

## GENE EXPRESSION OF SPERM ASSOCIATED ANTIGEN 8 AND RAN-BINDING PROTEIN 9 ON AZOOSPERMIC MALE: ITS ASSOCIATION WITH SPERMATOGENIC ARREST

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### ARTIKEL INFO *ABSTRAK*

Diterima: 01 August 2022	<i>Proteins that play an important role in the transcription process during spermatogenesis are CREMs that bind to their ACT activators that are suspected to be regulated by SPAG8 and RANBP9. Until now the role of both genes in the spermatogenic arrest process is not known. This study aims to determine the relative expression of Spag8 and RanBP9 on spermatogenic arrest and to analyze the correlation of expression of both genes. This study is a cross sectional study using a sample of testicular biopsy with Johnsen 2 to 8 score. Relative expression analysis of Spag8 and RanBP9 using qRT-PCR technique with Livak calculation. The data obtained were analyzed statistically using ANOVA one way test for Spag8 and Kruskal Wallis test for RanBP9 with significance value <math>p &lt; 0,05</math>. The results of this study show that the relative expression of Spag8 and RanBP9 is highest on Johnsen 3 scores and is statistically significantly different (<math>p &lt; 0.05</math>). There is a positive correlation with a very strong correlation strength between SPAG8 and RANBP9 expressions. Based on the results of this study shows that both of these genes are candidates for spermatogenic arrest.</i>
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### Introduction

Infertility is one of the most high reproductive health problems today. In the general population, as many as 10-15% of couples have infertility, and 50% is caused by problems in men. One of the problems of male infertility is azoospermia, with a prevalence of 10-20%. Azoospermia defined as a condition in which there is no spermatozoa in semen or ejaculate on two separate cement analysis studies. One

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cause of azoospermia is a testicular disorder, may be a congenital aberration, acquired abnormality or an idiopathic disorder leading to a spermatogenic failure.

Spermatogenic failure includes spermatogenic arrest (maturation arrest). Spermatogenic arrest or arrest of germ cell maturation, may occur in the spermatogonia stage, primary spermatocytes, secondary spermatocytes and round spermatids. Spermatogenic arrest is a histopathological phenomenon with many possible causes. Patients with fertility disorder showed a spermatogenic arrest prevalence of about 4-30%, and approximately 23% showed spermatogenic arrest at the primary spermatocyte level and almost a third occurred in both testes (bilateral). Spermatogenic arrest is primarily caused by genetic problems such as trisomy, translocation, inversion or a deletion on the Y chromosome (Yq11). In addition, spermatogenic arrest may be caused by secondary factors, such as toxic (radiotherapy, chemotherapy, antibiotic), heat or common disease (liver or renal insufficiency, sickle cell anemia). Patients with complete spermatogenic arrest can be found in the azoospermic state, whereas in the case of partial spermatogenic arrest, oligoastenozoospermia occurs with varying degrees.

Spermatogenic arrest is diagnosed by examination of testicular biopsy, ie examining testicular histopathology and assessing by Johnsen assessment. Testicular biopsy may help predict the possibility of sperm finding in testicular microdissection. In a study conducted by Ramasamy et al, of 135 men examined, spermatozoa were found in 51% of men with

Sertoli-cell-only syndrome pattern, 83% in men with maturation arrest and 100% in men with hypospermatogenesis. In severe azoospermia and oligoastenozoospermia with normal FSH serum and normal testicular volume, Testicular biopsy is needed to distinguish the arrest from excurrent duct obstruction. Accurate biopsy interpretation is important in determining both the prognosis in terms of reproduction and treatment considerations for men with azoospermia.

Blendy et al. And Nantel et al (1996) identified that cAMP Response Element Modulator (CREM) as a key component of spermatogenic development in mice. CREM gene is expressed also in human germ cells and CREM $\tau$  mRNA is present in the round spermatid cytoplasm. The change in expression of the CREM repressor to the activator occurs in men with normospermic. According to Weinbauer et al (1998), no CREM expression or CREM expression was significantly reduced in patients with spermatogenic arrest at the spermatid round level, resulting in a lack of signal transduction factors. In the seminiferous tubules containing spermatid elongation, CREM expression can be detected consistently Novitasari research (2015) on testicular tissue with spermatogenic arrest, it is shown that increased CREM expression, ie CREM isoform  $\tau$  at the spermatid stage has no direct effect on triggering Spermatogenic arrest. CREM binds to the CAMP response element, further

modulating the transcription of genes reacting to cAMP, and regulating gene expression in spermatids. Inactivation of CREM gene in mice causes round spermatids to form, but these spermatids fail to elongate resulting in infertility. CREM transcription activity in the testis, independent of the presence of phosphorylation activity, but by interaction with a coactivator is the activator of CREM in the testis (ACT). Fimia et al (1999) reported that during spermatogenesis in mice and humans, there was an increase in the expression of CREM and ACT in spatial and temporal pairs. The CREM-ACT complex will mediate the expression of post-meiotic genes, which are important in normal spermatogenesis. The CREM-ACT complex regulatory pathway is essential for spermatogenesis. Wu et al (2010) reported a new regulator for ACT that is Sperm associated antigen 8 (SPAG8). SPAG 8 is a testicular specific protein produced during germ cell differentiation.<sup>15</sup> Using immunohistochemistry, Miao et al (1995), found SPAG8 expression, which they referred to as BS-84 (84-kD Beijing sperm) in the testes but not found in the brain, liver or kidney Wang et al (1999), analyzed using FISH, and successfully mapped the SPAG8 gene on chromosome 9p13-p12. Studies by Wu et al (2010) indicated that SPAG8 localized in spermatids as well as to the head and tail of sperm on mouse testis. This suggests the role of SPAG8 in germ cell differentiation. In spermatogenesis, SPAG8 plays a role in enhancing or strengthening CREM tau (CREM $\tau$ ) bonds with ACT co-activators or increasing transcriptional activation regulated by ACT activator of

CREM in testis. This suggests that SPAG8 acts as a regulator for ACT and plays an important role in mediating gene transcription during spermatogenesis.<sup>18</sup> SPAG8 is found to have a functional relationship close to microtubules, based on a study by Rong Li et al (2009), which suggests that SPAG8 is concentrated in microtubule-organizing center (MTOC) during prophase, on the microtubule spindle during metaphase, in the microtubule and central astral during anaphase, and remain in the middle region during telofase. During cytokinesis, SPAG8 is located on MTOC. This SPAG8 immunoreactivity pattern shows that SPAG8 localization is regulated during the cell cycle. The interaction between SPAG8 and ACT is estimated because of nuclear transport in microtubule-dependent patterns, in which SPAG8 is confirmed to be associated with RanBPM.

RANBP9 (RAN-Binding Protein 9) which is also called RanBPM (Ran Binding Protein microtubule organizing center), the size of 90 kD protein that plays a role in the transport nukleositolasmik, cell cycle regulation and the establishment of the aster microtubules during mitosis. With system yeast two-hybrid applications, have identified proteins that interact with HSMP-1 (SPAG8), and one of its components is RanBPM. In mice testes, RANBP9 expressed mostly in pachytene spermatocytes and spermatids. Weak staining was detected in somatic cells (Sertoli and Leydig cells). In human

testis, RanBP9 produced at the stage of spermatogonia and primary spermatocytes, indicating that this gene is expressed during the early stages of spermatogenesis, while in mice testes, the gene is located on the round spermatids and spermatid elongation, as well as SPAG8, indicating that the two components are expressed during spermiogenesis.

RanBPM has a crucial role in the initiation and maintenance of spermatogenesis. After completing gonococcal migration of the gonads, RanBPM is expressed in germ cells. At birth, RanBPM expression is primarily confined to gonocytes. At two weeks of age, expression is detected in both spermatogonia and sertoli cells. In adult mice, there is a pattern of expression that depend on the stage of the seminiferous tubules, thus showing RanBPM expression is dynamically regulated during spermatogenic wave.

Through this research, we want to know how expression of SPAG8 and RANBP9 on spermatogenic arrest events and analyze the relationship between the expression of these genes with the criteria Johnsen and expression in the seminiferous tubules in the testis biopsy preparations.

## Research methods

### A. Deparaffinization

Tissue samples from testicular biopsy in the paraffin blocks prepared and had been classified by histology based on Johnsen assesment. The procedure of sample deparaffinization in paraffin block is as follows: the tissue in the paraffin block is cut with the scapel and then inserted into 1.5 mL microcentrifuge tube, then added xylol as much as 800  $\mu$ L and left for 5 minutes.

Subsequently, 400  $\mu$ L of absolute ethanol was added to the mixture, centrifuged for two minutes at a maximum speed (13,500 rpm). Centrifugation results will be obtained pellets and supernatant. The supernatant was discarded and then 400  $\mu$ L of absolute ethanol was added and centrifugation was continued for two minutes at maximum speed and discarded the supernatant. The tube is turned briefly on tissue paper to remove the ethanol residue. The tissue pellet is then dried for 10 minutes at 65°C and the tissue is ready to proceed to the RNA isolation stage.

### B. Isolation of RNA

RNA isolation was performed using Rneasy FFPE kit (Qiagen, Germany) according to the protocol. As for the RNA isolation procedure from the deparaffinization tissue as follows: Added 150  $\mu$ L or 240  $\mu$ L PKD buffer, then mixed by vortex, then centrifuged for 1 minute at 11,000 xg (or 10,000 rpm). Next, 10  $\mu$ L proteinase K was added to the clear phase at the bottom of the tube and then mixed by pipetting up and down. It was incubated at 56°C for 15 minutes, then at 80°C for 15 minutes. The bottom part of the colorless phase is transferred into a new 1.5 mL microcentrifuge tube then incubated on ice for 3 minutes and centrifugation for 15 minutes at 20,000 xg (13,500 rpm). The supernatant is transferred into a new microcentrifuge tube carefully not to have any pellets removed. Dnase booster buffer was

added to the amount of volume obtained from the previous stage with total sample volume approx 16  $\mu\text{L}$  and 10  $\mu\text{L}$  Dnase 1 stock solution. Mix it by flipping the tube, then spindown to collect the liquid residue from the sides of the tube. Next incubate at room temperature for 15 min, then add 320  $\mu\text{L}$  RBC buffer, and mix the lysates. Next, add 720  $\mu\text{L}$  ethanol (100%) to the sample, mixed with a pipette. Subsequently, 700  $\mu\text{L}$  samples were transferred to the Rneasy MinElute spin column, the tube was closed and centrifuged for 15 seconds at a speed of  $\geq 8000 \text{ xg}$  ( $\geq 10,000 \text{ rpm}$ ). The filtrate inside the collect tube is discarded. The stages are repeated until the entire sample passes through the Rneasy MinElute spin column. 500  $\mu\text{L}$  RPE buffer was added to the Rneasy MinElute spin column, centrifuged at  $\geq 10,000 \text{ rpm}$  for 15 seconds, then the filtrate was discarded and the collect tube reused, adding 500  $\mu\text{L}$  RPE Buffer into the Rneasy MinElute spin column and centrifugation  $\geq 10,000 \text{ rpm}$  for 2 minutes to clean the spin column membrane. Then place the Rneasy MinElute spin column on a new 2 mL collect tube, spin column cover opened and centrifuged full speed for 5 minutes. Place Rneasy MinElute spin column on a 1.5 mL or 2 mL tube, add 14-30  $\mu\text{L}$  Rnase-free water into the spin column, cover slowly and centrifuge for 1 min at maximum speed to elute the RNA.<sup>61</sup> RNA elution can be directly used for PCR process or store at  $-20^\circ\text{C}$  or  $-80^\circ\text{C}$  for old storage. To check the concentration and purity of the RNA, RNA elute first centrifuged for 2 minutes at maximum speed then transfer the supernatant to the new 1.5 mL reaction tube.

Checking the concentration and purity of RNA using Nanodrop Maestrogen is quite important, before performing cDNA synthesis. RNA is considered pure if the purity is  $\geq 2$ . Electrofresis is performed to determine the quality of RNA. A good RNA result will produce two bands of 28S and 18S.

### C. cDNA synthesis

The total amount of RNA from the testicular tissue is extracted with ReverTra Ace® qPCR reagent RT Master Mix with gDNA remover (Toyobo) in accordance with the protocol. The kit-based procedure for performing cDNA synthesis is as follows: the whole kit the synthesis of divortex and dipentrifus cDNAs before use. Samples in the form of RNA, a number of calculations based on RNA concentration, were fed into 0.2 mL PCR tube and incubated at  $65^\circ\text{C}$  for 5 mins. The RNA sample was then added with 4xDN Master Mix which was mixed with gDNA remover, also added with Nuclease Free Water up to total volume of 16  $\mu\text{L}$ , then incubated at  $37^\circ\text{C}$  for 5 mins. 5xRT Master Mix II was added, then incubated at  $37^\circ\text{C}$  for 15 mins, followed by incubation at  $50^\circ\text{C}$  for 5 mins then the reaction was stopped by incubation at  $98^\circ\text{C}$  for 5 mins, then chill on ice. Samples are then stored at  $-20^\circ\text{C}$  for prolonged storage or as soon as possible with qPCR.

### D. Primer Design

The primers used for SPAG8 and RanBP9 genes are designed based on

sequences derived from the ncbi database. Primer used to amplify SPAG8 genes using Primerquest software from IDT by considering good primer conditions such as primary length 18-30 bp, G/C base content composition between 40%-60%, avoiding dimer-hairpin formation, and primer produced must be specific. Primary specificity is confirmed by using the Basic Local Alligment Tool (BLAST).

Primers obtained for SPAG8 gene are forward (F) 5'-GAC-ATT-AAA-GGG-AAG-T-ATG-G-3' and reverse (R) 5'-TGG-C-T-T-C-3'. Primary obtained for RanBP9 gene ie forward (F) 5'-G-TCC-ATT-AAG-AAT-AGA-CA-3' and reverse (R) 5'-TAC-CCA-AGT-TTA-T-GAA-AG-3'. GAPDH primer (glyceraldehyde-3-phosphate dehydrogenase) gene as a reference gene that functions for the normalization of the value (Cycle threshold), with the primer sequence of the Forward (F) is 5'-GAA-ATC-CCA-TCA-CCA-T-TCC-AGG-3' and the reverse (R) primer is 5'- GAG-CCC-CAG-CT-CCA-TG-3' (Bao et al., 2014).

#### **E. Gene expression analysis of SPAG8 and RanBP9 genes with qPCR technique**

The qPCR technique is used to measure the quantity of SPAG8 and RANBP9 mRNAs in each study sample group expressing the mRNAs of both genes. Primers used in qPCR for SPAG8 and RANBP9 gene expression as target genes are primers that produce 75 pb and 104 bp amplicons of cDNA. The GAPDH gene primer produces a 120 bp amplicon cDNA. Amplification of qPCR expression is done using KAPA SYBR® FAST qPCR

Universal Master Mix (2X) Kit. The qRT-PCR process begins by performing a singleplex reaction with two replicates (duplo) for each with a total volume of 20 µL in a 96-wells optical reaction plate. Each qPCR reaction consists of 10 µL 2X KAPA SYBR® FAST qPCR Master Mix2 Universal, 0.4 µL 50X ROX Low, 0.4 µL primary forward, 0.4 µL reverse primer and 3 µL cDNA template. The primary addition depends on the type of gene to be detected.

All qPCR components are incorporated into 96-wells optical reaction plate and covered with optical adhesive cover which is then detected qPCR using Real Time PCR Applied Biosystems® 7500 machine. As controls are included negative controls without no template control (NTC) for knowing the occurrence of contamination during mixing of qPCR reagent, then amplification process begins with one denaturation cycle at 95 ° C for 3 minutes, then continued with one cycle 95 ° C for 3 second, followed 40 repeat cycle, and annealing process At 60 ° C for 30 seconds and elongation at 72 ° C for 30 seconds (Zhu et al., 2015).

The mRNA expression on each sample transcript was calculated using the mean value and predetermined efficiency, then normalized with the efficiency of the reference gene PCR (GAPDH) and the sample used as the control / calibrator (rating 8) in each sample, using the Livak formula, ie

with the formula  $N = 2^{-\Delta\Delta Ct}$ . N is the ratio of expression of the target gene to the reference gene, the value 2 indicates the level of efficiency established in the Livak expression method. The value of  $\Delta\Delta Ct$  is obtained from the difference of sample  $\Delta Ct$  value with the value of  $\Delta Ct$  control and  $\Delta Ct$  value obtained from the difference of target gene value with the reference gene value.

The research sample used is testicular tissue in azoospermic men who had done testicular biopsy at Urology Department of RSCM-FKUI Jakarta, which then made histology preparation for Johnsen assessment in Department of Medical Biology Faculty of Medicine during period of 2014 - 2017. The sample groupings were based on Johnsen's assessment criteria based on the type of cells present in each seminiferous tubule.

## Results and Discussion

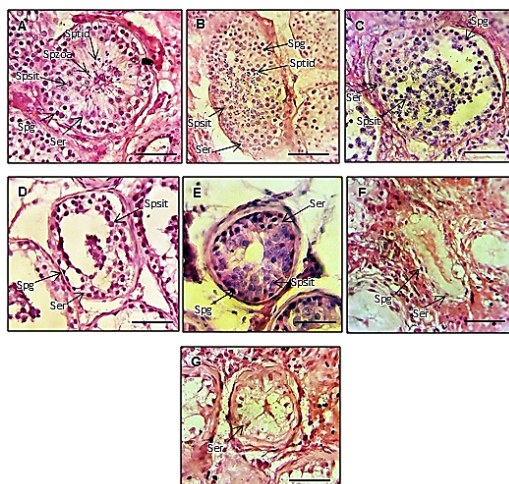
### A. Characteristic of Research Sample

**Table 1**  
**Characteristics of research sample**

<b>Sample assesment</b>	<b>Average samples age</b>	<b>Samples amount</b>
Scoring 2	34 y.o	5 Samples
Scoring 3	36 y.o	5 Samples
Scoring 4	45 y.o	4 Samples
Scoring 5	35 y.o	8 Samples
Scoring 6	36 y.o	8 Samples
Scoring 7	40 y.o	9 Samples
Scoring 8(C)	36 y.o	2 Samples
<b>TOTAL</b>		<b>41 Samples</b>



## B. Seminiferus tubules histopathology based on Johnsen assessment



**Figure 1**  
**Seminiferus tubule based on Johnsen assessment**

## C. Molecular analysis RNA Isolation

RNA isolation has been performed from 41 samples of testicular tissue in paraffin blocks derived from men with azoospermia. The concentration and purity of RNA was measured using Nanodrop Maestrogen at wavelengths of 260 and 280 and yielded a mean RNA purity of all samples that was 1.75. Eluat of RNA was stored at  $-80^{\circ}\text{C}$  for further processing.

## D. cDNA synthesis

Synthesis of cDNA from the previous RNA isolation process and the result is stored at  $-20^{\circ}\text{C}$  for further use in qPCR technique. The average cDNA concentration obtained from all samples in this study was  $994\text{ ng} / \mu\text{L}$  with mean purity of cDNA 2.49

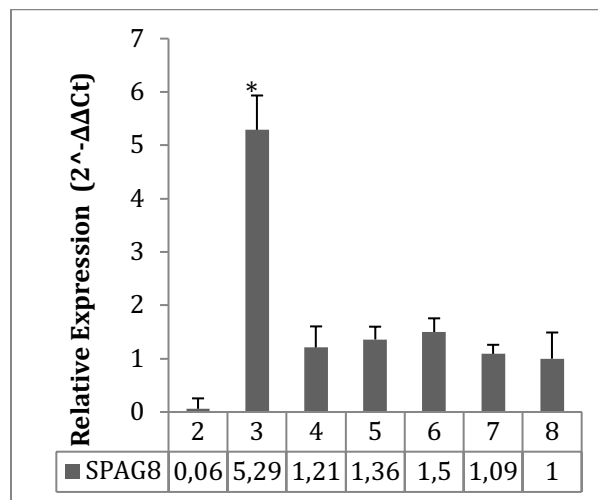
## E. Expression Change Analysis in Spag8 and Ranbp9 Genes by qPCR technique

mRNA expression of Spag8 gene generated 5.3 times more than others at Johnsen's assessment (Kierszenbaum et al., 2002) These results are consistent with studies conducted by Zhang et al (2000) that in human testes, hSMP-1 or SPAG8 is detected in spermatogonia, which means that SPAG8 is involved in the early stages of spermatogenesis. In spermatogenesis process, scoring 3 is a stage where only spermatogonia as germ cells (Fimia et al., 1999). Diploid spermatogonia cells develop into primary spermatocytes via mitosis.<sup>24</sup> Mitosis consists of five phases, namely prophase, prometaphase, metaphase, anaphase and telophase. Immunofluorescence with confocal microscope shows that localization of SPAG8 and microtubules very closely regulated by the cell cycle

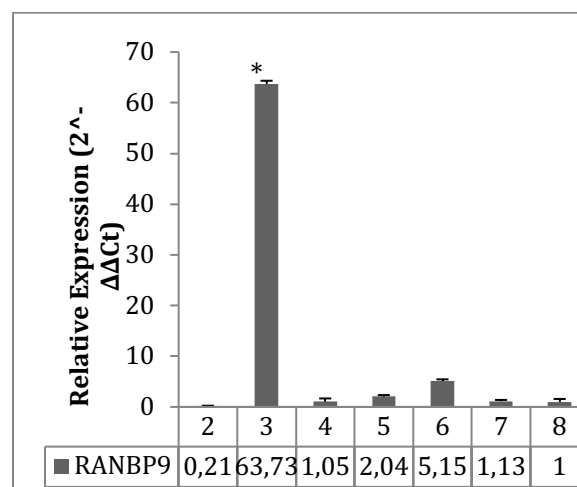


shows that SPAG8 is involved in spindle formation during cell division processes. This SPAG8 localization shift is associated with dynamic  $\alpha$ -tubulin relationships temporally and spatially during cell cycles (Monaco et al., 2004). Data for both of genes (Spag8 and Ranbp9) gene expression has normal distribution, so ANOVA oneway test is done. For Spag8 gene, the significance value with ANOVA

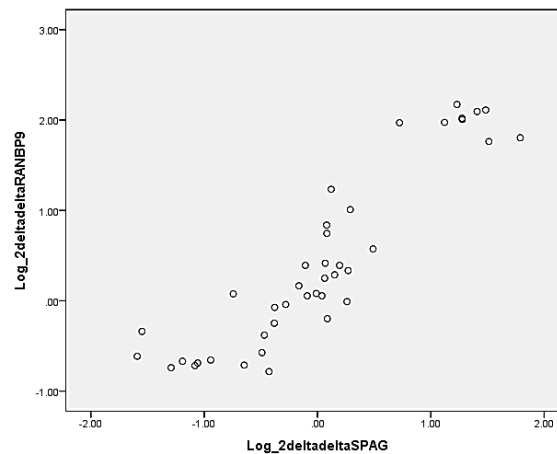
indicates the number  $p < 0.05$  which means that there are significant differences in each Johnsen assessment. For ranbp9 gene, with ANOVA oneway obtained  $p < 0.05$ . To know the correlation between the expression of the two genes, used Pearson correlation test, and the results show that there is a positive correlation with a strong correlation strength (Wang et al., 1999)



**Figure 2**  
Relative expression of Spag8 gene mRNA



**Figure 3**  
Relative expression of Ranbp9 gene mRNA



**Figure 4**  
**Positive correlation and strength correlation**  
**between SPAG8 gene and RANBP9 gene**

## Discussion

Statistical analysis showed that there were significant differences in Spag8 gene expression in each Johnsen rating ( $p < 0.05$ ). These results are consistent with the study conducted by Zhang et al (2000) that in human testes, hSMP-1 or SPAG8 is detected in spermatogonia, which means that SPAG8 is involved in the initial stages of spermatogenesis. In the process of spermatogenesis, assessment 3 is the stage where there is only spermatogonia as germ cells. Spermatogenesis involves a series of cell division phases in which diploid spermatogonia cells develop into primary spermatocytes through the process of mitosis. Mitosis involves the proliferation of spermatogonium and spermatocytes in the basal section. In the mitosis phase, primitive spermatogonia develops to produce several generations of successive spermatogonia (Murrin & Talbot, 2007) Mitosis consists of five phases namely prophase, prometaphase,

metaphase, anaphase and telophase. Immunofluorescence with Confocal microscopy shows that SPAG8 is concentrated in the central organizing microtubules, Microtubules-Organizing Center (MTOC) during prophase. As the cell develops into metaphase, SPAG8 immunoreactivity is along the spindle of the microtubules and joins completely with  $\alpha$ -tubulin on its spindles and poles. During anaphase, SPAG8 is detected in both the astral microtubules and the middle zone. At the time of telophase, SPAG8 was still in the middle zone. After cytokinesis, SPAG8 returned to MTOC. In other words, the localization of SPAG8 and microtubules is very tightly regulated by the cell cycle indicating that SPAG8 is involved in spindle formation during the process of cell division. This shift of localization of SPAG8 is associated with dynamic  $\alpha$ -tubulin relationships temporally and spatially during

the cell cycle. The central event during mitosis is chromosome segregation, depending on the mitotic spindle. The core of the mitotic spindle is a microtubule composed of bipolar, with negative ends (minus ends) focused on the two poles of the spindle, and positive ends (plus ends) radiating out from the poles. The positive ends of several microtubules, called interpolar microtubules, interact with the ends of the microtubules from other poles, producing an antiparallel arrangement in the middle of the spindle. The positive end of the other microtubules, the kinetochore microtubules, attach to sister chromatids in pairs on large protein structures called kinetochores, which are located in the centrosomes of each sister chromatid. Finally, many spindles contain astral microtubules which radiate out of the pole and make contact with the cell cortex, helping position the spindle in the cell. Each spindle pole is focused on organelle proteins called centrosomes (Bao et al., 2014) During spermatogenesis, an extraordinary rearrangement of the microtubule cytoskeleton to transform apolar spermatogonia into polarized spermatozoa. Li et al. research shows that the localization of SPAG8 in human testes is present at all stages of spermatogenesis. This suggests that SPAG8 plays a role in cell division during spermatogenesis.<sup>18</sup> Increased spermatogonium proliferation activity by involving this mitotic spindle is thought to increase the relative expression of SPAG8 genes. Research by Wu et al. (2010) shows that SPAG8 has the potential to increase ACT binding in CREM $\tau$  and activate transcription. These results show that

SPAG8 is involved in transcription regulation pathways. CREM is known to modulate transcription of genes that react to cAMP and regulate gene expression in spermatids. CREM mediates the transcription of many postmeiotic genes such as Tnp1, Tnp2, Prm1 and Prm2 by binding to the cAMP response element (CRE) of the promoter. These gene products are responsible for chromatin remodeling and spermatid elongation.<sup>21</sup> In experimental animals, the expression of CREM (activator) occurs postmeiosis of germ cells, whereas at the time of premeiosis, what is expressed is the CREM repressor. CREM repressors act by binding unproductively CRE or by forming inactive heterodimers with CREB. Research by Peri et al (1998) shows that loss of transition from CREM repressor to CREM activator  $\tau$ 1 is characterized by impaired spermatogenesis or in other words, the absence of CREM switch can be associated with spermatogenic arrest. In a preliminary study by Novitasari (2015) it was found that CREM isoform expression  $\tau$  increased during Johnsen 7 assessment or at the spermatid stage in the process of spermatogenesis. The transcription activity played by CREM $\tau$  could take place by an increase in the expression of the gene, but this activity did not occur. The results of this study indicate that there is a relatively low expression of the SPAG8 gene mRNA at the spermatid stage of the spermatogenesis process or on the Johnsen 7 evaluation. Ranbp9 gene mRNA expression increased at Johnsen 3 assessment by 93.3 times. Statistical analysis showed that there were significant differences in Ranbp9 expression in each Johnsen assessment. The results of

the study of the relative expression of mRNA from the Ranbp9 gene are consistent with results submitted by Tang et al. (2004) that Ranbp9 is also expressed in human testicular germ cells and is formed during the division of spermatogonia mitosis and its differentiation process into spermatozoa (Blendy et al., 1996)

RANBP9 is a family of proteins that bind Ran.<sup>22</sup> RANBP9 is also called RanBPM, which is a Ran binding protein at the center of the microtubule organization.<sup>48</sup> Ran, is a Ras protein associated with GTP-ase, needed to induce spindle formation by chromosomes in the mitotic phase. High RanGTP concentrations are expected to surround M phase chromatin.<sup>56</sup> During mitosis, there is an increase in microtubule instability, whereby individual microtubules can grow or shrink and stochastically switch between the two. The transition from growth to depreciation is called catastrophe, and the transition from shrinkage to growth is called rescue (Cerilli et al., 2010).

New microtubules are continuously created to balance their loss that is completely disappeared by depolymerization. The dynamics of microtubule polymerization are very important for biological function (Macho et al., 2002) This dynamic allows microtubules to adopt spatial arrangements that can change rapidly in response to cellular requirements and in some cases, to do mechanical work. Increased microtubule instability is coupled with an increase in the ability of the centrosome to nucleate microtubules, resulting in a very dense and dynamic array of microtubule spindles. ideal

for capturing sister chromatids (Gudeloglu & Parekattil, 2013)

Two classes of proteins regulate microtubule dynamics in mitosis. Catastrophic factor proteins that cause destabilization of the microtubule array by increasing the catastrophe frequency. One of these proteins is kinesin-related protein which does not function as a mobilizer (Nieschlag et al., 2010)

The second protein, microtubule-associated proteins (MAPs) has the opposite effect of stabilizing microtubules in various ways: it can increase the frequency of rescue, where microtubules switch from shrinkage to growth. Changes in catastrophic factors and MAPs can make microtubules become more dynamic in the M phase by increasing total depolymerization of microtubules and reducing total microtubule polymerization. In addition, Ran is also needed to transport proteins in and out of the nucleus during interphase, as well as for the unification of microtubules (Weinbauer et al., 1998)

Ran-GTPase provides free energy directly for nuclear transport. Cells regulate the transport of nuclear proteins and RNA molecules through nuclear pore complexes (NPC) by controlling the access of these molecules to the transport machine.<sup>67</sup> Imports of nuclear proteins via NPC in the nucleus increase the need in the cell. Cells obtain energy for these needs by hydrolyzing GTP. Ran is found both in the cytosol and the nucleus, and is needed for import and export of the nucleus. In addition to spermatogonia, RANBP9 is also produced in primary spermatocytes. This shows its expression during the early stages of

spermatogenesis.<sup>23</sup> But in this study the expression of Ranbp9 on Johnsen 4's assessment has decreased. Johnsen's assessment 4 is the stage in which there is little spermatocytes (Nieschlag et al., 1997)

The stages of spermatocytes are the stages of meiotic division. Crucial to the meiosis stage is producing haploid germ cells, which are genetically different from each other (Ramasamy & Schlegel, 2007). Decreased Ranbp9 expression at this stage seems to be due to the meiosis stage where the process of recombination of genetic material occurs and the reduction in the number of chromosomes. Diploid cells proliferate with the division of mitosis and haploid cells formed when meiosis does not proliferate. There was a positive correlation with the strength of a very strong correlation between the spag8 gene mRNA expression and the Ranbp9 gene (Li et al., 2009) A positive correlation shows that, the higher the relative expression of the Spag8 gene's mRNA, the higher the relative expression of the Ranbp9 gene's mRNA. This is consistent with what was stated by Tang et al (2004) that SPAG8 and RANBP9 were found to be associated both in vitro and in vivo conditions (Franco et al., 2015)

RANBP9 was involved in nucleating microtubules, where SPAG8 and microtubules were interrelated components at the center of the microtubule organization (Wu et al., 2010) This result is also in accordance with the conjecture of Wu et al (2010) which shows that the transition pattern of SPAG8 expression in germ cells is supported by the presence of shuttling activity because its association with RANBP9 which plays a role in nuclear

transport depends on Ran (Dohle et al., 2012)

The association between SPAG8 and RANBP9 occurs mainly at the beginning of spermatogenesis, ie at spermatogonia, or at the stage of premeiosis. In the spermatogonia stage, CRR isoform mRNA repressors are expressed in low levels, whereas during meiosis CREM activators are very high expressed. In the postmeiosis stage, only the CREM activator form is expressed (Martin-du Pan & Campana, 1993)

The size of the repressor isoform is shorter and the loss of exons (resulting from alternative cuts, alternative initiation codons, and alternative polyadenylation) contributes to the reactivation, terminus N of proteins. The CREM $\alpha$  repressor binds to the CRE site, which is defined by the palindromic site (TGACGTCA) on the promoter. Tenbrock et al. (2006) showed that CREM $\alpha$ , which is a CREM repressor, interacts with and recruits HDAC1 in gene promoters. The recruitment of HDAC1 leads to the occupation of the promoter region of at least two genes by nonacetylation histones, which are known to lead to the closure of the chromatin structure and limited transcription activity. In the Johnsen 2 assessment in this study a relatively low relative expression of the SPAG8 mRNA gene was obtained. In Johnsen 2's assessment, seminiferous tubules only contain Sertoli cells which are somatic cells (Miao, 1995) Transcription activity carried out by somatic cells is different from transcription activity by germ cells, which in germ cells do not require the presence of phosphorylation, but through bonding with the CREM activator in the

testis (ACT), so that the relative expression of the SPAG8 gene mRNA and the relative expression of mRNA the RANBP9 gene will start to increase if there are germ cells, especially if there are only spermatogonia cells in the seminiferous tubules ([Barbotin et al., 2017](#))

By the time spermatogonia has proliferated into primary spermatocytes at Johnsen 4's assessment, the relative expression of both genes begins to decrease. Primary spermatocytes are the stages of meiosis in the process of spermatogenesis. During the meiosis stage, only CREM activators are expressed. The increase in CREM activator expression should be followed by the increase in SPAG8 relative expression and RANBP9 relative expression. The low expression of both genes is thought to be due to the expression of other genes that help regulate the formation of primary spermatocyte cells ([Xu et al., 2007](#))

When spermatids begin to appear in the seminiferous tubules, the expression of both genes increases but not significantly, so it is not strong enough to increase the activity of the CREM activator. This is thought to result in spermatogenic arrest, because during the spermatid stage, proteins are needed for the transition to the spermatozoa, but there is no adequate protein formed during the premeiosis stage, due to the presence of CREM repressors that causes the closure of the chromatin structure. It is not yet known how the role of SPAG8 in CREM repressors is based on the available literature, SPAG8 is thought to only support the work of CREM activators.

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