Experimental Cell Research 442 (2024) 114281

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research article

In vitro obtainment of stem-like cells from gubernaculum testis biopsies of cryptorchid pediatric patients

Sara Vinco [a], Giulia Ambrosini [a], Andrea Errico [a], Nunzio Marroncelli [a], Elisa Dalla Pozza [a], Eleonora Matranga [a], Nicola Zampieri [b,c], Ilaria Dando [a,*]

[a] Department of Neurosciences, Biomedicine and Movement Sciences, Biochemistry Section, University of Verona, Verona, Italy
[b] Department of Engineering for Medicine Innovation, Pediatric and Fertility Lab, Woman and Child Hospital, Division of Pediatric Surgery, University of Verona, Verona, Italy
[c] UNICAMILLUS, International Medical University, Rome, Italy

ARTICLE INFO

Keywords:
Cryptorchidism
Gubernaculum testis
Stem cells
Regenerative medicine
Pediatric andrology

ABSTRACT

Testicular descent is a crucial event in male sexual development, and alterations in this process during gestation can lead to reduced fertility in adulthood. Cryptorchidism, i.e., failure of one or both testicles to descend into the scrotum, is one of the most common birth defects and represents a principal cause of infertility in adulthood. Therefore, identifying effective approaches for preserving fertility in childhood is of primary importance. In this context, the key role played by the gubernaculum testis during the placement of the testes in the scrotal bursa emerges. Given its close affinity to testicular tissue and its richness in mesenchymal cells, our prime aim is to characterize this para-testicular tissue to explore its potential ability to differentiate into testicular cells for fertility preservation. The first step of our task is represented by the present study that aimed to obtain *in vitro* stem-like cells starting from gubernaculum testis biopsies of four pediatric patients affected by cryptorchidism, aiming to differentiate them into testicular functioning cells. Our results show that the obtainment of aggregates with stem features is not dependent on the age of the patients and, therefore, not even on the damage suffered by the testis during its stay in the abdomen. This study opens the possibility of extending this approach to older patients, offering a new potential approach to support their fertility potential.

1. Introduction

Cryptorchidism is the absence at birth of one or both testicles in the scrotum and is the most common birth defect in male children. A cryptorchid testis may descend spontaneously in the first 3 months of a child's life; otherwise, it is necessary to proceed surgically. Orchidopexy, the surgical procedure by which the gonad is placed in the correct physiological position, is recommended between 6 and 12 months of age [1,2] It has been shown that the patient's age at the time of surgery is a factor that strongly influences testicular volume and hormonal function in adulthood [3]. Indeed, it has been observed that the number of germ cells decreases dramatically between 9 months and 3 years of age in patients with cryptorchidism. The number of Sertoli cells and the diameter of the seminiferous tubules also decrease during this period, albeit to a lesser extent [4]. More frequently in bilateral forms of cryptorchidism, there is an impairment of spermatogenesis: approximately 80 % of men with a history of bilateral cryptorchidism present a spermiogram with abnormal data; in subjects with unilateral cryptorchidism, the percentage drops to 50 % [5]. Cryptorchidism, thus, appears to be closely related to the alteration of a child's fertility potential and is, in fact, the best-characterized risk factor for infertility and testicular cancer in adulthood [6,7].

The gubernaculum testis is a genitoinguinal ligament that directs the testis toward the scrotum during gestation [8]. It comprises an abundant extracellular matrix rich in glycosaminoglycans and mesenchymal cells, such as fibroblasts and smooth muscle cells. During the last steps of testis descent, the connective tissue of the gubernaculum undergoes an important remodeling, becoming a fibrous structure rich in collagen and elastic tissue. Throughout this transformation, the gubernaculum responds to testosterone, becoming enriched in vessels, supporting its contraction with the consequent descent of the testicle [8]. These and other data highlight that gubernaculum testis shares many features with the testicular cells, including a common embryonic origin [9].

Starting from these observations, we take advantage of their

* Corresponding author.
E-mail address: ilaria.dando@univr.it (I. Dando).

https://doi.org/10.1016/j.yexcr.2024.114281
Received 12 September 2024; Received in revised form 9 October 2024; Accepted 11 October 2024
Available online 11 October 2024
0014-4827/© 2024 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

common characteristics to obtain *in vitro* functioning testicular cells from a gubernaculum testis biopsy taken during orchiopexy, intending to support the patient's fertility potential in adulthood by applying *in vitro* technologies. For this objective, in this paper, we aimed to obtain stem-like cells from gubernaculum biopsy. We de-differentiated *in vitro* gubernacular cells derived from four pediatric patients suffering from cryptorchidism using a specific de-differentiation protocol [10,11]. The obtained cells were cultured at different times and characterized at the morphological level and at the expression of stem markers to identify the apex of stemness. Understanding when stem cells reach this peak is essential for developing effective differentiation strategies, and these data will be exploited in the next step to differentiate them into functional Leydig and Sertoli cells, as reported by other authors in diverse models [12,13]. The possibility of differentiating stem cells into functional testicular cells and cultivating them *in vitro* represents an important step forward in the clinical management of cryptorchidism and other testicular pathologies, making it possible to generate autologous cell models, such as organoids, for each patient to support spermatogenesis *in vitro*. Therefore, this project aims to preserve male fertility in the pediatric age, paving the way for new clinical strategies and broader applications.

2. Materials and methods

2.1. Patients

This study was approved by the Board of the "Pediatric-Adolescent Fertility Study Laboratory" and the Ethics Committee of the Verona University Hospital- AOUI (*Azienda Ospedaliera Universitaria Integrata*)-Project: ANDRO-PRO- 4206CESC. Informed consent was obtained from all patients/parents involved in the study. Samples are collected in our laboratory from patients with primary azoospermia, genetic syndromes associated with azoospermia, cryptorchidism, testicular torsion, testicular trauma, and testicular neoplasms. Each patient is cataloged with a progressive identification number. In this study, we performed *in vitro* studies of primary cells derived from the gubernaculum testis biopsy taken during the orchiopexy of four pediatric patients with cryptorchidism. Patients included in this study are.

- patient #2 (also referred as pt #2): 8 years old, unilateral cryptorchidism presenting an hypotrophic testicle;
- patient #20 (also referred as pt #20): 2 years old, unilateral cryptorchidism;
- patient #94 (also referred as pt #94): 10 years old, bilateral cryptorchidism and the gubernaculum testis connected to the right testicle was considered in this study;
- patient #99 (also referred as pt #99): 6 years old, cryptorchidism characterized by a retractile testicle.

2.1.1. Inclusion criteria

The inclusion criteria defined for patients enrolled in the study were.

- inguinal cryptorchidism;
- unilateral or bilateral orchidopexy;
- no metabolic, genetic, or chronic inflammatory diseases;
- only one surgery in life either of the genital organs or related to other districts (e.g., urinary system, gastrointestinal system) and without other associated diseases.

2.1.2. Exclusion criteria

We considered as exclusion criteria from the study.

- patients with intraabdominal testis (to avoid interpretive bias about the true position of the testis);
- patients undergoing orchidopexy surgery with the trans-scrotal technique, a surgical technique not uniformly approved by all surgeons and not standardized as the traditional technique;
- conversion to laparoscopic technique: essential for intra-abdominal mobilization of sperm vessels.

2.2. Processing and in vitro culture of testicular and gubernacular testis tissue

We processed gubernacular testis as our group had previously reported [14]. We cut the tissue with a scalpel on a Petri dish with 500 μL of phosphate-buffered saline (PBS) in sterile conditions in a biological safety cabinet. Then, the tissue fragments were enzymatically disrupted with compounds diluted in a cell culture medium containing type I collagenase and hyaluronidase. After this step, the cells were incubated at 37 °C and 5 % CO_2 until completely homogenized. Cell viability was assessed using the Trypan Blue assay. Ultimately, we cultured the cells with DMEM medium supplemented with 10 % fetal bovine serum (FBS), 4.5 g/L glucose, and 50 g/mL gentamicin sulfate (all from Gibco/Life Technologies) and maintained them in a 37 °C and 5 % CO_2 environment in cell culture flasks.

2.3. In vitro obtainment of stem-like cells

To obtain gubernacular stem-like cells (called GSC), we started with gubernacular cells (called control parental cells). The culture medium of the parental cells was aspirated, and cells were washed twice with 1X phosphate-buffered saline (PBS) to remove any residual serum. After trypsin, cells were collected and centrifuged at 300 g for 3 min. The supernatant was carefully removed, while a portion of the pellet was stored at −80 °C for later RNA extraction of parental control cells. The remaining pellet was resuspended in a stem-specific medium consisting of DMEM/F-12 without glucose supplemented with 1 g/L glucose, B27, 1 μg/mL fungizone, 1 % penicillin/streptomycin (all from Gibco/Life Technologies, USA), 5 μg/mL heparin (Sigma/Merck), 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL fibroblast growth factor (FGF) (both from PeproTech, United Kingdom) [9,10]. Cells were then amplified in a 37 °C incubator with 5 % CO_2 until maximum stemness was reached. Every two days, 2 mL of fresh medium supplemented with growth factors was added to promote the transition of differentiated cells to a stem state. Their ability to grow in suspension-forming spheres was analyzed, along with the expression of stem markers - two fundamental characteristics of stem cells - at different time points: 1 day (T1), 3 days (T3), 7 days (T7), 14 days (T14), and 21 days (T21) of culture. Once the peak of stemness has been reached, the obtained gubernacular stem cells are stored at −80 °C for subsequent experiments. For all the time points, we acquired images of the cells using a bright microscope (Axio Vert. A1, Zeiss, Germany).

2.4. Morphometric analysis of spheroid aggregates

To calculate the diameter of cell aggregates obtained from cultivating GSC, we used the computer program 'ImageJ' (NIH Image, Bethesda, MD, USA). This program enabled the digital processing of images acquired with the microscope and calculating morphological parameters, such as areas and diameters. Once the picture has been opened with 'ImageJ', the tool representing a segment was used, with which it was possible to draw the diameter from one end of the sphere to the other and obtain its length by clicking on 'Analyze' - 'Measure'. The procedure was repeated to obtain the length of the second diameter, which corresponded to the width of the sphere. From the two diameters obtained, the area of the aggregates was calculated using the formula: $A=(d_1/2) \times (d_2/2) \times \pi$. To prove that the area obtained was as close as possible to the real area, the tool representing an irregular oval allowed the perimeter of the sphere of interest to be drawn with the cursor. Clicking on "Analyze" - "Measure," the measurement of the area was

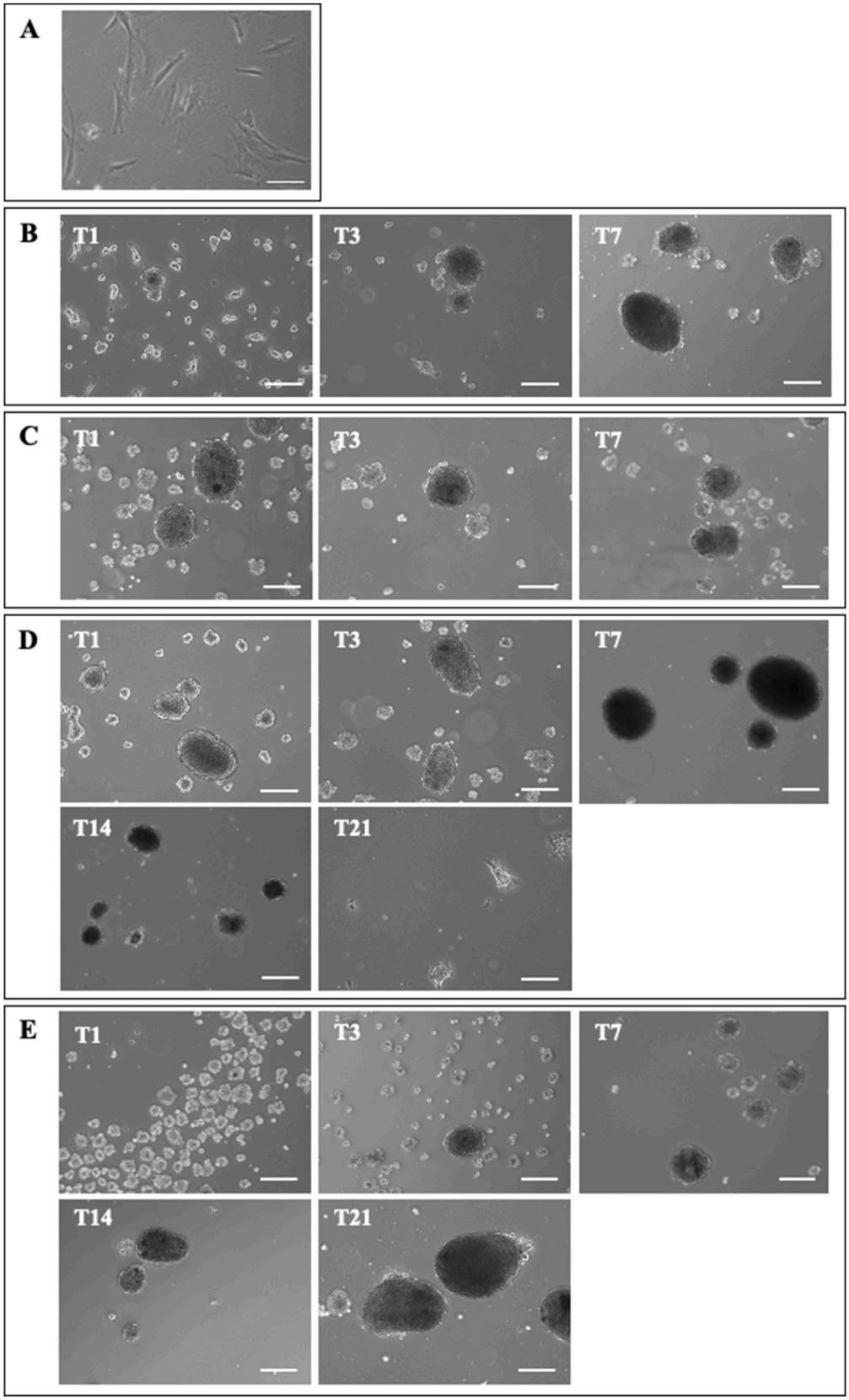

Fig. 1. Representative images of primary parental and stem-like cells. A) Gubernacular cell morphology of pt #2 at 10× magnification. Scale bar: 100 μm. B) Pt #2 GSC after 1 day (T1), 3 days (T3), and 7 days of culture. C) Pt #99 GSC at T1, T3, and T7. D) Pt #20 GSC at T1, T3, T7, T14, and T21. E) Pt #94 GSC at T1, T3, T7, T14, and T21. All the images of panels B–E were at 5× magnification, and the scale bar is 200 μm.

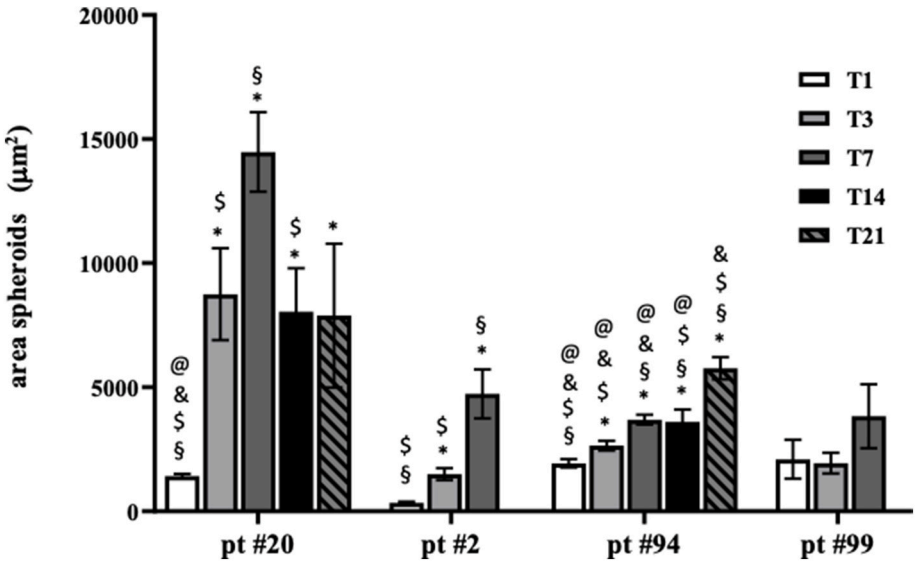

Fig. 2. Identification of the highest capacity of the cells to form suspension spheres. Average sphere diameters for each patient at the different timings after 1, 3, 7, 14 and 21 days (T1, T3, T7, T14, and T21, respectively) with the corresponding standard errors. Statistical analysis for GSC at the different timings: *p < 0.05 towards T1; §p < 0.05 towards T3; $ p < 0.05 towards T7; & p < 0.05 towards T14; @ p < 0.05 towards T21.

directly displayed. "ImageJ" enabled a precise and detailed analysis of the stem spheres' dimensions, guaranteeing the results' accuracy.

2.5. RNA extraction and real-time PCR

According to the manufacturer's instructions, total RNA was extracted from 6 × 10^5 cells using ZymoBIOMICS RNA Miniprep, and 1 μg of RNA was reverse transcribed using first-strand cDNA synthesis. qPCR was performed in triplicate samples by SYBR-Green detection chemistry with GoTaq qPCR Master Mix (Promega, USA) on a Quant-Studio 3 Real-Time PCR System (Thermo Fisher Scientific, USA). RNA integrity was determined by electrophoresis on an agarose gel. Total RNA (1 μg) was used to synthesize first-strand cDNA by RT-PCR.

The primers used in this study were:
CD29 forward, 5′- GACGCCGCGCGGAAAA -3';
CD29 reverse, 5′- ACATCGTGCAGAAGTAGGCA -3';
CDH3 forward: 5′- TCCTTCTCCAGGTTTGCTGG -3';
CDH3 reverse: 5′- GAATACTTTCCCCAGCGCCT -3';
VAP1 forward, 5′- ACTTTTGGGAGGGGAACCAG -3';
VAP1 reverse, 5′- GGTCCCATTCCTTAACCCACT -3';
ZEB1 forward, 5′- GTTACCAGGGAGGAGCAGTGAAA -3′,
ZEB1 reverse, 5′- GACAGCAGTGTCTTGTTGTTGTAGAAA -3';
CDH1 forward, 5′- GACACCAACGATAATCCTCCGA -3′,
CDH1 reverse, 5′- GGCACCTGACCCTTGTACGT -3';
NANOG forward, 5′- AGTCCCAAAGGCAAACAACCCACTTC -3′,
NANOG reverse, 5′- TGCTGGAGGCTGAGGTATTTCTGTCTC -3';
SOX2 forward, 5′- GGGAAATGGGAGGGGTGCAAAAGAGG -3′,
SOX2 reverse, 5′- TTGCGTGAGTGTGGATGGGATTGGTG -3';
OCT3/4 forward, 5′- GACAGGGGGAGGGGAGGAGCTAGG -3′,
OCT3/4 reverse, 5′- CTTCCCTCCAACCAGTTGCCCCAAAC -3';
SDHA forward 5′-GGACCTGGTTGTCTTTGGTC-3';
SDHA reverse 5′-CCAGCGTTTGGTTTAATTGG-3'.

The cycling conditions used were: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. Each triplicate's average cycle threshold was analyzed according to the $2^{-\Delta\Delta Ct}$ method, using SDHA as an endogenous control. Real-time PCR data were calculated as fold change relative to control parental cells for each patient-derived cell.

2.6. Statistical analysis

The presented results are the mean ± SE of at least three biological replicates for each condition. We determined statistically significant differences with the *t*-test. We analyzed the data using the GraphPad Prism program and reported statistical significance in Figure Legends.

3. Results

3.1. In vitro culture of gubernacular cells and stem-like cells

Four pediatric patients with cryptorchidism were selected for this study, whose gubernaculum testis biopsies were used for *in vitro* culture. The biopsies of gubernaculum testis have a volume average of approximately 10–20 mm^3. They were taken from each of the four patients and were disrupted and *in vitro* cultured, as reported in the Materials and Methods section. During the *in vitro* culture period, considerable heterogeneity emerged in the cells' adaptation rate to *in vitro* growth, which was patient-dependent. Some patients' cells showed rapid adaptation within a short time after culture (1–2 days), while others required a more extended period to develop significant adherence and growth (approx. 30 days). A representative image of the *in vitro* growth of the gubernaculum testis is shown in Fig. 1 A, where it can be seen that this tissue is composed of different cell types reflecting its complexity, with some cells showing a morphology typical of fibroblasts, such as elongated shape and filiform extensions, and others showing the characteristic stripes of smooth and striated muscle cells.

Then, gubernacular cells from all four patients grown in culture were placed in a stem cell-specific medium to obtain gubernaculum stem-like cells (GSC). After 24 h of incubation (T 1), cells of all patients show an evident morphological change from an elongated structure typical of adherent cells (as shown in Fig. 1A) to a growth in suspension-generating aggregates (T1 in Fig. 1 B-E). After 3 days (T 3) of culture in stem cell-specific medium, the cell aggregates tend to become larger (T 3 in Fig. 1B–E), reaching an increasingly defined spherical shape after 7 days (T7), especially for patients #2 and #20, (T7 in Fig. 1 B and D). Following the first week of culture, the cells from the various patients show morphological differences. In the case of patients #2 and #99 (Fig. 1B and C, respectively), cell cultures were stopped at day seven due to the low viability of the cells that had difficulty progressing, and the de-differentiation process further increased cell death. Conversely, cells

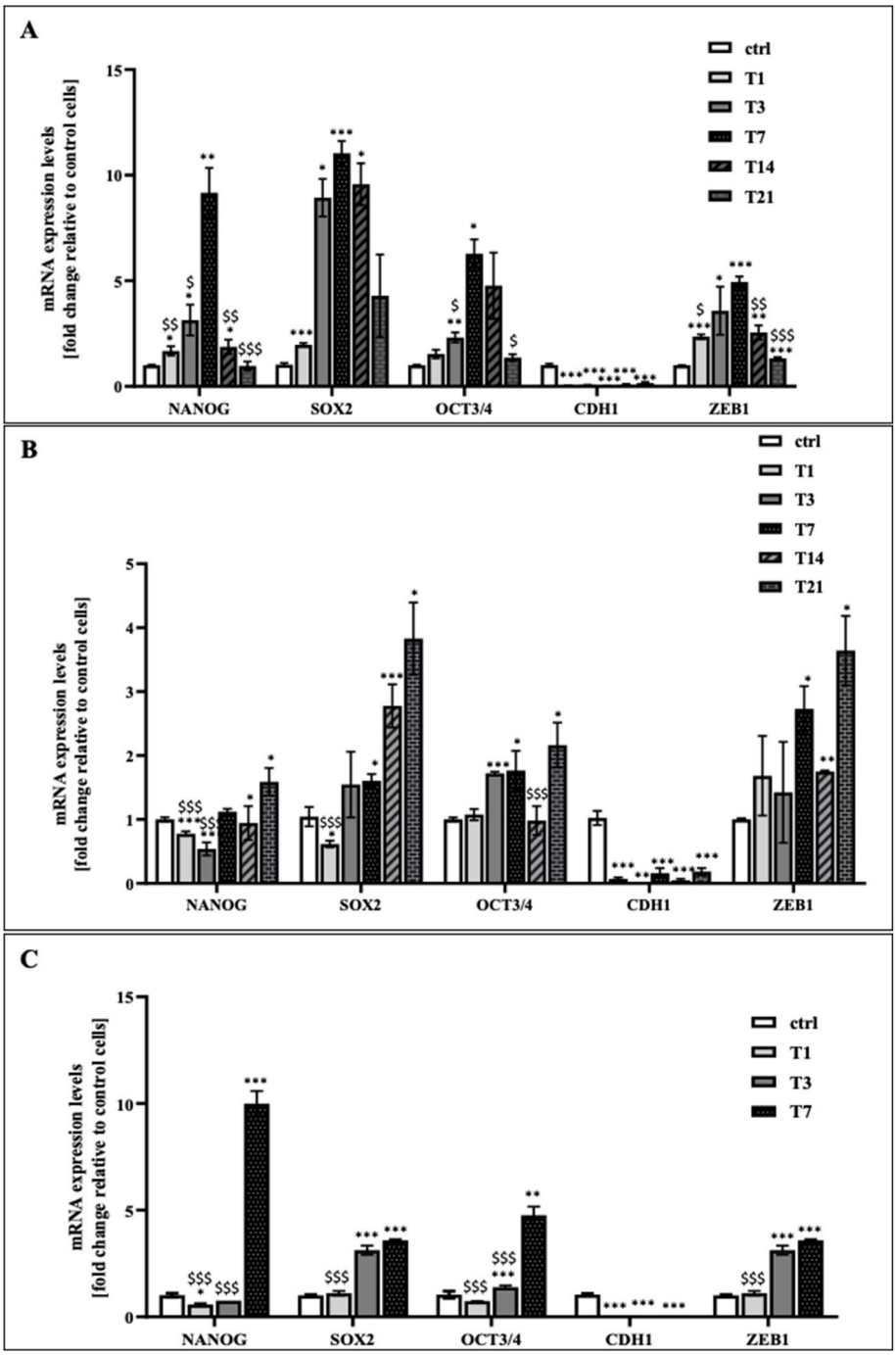

Fig. 3. Analysis of stem and EMT marker expression level. Real-time PCR analysis of the basal expression of the stem markers NANOG, SOX2 and OCT3/4 and the EMT markers CDH1 and ZEB1 in parental gubernaculum cells (CTRL) and GSC at different culture times (T1, T3, T7, T14, and T21) of patients #20 (A), #94 (B) and #99 (C). Values are reported as fold change compared to parental gubernaculum cells. Statistical analysis: *p < 0.05, **p < 0.01, ***p < 0.001 for parental cells to GSC; $ p < 0.05, $$ p < 0.01, $$ p < 0.001 for GSC to GSC at T7 (A and C) and for GSC to GSC at T21 (B).

derived from patient #20, after 14 days (T 14) and, even more drastically, after 21 days (T 21) of culture, progressively lose their spheroidal structure, disaggregating and adhering again to the surface of the flasks (Fig. 1 D). Finally, GSC derived from the tissue of patient #94 showed a distinct behavior compared to all other patients. Cells started to form aggregates with significant size after 14 days of culture, reaching their maximum capacity to form spheres after 21 days (Fig. 1 E).

All these data were established by the sphere size analysis (Fig. 2). This measurement statistically confirmed the stemness peak of each patient, which corresponds to T7 for pt #20 and #2 (despite the latter patient's cells were not able to grow for a more extended period), T21 for pt # 94, and for pt# 99 the sphere size was maintained during the culture period.

3.2. Analysis of the expression of stem markers in GSC

To determine whether increased sphere size correlates with the acquisition of stem characteristics, we analyzed the expression of three stem marker genes, i.e., *NANOG*, *SOX2*, and *OCT3/4*, and two epithelial-mesenchymal transition (EMT) marker genes, i.e., *ZEB1* and *CDH1*.

Table 1
Mean values ± SE of gene expression analyzed through real-time PCR in gubernacular cells and GSCs derived from patient #20, #94, and #99.

Patient #20						
	CTRL	T1	T3	T7	T14	T21
NANOG	1.002 ± 0.025	1.679 ± 0.220	3.143 ± 0.735	9.165 ± 1.178	1.875 ± 0.333	0.968 ± 0.209
SOX2	1.029 ± 0.079	1.974 ± 0.079	8.936 ± 0.888	11.039 ± 0.578	9.574 ± 0.988	4.294 ± 1.954
OCT3/4	1.002 ± 0.032	1.536 ± 0.200	2.318 ± 0.238	6.281 ± 0.678	4.768 ± 1.568	1.363 ± 0.157
CDH1	1.010 ± 0.065	0.029 ± 0.016	0.059 ± 0.018	0.008 ± 0.002	0.052 ± 0.063	0.157 ± 0.057
ZEB1	1.000 ± 0.005	2.358 ± 0.102	3.585 ± 1.138	4.940 ± 0.259	2.557 ± 0.335	1.330 ± 0.054
Patient #94						
	CTRL	T1	T3	T7	T14	T21
NANOG	1.002 ± 0.031	0.778 ± 0.035	0.540 ± 0.101	1.123 ± 0.045	0.945 ± 0.265	1.591 ± 0.213
SOX2	1.044 ± 0.151	0.616 ± 0.053	1.547 ± 0.515	1.607 ± 0.103	2.776 ± 0.337	3.830 ± 0.564
OCT3/4	1.002 ± 0.029	1.076 ± 0.089	1.720 ± 0.026	1.766 ± 0.307	0.983 ± 0.226	2.164 ± 0.349
CDH1	1.023 ± 0.109	0.071 ± 0.022	0.019 ± 0.001	0.161 ± 0.078	0.048 ± 0.022	0.185 ± 0.055
ZEB1	1.001 ± 0.016	1.683 ± 0.623	1.425 ± 0.790	2.730 ± 0.355	1.749 ± 0.049	3.642 ± 0.542
Patient #99						
	CTRL	T1	T3	T7		
NANOG	1.013 ± 0.094	0.573 ± 0.064	0.755 ± 0.001	9.997 ± 0.585		
SOX2	1.002 ± 0.035	1.109 ± 0.103	3.124 ± 0.217	3.588 ± 0.053		
OCT3/4	1.039 ± 0.164	0.738 ± 0.005	1.389 ± 0.075	4.763 ± 0.408		
CDH1	1.036 ± 0.052	0.010 ± 0.001	0.025 ± 0.008	0.009 ± 0.005		
ZEB1	1.002 ± 0.035	1.109 ± 0.103	3.124 ± 0.217	3.588 ± 0.053		

During EMT, a process that is strictly connected with the acquisition of stem properties [15,16], the expression of *ZEB1*, a mesenchymal marker, is increased; in contrast, the expression of *CDH1*, the gene that transcribes for E-cadherin, is decreased [17,18]. We analyzed these genes' expression on parental control gubernacular cells and GSC at the different culture time points (T1, T3, T7, T14, and T21, where applicable) of three patients #20, #94, and #99. However, regarding patient #2, the analysis of stem marker expression was not performed due to the difficulty of these cells growing *in vitro* and thus obtaining biological replicates for data robustness. Globally, for all three patients, the mRNA expression levels of *NANOG*, *SOX2*, *OCT3/4*, and *ZEB1* genes increased at various times in GSC compared to the parental control cells (Fig. 3A–C). Notably, the peak of expression of all these markers is after 7 days of culture (T7) for GSC of patients #20 (Fig. 3 A) and #99 (Fig. 3 C) and after 21 days of culture (T21) for GSC of patient #94 (Fig. 3 B), confirming that at these times gubernacular cells acquire the highest degree of stemness, in line with the larger size of the spheres (Fig. 2). Regarding *CDH1*, its expression in GSC of all patients decreases compared to their parental control cells and these low expression levels are maintained throughout the entire GSC culture period (Fig. 3A–C). All the values of gene expression are reported in Table 1. Altogether, these data confirm the peak of stemness identified through the morphological analysis of the spheres, supporting a strict correlation between morphology and the acquisition of stem features.

3.3. Analysis of the expression of adhesion markers in GSC

To better understand GSC's molecular characteristics and identify possible changes in cell adhesion during the de-differentiation process, we analyzed the expression of CDH3, VAP1, and CD29, which are associated with cell adhesion, maintenance of the stem state and may also be connected with spermatogenesis. The *CDH3* gene encodes for a protein that plays a role in cell cohesion, tissue organization, and regulation of germ-cell development. Its presence maintains the spatial organization of cells within the testicular tissue by facilitating the interaction and communication between Sertoli cells and germ cells during spermatogenesis, creating an optimal environment for sperm development [19,20]. On the other hand, the *VAP1* gene is a known regulator of cell adhesion and interaction with other adhesion molecules. Finally, the *CD29* gene is involved in the formation and stability of adherent junctions between germ cells and Sertoli cells, contributing to the cohesion and functionality of testicular tissue, and regulates the movement of germ cells within the testicular tissue, ensuring proper migration to the lumen of the seminiferous tubule for the production of mature spermatozoa [21,22]. To assess whether there are significant

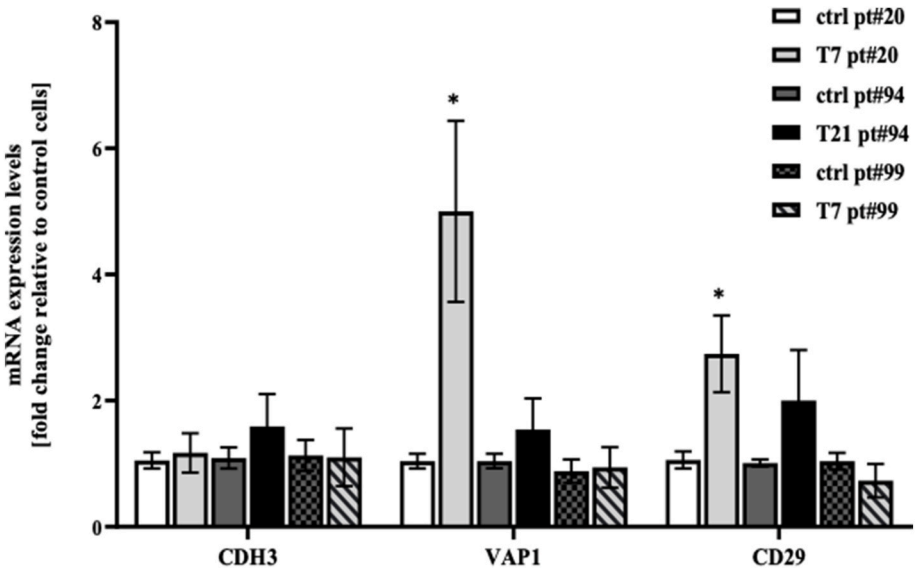

Fig. 4. Analysis of adhesion markers expression. Real-time PCR analysis of the basal expression of the CDH3, VAP1, and CD29 in parental gubernaculum cells (ctrl) and GSC at the respective peak of stemness (T7 for pt #20 and #99, and T21 for pt #94). Values are reported as fold change concerning parental gubernaculum cells of individual patients. Statistical analysis: *$p < 0.05$ for parental cells to GSC.

differences in the expression of these genes during the de-differentiation process of gubernaculum cells, we analyzed their expression in parental and GSC patients' derived cells. Regarding patient #20, a significantly increased expression of the adhesion markers VAP1 and CD29 has been observed in GSC at the peak of stemness compared to the parental gubernacular cells (Fig. 4). Conversely, GSC derived from patient #94 present a slight trend of increase of the three markers, whereas GSC from patient # 99 do not present a significant difference in their expression in GSC compared to parental control cells (Fig. 4).

4. Discussion

Cryptorchidism is a common clinical condition in pediatric patients that, in the long term, can lead to infertility, testicular neoplasms, and hypogonadism. Since malfunctioning testicular cells, as a result of the testicle remaining in the abdomen, lead to infertility and low testosterone levels, this project represents the first step in regenerating autologous *ex-novo* functioning testicular cells capable of producing testosterone. To this end, this study focused on a para-testicular tissue, the gubernaculum testis, which is rich in mesenchymal cells and closely related to testicular tissue. Our aim is to de-differentiate cells from GSC and then transform them into Leydig-like testicular cells, which play a crucial role in supporting spermatogenesis and testosterone synthesis. In this project, we focused on the de-differentiation of gubernaculum cells into GSC to identify the stemness peak of cells to differentiate them into testicular cells. We used gubernaculum testis biopsies derived from four different patients aged between 2 and 10 years, all suffering from cryptorchidism. During the de-differentiation procedure, the cells of all patients showed similar behavior in the early stages, switching from an *in vitro* growth in adhesion to the formation of aggregates in suspension already after the first day of culture in a stem cell-specific medium. This trend was maintained until the first week of culture, after which differences between the cells of the various patients became apparent. Indeed, patient #20, aged 2 years, showed an increased ability to form spheres around day 7 (T7) of culture. The identification of T7 as the timeframe in which this patient's cells reach the peak of stemness was supported by the analysis of stem and EMT markers, with NANOG showing the peak of expression 7 days after the start of culture and a tendency for increased expression of SOX2, OCT3/4, and ZEB1 during the same period. After the first week of culture, the cells of patient #20 tended to disaggregate and adhere again, followed by the decreased expression trend of the analyzed mRNAs. Conversely, patient #2, aged 8, showed a progressive ability to form large spheres as days passed, reaching a significant peak in aggregate size at 7 days after culture. However, no further analysis was possible due to the difficulty of growing cells *in vitro*. In contrast, patient #94, who was 10 years old and the oldest in this study, showed a delay in the ability to form spheres, with the peak size reaching 21 days of culture (T21). Marker expression analysis confirmed this peak, with an increasing trend in NANOG, SOX2, OCT3/4, and ZEB1 between T14 and T21. Finally, 6-year-old patient #99 showed the ability to form round-shaped spheres after 1 day of culture and maintained this capacity until day 7. However, the subsequent timing needed to be assessed due to difficulties in growing cells *in vitro*. Regarding markers, NANOG and OCT3/4 show a peak expression at T7, whereas ZEB1 and SOX2 show an increasing trend in the first three days of culture. Altogether, these results indicate that the behavior of the cells is not strictly related to the permanence of the testicle in the abdomen but rather to the type of damage suffered and individual genetic predispositions that influence the response of the urogenital cells. This is of particular interest, as it shows that even a testis that has suffered long-term damage by remaining in the abdomen for so long, as in the case of patient #94, may have a gubernaculum testis that is still functional and responsive to stimuli *in vitro*, capable of generating stem cells perhaps after longer culture times.

Once the stem apex of the gubernacular stem cells had been identified, we analyzed the expression of 3 adhesion markers (CDH3, VAP1, and CD29) to characterize the molecular features of GSC for the enrolled patients. This evaluation was performed on 3 of the 4 patients due to the difficulty of growing tissue from patient #2 *in vitro*. Initially, the focus was on CDH3, which not only participates in cell adhesion and the creation of junctions between cells but also contributes to the spatial organization of cells in testicular tissue, facilitating the interaction and communication between Sertoli cells and germ cells during the process of spermatogenesis [19,20]. However, there are no in-depth studies on its specific role in the pediatric context. None of the 3 patients showed a significant increase in the expression of this gene in the stem state. Regarding VAP1, its expression shows an increased peak only in GSC of patient #20. Finally, CD29, which contributes to the cohesion and function of testicular tissue, shows an increased expression in GSC from patient #20 and a trend of increase in GSC of patient #94. The results highlight that the expressions of VAP1 and CD29 are increased in stem-like cells from 2-year and 10-year patients, #20 and #94 respectively, supporting that these proteins may have a role in the formation of spheroids, however further studies are needed to deeper investigate their role linked to the function of testicular cells.

These data open the way for the search for other markers of adhesion in the pediatric context to predict the ability to recover fertile potential in children using specific stimuli induced in the laboratory.

5. Conclusion

This study aims to investigate an innovative regenerative medicine technique for preserving fertile potential in the pediatric age, focusing on the generation of stem cells from the gubernaculum testis, which is indispensable for the correct occurrence of testicular descent. Our results open new perspectives in tissue regeneration, highlighting that the *in vitro* obtainment of autologous stem-like cells could also be applied to those cryptorchid patients surgically treated in advanced age, supporting the future fertility potential of a broader set of patients.

CRediT authorship contribution statement

Sara Vinco: Writing – original draft, Methodology, Data curation, Conceptualization. **Giulia Ambrosini:** Methodology, Investigation. **Andrea Errico:** Writing – review & editing, Investigation. **Nunzio Marroncelli:** Writing – review & editing, Methodology. **Elisa Dalla Pozza:** Writing – review & editing, Supervision, Methodology. **Eleonora Matranga:** Methodology, Investigation. **Nicola Zampieri:** Writing – review & editing, Funding acquisition, Conceptualization. **Ilaria Dando:** Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Verona University Hospital- AOUI (*Azienda Ospedaliera Universitaria Integrata*)- Project: ANDRO-PRO- 4206CESC; date: April 26, 2024.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Funding information

This work was supported by Ministero dell'Università e della Ricerca (MUR), Rome, Italy.

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.

Acknowledgement

We thank Lions Clubs International- Isola della Scala and Bovolone (Italy)- for supporting our research. We also thank "Centro Piattaforme Tecnologiche" of the University of Verona (Italy) and American Academy of Pediatrics (Surgery Section) for the support.

Data availability

Data will be made available on request.

References

[1] Y. Wei, S.-d. Wu, Y.-c. Wang, T. Lin, D.-w. He, X.-l. Li, J.-h. Liu, X. Liu, Y. Hua, P. Lu, D.-y. Zhang, S. Wen, G.-h. Wei, A 22-year retrospective study: educational update and new referral pattern of age at orchidopexy, BJU Int. 118 (2016) 987–993, https://doi.org/10.1111/bju.13588.

[2] E. Martin Ritzén, A. Bergh, R. Bjerknes, P. Christiansen, D. Cortes, S. Haugen, N. Jörgensen, C. Kollin, S. Lindahl, G. Läckgren, K. Main, A. Nordenskjöld, E. Rajpert-De Meyts, O. Söder, S. Taskinen, A. Thorsson, J. Thorup, J. Toppari, H. Virtanen, Nordic consensus on treatment of undescended testes, Acta Pædiatrica 96 (2007) 638–643, https://doi.org/10.1111/j.1651-2227.2006.00159.x.

[3] N. Zampieri, D. Caridha, S. Patanè, F. Bianchi, E. Vestri, C. Bruno, F. Camoglio, Elastosonographic evaluation of the post-operative morpho-volumetric recovery of the gonad in the cryptorchid patient, American journal of clinical and experimental urology 7 (3) (2019) 182–187.

[4] C. Kollin, J.B. Stukenborg, M. Nurmio, E. Sundqvist, T. Gustafsson, O. Söder, J. Toppari, A. Nordenskjöld, E.M. Ritzén, Boys with undescended testes: endocrine, volumetric and morphometric studies on testicular function before and after orchidopexy at nine months or three years of age, J. Clin. Endocrinol. Metabol. 97 (12) (2012) 4588–4595, https://doi.org/10.1210/jc.2012-2325.

[5] G. Cobellis, C. Noviello, F. Nino, M. Romano, F. Mariscoli, A. Martino, P. Parmeggiani, A. Papparella, Spermatogenesis and cryptorchidism, Front. Endocrinol. 5 (2014) 63, https://doi.org/10.3389/fendo.2014.00063.

[6] C.I. Ciongradi, I. Sârbu, C.O. Iliescu Halițchi, D. Benchia, K. Sârbu, Fertility of cryptorchid testis-an unsolved mistery, Genes 12 (12) (2021) 1–11, https://doi.org/10.3390/genes12121894.

[7] K. Banks, E. Tuazon, K. Berhane, C.J. Koh, R.E. De Filippo, A. Chang, et al., Cryptorchidism and testicular germ cell tumors: comprehensive meta-analysis reveals that association between these conditions diminished over time and is modified by clinical characteristics, Front. Endocrinol. 3 (2012) 182, https://doi.org/10.3389/fendo.2012.00182.

[8] J.M. Hutson, T. Nation, A. Balic, B.R. Southwell, The role of the gubernaculum in the descent and undescent of the testis, Therapeutic advances in urology 1 (2) (2009) 115–121, https://doi.org/10.1177/1756287209105266.

[9] K.S. Ludwig, The development of the caudal ligaments of the mesonephros and of the gonads: a contribution to the development of the human gubernaculum (Hunteri), Anat. Embryol. 188 (6) (1993) 571–577, https://doi.org/10.1007/BF00187012.

[10] G. Ambrosini, E. Dalla Pozza, G. Fanelli, C. Di Carlo, A. Vettori, G. Cannino, C. Cavallini, C.A. Carmona-Carmona, J. Brandi, S. Rinalducci, M.T. Scupoli, A. Rasola, D. Cecconi, M. Palmieri, I. Dando, Progressively de-differentiated pancreatic cancer cells shift from glycolysis to oxidative metabolism and gain a quiescent stem state, Cells 9 (7) (2020) 1572, https://doi.org/10.3390/cells9071572.

[11] E. Dalla Pozza, I. Dando, G. Biondani, J. Brandi, C. Costanzo, E. Zoratti, M. Fassan, F. Boschi, D. Melisi, D. Cecconi, M.T. Scupoli, A. Scarpa, M. Palmieri, Pancreatic ductal adenocarcinoma cell lines display a plastic ability to bi-directionally convert into cancer stem cells, Int. J. Oncol. 46 (3) (2015) 1099–1108, https://doi.org/10.3892/ijo.2014.2796.

[12] X. Li, Z. Wang, Z. Jiang, et al., Regulation of seminiferous tubule-associated stem Leydig cells in adult rat testes, Proc. Natl. Acad. Sci. U. S. A. 113 (10) (2016) 2666–2671, https://doi.org/10.1073/pnas.1519395113.

[13] X. Chen, C. Li, Y. Chen, et al., Differentiation of human induced pluripotent stem cells into Leydig-like cells with molecular compounds, Cell Death Dis. 10 (2019) 220, https://doi.org/10.1038/s41419-019-1461-0.

[14] Andrea Errico, Francesco Saverio Camoglio, Nicola Zampieri, Ilaria Dando, Testicular torsion: preliminary results of in vitro cell stimulation using chorionic gonadotropin, Cells 11 (3) (2022) 450, https://doi.org/10.3390/cells11030450.

[15] T.T. Liao, M.H. Yang, Revisiting epithelial-mesenchymal transition in cancer metastasis: the connection between epithelial plasticity and stemness, Mol. Oncol. 11 (7) (2017) 792–804, https://doi.org/10.1002/1878-0261.12096.

[16] M. Angela Nieto, Ruby Yun-Ju Huang, Rebecca A. Jackson, Jean Paul Thiery, Emt, Cell 166 (1) (2016) 21–45, https://doi.org/10.1016/j.cell.2016.06.028. ISSN 0092-8674.

[17] J.E. Larsen, V. Nathan, J.K. Osborne, R.K. Farrow, D. Deb, J.P. Sullivan, et al., ZEB1 drives epithelial-to-mesenchymal transition in lung cancer, J. Clin. Invest. 126 (9) (2016) 3219–3235, https://doi.org/10.1172/JCI76725.

[18] S.J. Serrano-Gomez, M. Maziveyi, S.K. Alahari, Regulation of epithelial-mesenchymal transition through epigenetic and post-translational modifications, Mol. Cancer 15 (2016) 18, https://doi.org/10.1186/s12943-016-0502-x.

[19] H.T. Nguyen, L.J. Martin, Classical cadherins in the testis: how are they regulated? Reprod. Fertil. Dev. 35 (14) (2023) 641–660, https://doi.org/10.1071/RD23084.

[20] H.T. Nguyen, L.J. Martin, The transcription factors Junb and Fosl2 cooperate to regulate Cdh3 expression in 15P-1 Sertoli cells, Mol. Reprod. Dev. 90 (1) (2023) 27–41, https://doi.org/10.1002/mrd.23656.

[21] M.K. Siu, D.D. Mruk, W.M. Lee, C.Y. Cheng, Adhering junction dynamics in the testis are regulated by an interplay of beta 1-integrin and focal adhesion complex-associated proteins, Endocrinology 144 (5) (2003) 2141–2163, https://doi.org/10.1210/en.2002-221035.

[22] J. Schaller, H.J. Glander, J. Dethloff, Evidence of beta 1 integrins and fibronectin on spermatogenic cells in human testis, Hum. Reprod. (Oxf.) 8 (11) (1993) 1873–1878, https://doi.org/10.1093/oxfordjournals.humrep.a137952.