly involved in maintaining stemness and controlling stem cell development [72]. However, our knowledge regarding the expression levels of YY2 and its biological functions and associations with diseases remains limited. Earlier research indicates that YY2, unlike YY1, may function as a tumor suppressor [73,74]. Likewise, it was observed that YY2 exhibited downregulation in the current study for both conditions.

In PCa, the transcriptional co-regulator Ddx5 acts as an androgen receptor co-activator. Its overexpression is often negatively correlated with aggressive tumor development and treatment resistance. YY1's interaction with DDX5 influences the transcriptional regulation of genes involved in cell proliferation [75]. Downregulation of DDX5 may disrupt these regulatory pathways, affecting tumor growth and progression. Our findings demonstrate that PCSCs exposed to an embryonic microenvironment exhibited reduced expression of Ddx5, suggesting a potential new strategy to target CSCs by reprogramming [76,77]. Gwak et al. also discovered that YY1 is associated with the promoters of E3 ubiquitin ligase RNF2, a member of the Polycomb group proteins. Although the role of RNF2 in the progression of prostate cancer has not yet been determined, siRNA-mediated knockdown of RNF2 resulted in the elimination of SNAIL-mediated repression of E-cadherin and an increase in cell motility in pancreatic cancer [55]. Our results add to the existing evidence indicating that the embryonic microenvironment can inhibit Rnf2 expression in PCSCs.

The protein-protein interaction network of YY1 and YY2 proteins has revealed several potential targets for advanced cancer therapies. However, further research and assays are necessary to confirm gene and protein expression levels in the implicated proteins and pathways. In vivo studies must also be conducted. The potential impact of YY1 and its associated genes' downregulation on tumor suppression in prostate cancer stem cells (PCSCs) may be influenced by epigenetic mechanisms. To deepen our understanding of the reprogramming impacts occurring within this embryonic microenvironment system, it is necessary to do more research. This might involve directing attention towards cell cycle analysis, CHIP-seq analysis, and in vivo investigation of xenograft models. Furthermore, we want to validate the transcriptome analysis results and investigate the underlying mechanistic regulations in future research. The investigations will provide significant insights into the functioning of antitumor effectors within the context of the embryonic microenvironment. YY1 and its associated genes, implicated in the previously discussed epigenetic silencing processes, are downregulated in the present study and may contribute to the reprogramming of malignant PCSCs into a benign phenotype.

To summarize, YY1 is vital in controlling gene expression, particularly in chromatin remodeling PCa. It can activate or repress genes depending on the cellular context. In this study, our findings suggest that the relationship between YY1 and certain transcriptional factors involved in PCa can be reprogrammed by the embryonic microenvironment. The ability of the embryonic environment to suppress YY1 and YY1-related gene expressions in PCSCs shows promise for new therapeutic approaches in targeted prostate cancer therapy.

5. Conclusion

In conclusion, this comprehensive study sheds light on the intricate role of YY1 in PCa progression and the potential for reprogramming CSCs within the embryonic microenvironment. Through histopathological analyses, immunostaining techniques, and transcriptome profiling, we elucidate the elevated expression of YY1 in high-grade PCa tissues and CSCs, underscoring its significance in tumor aggressiveness. Moreover, our exploration into the embryonic microenvironment reveals its ability to downregulate YY1 and associated genes in PCSCs, offering novel insights into therapeutic avenues for targeted PCa treatment. The findings presented here deepen our understanding of YY1's involvement in PCa pathogenesis and highlight the potential of leveraging embryonic microenvironmental cues to reprogram cancer cells toward a less aggressive phenotype. Regarding the study's limitations, the data collected will significantly contribute to future research. This includes extensive in vitro and in vivo investigations to determine the impacts of micro-environmental factors on PCSCs. Furthermore, integrating transcriptome data with other omics datasets, such as proteomics or epigenomics, can offer a more comprehensive view of the regulatory networks involving YY1. This integrative approach can uncover potential feedback loops or compensatory mechanisms that might be activated in response to YY1 downregulation, thereby enhancing our understanding of its role in cancer biology. Moving forward, further investigations into the molecular mechanisms underlying YY1 regulation and its implications in PCa therapy will be crucial for translating these discoveries into clinical applications to improve patient outcomes.

Ethics approval and consent to participate

Experimental methods concerning human tissues and animal experiments have received approval from the Ege University Medical Research Ethics Committee (24–4 T/73) and the Ege University Ethics Committee for Animal Experiments (2015–024), respectively.

Patient consent for publication

Yes.

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Author statement

We declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

CRediT authorship contribution statement

Gulperi Oktem: Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Aleyna Demir:** Writing – original draft, Visualization, Validation. **Fatih Oltulu:** Visualization, Validation, Resources. **Emine Ozcinar:** Methodology, Investigation. **Fahriye Duzagac:** Methodology, Investigation. **Ummu Guven:** Methodology, Investigation. **Emre Karakoc:** Formal analysis. **Asli Cakir:** Methodology, Investigation. **Sule Ayla:** Methodology, Investigation. **Selcuk Guven:** Methodology, Investigation. **Eda Acikgoz:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization. **Aysegul Taskiran:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

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