From the Department of Biomedical Science

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The relationship between proAKAP4 concentration and sperm fertilization ability in mice

Bachelor Thesis

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Summary

The sperm protein AKAP4 is a highly conserved scaffold protein in mammals. It is responsible for the anchoring of cAMP-dependent protein kinases and plays an essential role in the assembly of the fibrous sheath of the mouse sperm principal piece.

AKAP4 and its precursor protein proAKAP4 have been localized in reptiles and different mammalian species, including humans and have been specifically studied in their role in sperm motility and fertilization capacity. Some of these studies showed a positive correlation between proAKAP4 concentration in sperm and sperm motility and fertility, which is why proAKAP4 has been suggested as a possible fertility marker.

This study aims to reveal if proAKAP4 could be used as a fertility marker in mice. For this purpose, frozen epididymal mouse sperm samples from four different strains (C57BL6/N, BALB/c, B6D2F1 and SWISS) were thawed and used in *in vitro* fertilization (IVF) assays. Respective sperm samples were classified as having either a low, medium, or high proAKAP4 concentration, and it was tested whether IVF outcome (two-cell rate and blastocyst rate) was predicted by proAKAP4 concentration group. We hypothesized that there would be a positive relationship between proAKAP4 concentration and sperm fertilization ability and that proAKAP4 might represent a suitable marker for male fertility in mice.

We found a significant effect of proAKAP4 concentration group on IVF outcome. Two-cell rates were significantly reduced in the low compared to the medium or high concentration group. We found no difference in IVF outcome between the medium or high concentration group. Thus, our results show that proAKAP4 could be used as an indicator of the fertilization ability in mouse sperm.

Zusammenfassung

Das Spermienprotein AKAP4 ist ein, in Säugetieren, hochkonserviertes Gerüstprotein. Es ist für die Verankerung von cAMP abhängigen Protein Kinasen verantwortlich und spielt eine wesentliche Rolle bei der Zusammensetzung der fibrösen Hülle des Hauptstück des Mäusespermiums.

AKAP4 und sein Vorläuferprotein proAKAP4 wurden in Reptilien, verschiedenen Säugetierarten, inklusive dem Menschen lokalisiert und besonders in ihrer Rolle in der Spermienmotilität und Befruchtungsfähigkeit untersucht. Einige dieser Studien zeigten eine positive Korrelation zwischen der proAKAP4 Konzentration in den Spermien und der Spermienmotilität und -fertilität, weswegen proAKAP4 als möglicher Fertilitäts-Marker vorgeschlagen wurde.

Diese Studie soll zeigen, ob proAKAP4 als Fertilitäts-Marker bei Mäusen verwendet werden kann. Dazu wurden gefrorene Mausspermaproben von vier verschiedenen Stämmen (C57BL6/N, BALB/c, B6D2F1 und SWISS) aufgetaut und für *in-vitro* Fertilisation (IVF) verwendet. Die Spermaproben wurden entsprechend ihrer proAKAP4 Konzentration als niedrig, mittel oder hoch klassifiziert, und es wurde getestet, ob das IVF-Ergebnis (Zweizellrate und Blastozystenrate) von der proAKAP4-Konzentrationsklasse prognostiziert werden kann. Wir stellten die Hypothese auf, dass es eine positive Beziehung zwischen der proAKAP4-Konzentration und der Spermienbefruchtungsfähigkeit geben würde und dass proAKAP4 ein geeigneter Marker für die männliche Fertilität bei Mäusen sein könnte.

Wir fanden einen signifikanten Effekt der proAKAP4-Konzentrationsklasse auf die IVF-Ergebnisse. Die Zweizellraten waren in der niedrigen Konzentrationsklasse signifikant geringer als in der mittleren oder hohen Konzentrationsklasse. Wir fanden keine Unterschiede in den IVF-Ergebnissen zwischen der mittleren und der hohen Konzentrationsklasse. Somit zeigen unsere Ergebnisse, dass proAKAP4 als Indikator für die Befruchtungsfähigkeit von Mäusespermien eingesetzt werden könnte.

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1 Introduction

1.1 Mouse models and fertility

Laboratory mice (*Mus musculus*) share 99 % of their genes with humans and are easy to breed and maintain in captivity, making them a well-suited model organism to study human diseases and conditions. Nowadays, a lot of disease models can be generated using modern genetic manipulation techniques (1,2). Many different inbred, hybrid and outbred mouse strains, including genetically modified mouse lines, are now available thanks to assisted reproductive techniques such as embryo transfer, sperm or embryo cryopreservation and *in vitro* fertilization (IVF). The effectiveness of these techniques continues to improve as our understanding of the processes responsible for male fertility, particularly sperm function, increases. Epidemiological studies have shown that the rate of infertility in humans has increased in recent decades, with male subfertility being the cause of approximately 40-50 % of childless couples (3). Most advances in understanding the genetics and pathophysiology of human infertility have been made by studying the reproductive physiology and genetics of mice (3,4). Reduced sperm motility (asthenozoospermia) is one of the predominantly contributing factors to male infertility and therefore it is crucial to understand the mechanisms that regulate flagellar movement (5).

1.2 A-kinase-anchoring proteins (AKAP)

Over 50 different A-kinase-anchoring proteins (AKAP) have been discovered and named (6). They are a group of scaffolding proteins diverse in structure but similar in function. They assist the organization of phosphorylation and dephosphorylation events by sequestering enzymes like protein kinases and phosphatases and combine signal transduction and signal termination molecules. AKAPs form multi-protein complexes as one of the molecules that accompanies the activation of protein kinase A (PKA) which is activated by cyclic adenosine monophosphate (cAMP) (7). They consist of a conserved amphipathic helix with a hydrophobic face that binds PKAs through interaction with an N-terminal four-helix bundle, found in the regulatory subunit dimer (6).

For the male reproductive system, AKAPs are very important, especially during gametogenesis. Several isoforms, some of them unique to the testis, have been found in male germ cells and

mature spermatozoa. They are involved in spermatogenesis and hold functions of mature spermatozoa, like sperm motility (8). AKAP3 and AKAP4 are structural proteins located in the principal piece of mature mouse spermatozoa (9). AKAP3 plays a role in the basic structure, while AKAP4 is important for the complete assembly of the fibrous sheath (10).

1.2.1 *Akap4*, AKAP4 and proAKAP4

AKAP4 (formerly known as p82 and AKAP82) is highly conserved in mammals as a scaffold protein for the anchoring of cAMP-dependent protein kinases, plays an essential role in the assembly of the fibrous sheath, a unique cytoskeletal structure surrounding the axoneme and outer dense fibers in the principal piece region of the flagellum (9,11,12) and is the product of the X-linked gene *Akap4* (13). *Akap4* is only expressed in spermatids in the postmeiotic phase of spermatogenesis (14,15). AKAP4 leads PKA to the fibrous sheath to facilitate phosphorylation of neighboring proteins that regulate flagellar function (16). ProAKAP4 (formerly known as proAKAP82) is the precursor to AKAP4 and owns an extra sequence called the prodomain (17). This prodomain contains 188 amino acids and is released during sperm differentiation in mice (18).

1.2.2 AKAP4 and proAKAP4 as fertility biomarkers

AKAP4 and its precursor protein proAKAP4 were localized in several animal species (equine, bovine, camelid, canine) including humans using immunohistochemistry and ELISA, and in particular, their relationship to sperm motility and fertility was examined. Several studies showed a positive correlation between proAKAP4 level and sperm motility and even fertility (19–24). When post-thawed stallion semen samples were investigated, the concentration of proAKAP4 was correlated to both total and progressive motility (21). This observation was also made in dog spermatozoa (19) and Malo et al. stated a strong positive correlation between proAKAP4 concentration and total motility in dromedary sperm (23). Similarly, the expression of AKAP4 was significantly higher in high fertility bull spermatozoa (22) and proAKAP4 concentration was significantly higher in bulls with a higher Non-Return-Rate (the proportion of cows that are not subsequently re-bred within a specified period of time after an

insemination) (24). Dobrynin et al. suggest that AKAP4 dysfunction might cause extremely high rates of pleiomorphic sperm in cheetahs (20). Human AKAP4, formally known as human A-kinase-anchoring protein 82 (hAKAP82), and proAKAP4 were characterized as major proteins of the sperm fibrous sheath. In contrast to mice, there was no detection of a phosphotyrosine-containing hexokinase in human sperm (25). The absence or low expression of proAKAP4 and AKAP4 has been linked to weak sperm motility and fibrous sheath dysplasia in men (26). Defective AKAP4 was also reported to be related to asthenozoospermia in men and may explain cases of infertility with multiple morphological abnormalities of the flagellum (27). Therefore, it was suggested that proAKAP4 could be a fertility marker (21,22,28–33).

1.3 The 3 R's and the need for Biomarkers

Since William Russel and Rex Burch presented their concept of the 3Rs (replacement, reduction, and refinement) in 1959, their idea has gained acceptance worldwide and has become the foundation for all national and international regulations to protect laboratory animals. Besides the replacement of living animals used in experiments whenever possible, their work called for the reduction of the number of animals used in an experiment and the refinement of the experimental procedure itself (i.e., minimizing the pain, suffering and distress imposed on the animals) (34).

If there is a positive correlation between proAKAP4 concentration in epididymal sperm and IVF outcomes, proAKAP4 concentration could be used in mice as a marker for the suitability of a sperm sample for IVF. This would be an important addition to the existing predictive capabilities based on fresh sperm motility values, which are routinely determined during cryopreservation of mouse sperm. The exclusion of unsuitable sperm samples for IVF or the selection of highly fertile samples would avoid an unnecessary use of females as oocyte donors and would make an important contribution to reducing the number of laboratory animals.

1.4 Aim of study

In mice, it was demonstrated in an AKAP4 knock-out mouse model that the protein plays an indispensable role in spermatogenesis (35), but to our knowledge, no study has investigated the

relationship between proAKAP4 concentration and fertilization ability in laboratory mice, the most used model organism in biomedical research. Given the broad application of *in vitro* fertilization in mice, a reliable sperm quality marker would allow selecting highly fertile males as sperm donors, thereby reducing the number of females needed and contributing to 3R.

In this study, we performed IVF assays using sperm samples of males with known proAKAP4 levels. We classified sperm samples as having either a high, medium or low level of proAKAP4 and assessed whether IVF outcome differ between proAKAP4 groups. We hypothesized that IVF rates would be lowest in sperm of the low proAKAP4 concentration group and highest in sperm of the high proAKAP4 concentration group and that proAKAP4 levels could be used as a fertility marker.

2 Materials and Methods

2.1 Study design

In this study, we investigated sperm fertilization ability of frozen-thawed epididymal mouse sperm in relation to their proAKAP4 concentration. Fertilization ability was determined by IVF assays (36), of which the outcome was evaluated as the rate of oocytes developing into two-cells after addition of sperm (two-cell rate) and as the rate of two-cells developing into blastocysts (blastocyst rate). In a previous study, the proAKAP4 concentration of 46 frozen-thawed mouse sperm samples consisting of the strains SWISS (N=10), C57BL/6N (N=12), BALB/c (N=12) and B6D2F1 (N=12) was measured with a commercial ELISA Mouse 4MID® Kit (37). Here we classified additional sperm samples from these males according to their proAKAP4 concentration into three groups: low (< 25 ng/ml; N=4), medium (> 25ng/ml < 50 ng/ml; N=21) and high (> 50 ng/ml; N=8).

A frozen aliquot of each sperm sample (cryopreserved from the previous study and stored in a nitrogen tank) was then used in IVF assays and the two-cell rate and blastocyst rate was compared between proAKAP4 groups to see if there is any effect of proAKAP4 level on IVF outcome.

We performed 46 IVFs in total. As we could not perform all IVFs at the same time, we performed them at 13 different days over the course of one month (Figure 1). We balanced female strain across IVF days and randomized the order in which strains were used for IVF on respective days.

Animal delivery	21.04.2022	Thursday					
	22.04.2022	Friday					
	23.04.2022	Saturday					
	24.04.2022	Sunday	PMSG injection				
	25.04.2022	Monday	PMSG injection				
	26.04.2022	Tuesday		hCG injection			
	27.04.2022	Wednesday		hCG injection	IVF assay		
Animal delivery	28.04.2022	Thursday			IVF assay	Two-cell evaluation	
	29.04.2022	Friday	PMSG injection			Two-cell evaluation	
	30.04.2022	Saturday	PMSG injection				
	01.05.2022	Sunday	PMSG injection	hCG injection			Blastocyst evaluation
	02.05.2022	Monday	PMSG injection	hCG injection	IVF assay		Blastocyst evaluation
	03.05.2022	Tuesday		hCG injection	IVF assay	Two-cell evaluation	
	04.05.2022	Wednesday		hCG injection	IVF assay	Two-cell evaluation	
Animal delivery	05.05.2022	Thursday			IVF assay	Two-cell evaluation	
	06.05.2022	Friday				Two-cell evaluation	Blastocyst evaluation
	07.05.2022	Saturday	PMSG injection				Blastocyst evaluation
	08.05.2022	Sunday	PMSG injection				Blastocyst evaluation
	09.05.2022	Monday	PMSG injection	hCG injection			Blastocyst evaluation
	10.05.2022	Tuesday		hCG injection	IVF assay		
	11.05.2022	Wednesday		hCG injection	IVF assay	Two-cell evaluation	
Animal delivery	12.05.2022	Thursday			IVF assay	Two-cell evaluation	
	13.05.2022	Friday	PMSG injection			Two-cell evaluation	
	14.05.2022	Saturday	PMSG injection				Blastocyst evaluation
	15.05.2022	Sunday	PMSG injection	hCG injection			Blastocyst evaluation
	16.05.2022	Monday	PMSG injection	hCG injection	IVF assay		Blastocyst evaluation
	17.05.2022	Tuesday		hCG injection	IVF assay	Two-cell evaluation	
	18.05.2022	Wednesday		hCG injection	IVF assay	Two-cell evaluation	
	19.05.2022	Thursday			IVF assay	Two-cell evaluation	
	20.05.2022	Friday				Two-cell evaluation	Blastocyst evaluation
	21.05.2022	Saturday					Blastocyst evaluation
	22.05.2022	Sunday					Blastocyst evaluation
	23.05.2022	Monday					Blastocyst evaluation

Table 1: Timetable of the experiment. A total of 46 IVFs were performed at 13 different days. Depicted is the temporal distribution of the respective IVFs with their preceding hormone injection required for superovulation, as well as their assessment of fertilization success one and four days later.

2.2 Animals and ethical statement

For IVF, we used a total of 145 female mice from four different strains, purchased from Janvier Labs, France. SWISS (N=30), B6D2F1 (N=36) and BALB/c (N=40) mice, were seven weeks old at arrival and C57BL/6N (N=39) were 3 weeks old. Age classes of females were chosen to respond optimally to superovulation. After arrival all females had four to ten days to habituate before being used as oocyte donors in the experiment.

The females were kept in groups of three in Makrolon® III cages containing wood granulate bedding (SAFE® select, SAFE Complete Care Competence, France), nesting material (Pur-Zellin® tissue swabs, Paul Hartmann GmbH, Austria) and enrichment (GLP Fun Tunnels Standard, LBS Biotechnology, UK). All mice had *ad libitum* access to food (mouse maintenance diet, ssniff Spezialdiäten GmbH, Germany) and free access to water.

Since this project is classified as animal testing according to the Tierversuchsgesetz 2012 (38), it was reported to and approved by the Bundesministerium für Bildung, Wissenschaft und Forschung, Austria. The approval was filed under the number 2022-0.109.869.

2.3 *In vitro* fertilization assays

2.3.1 Oocyte preparation

For IVF, oocyte donors were superovulated. Groups of three to four females were used as donors in each IVF assay. To stimulate follicle formation Pregnant Mare Serum Gonadotropin (PMSG) (Folligon® 1000 I. E., MSD, Austria) (0.1 ml i. p.) was administered, followed by human Chorionic Gonadotropin (hCG) (Chorulon® 1500 I. E., MSD, Austria) (0.1 ml i. p.) 50 hours later to trigger ovulation. Both substances are non-irritating and are carried out with the smallest possible cannula (24 G, 0.5 ") only by trained personnel.

Approximately 15 hours after hCG injection, females were sacrificed by cervical dislocation to retrieve oocytes for IVF assays. Therefore, an incision was made on the female's abdomen. The gastrointestinal tract was moved cranially and the fat pads caudally to expose the uterus horns and ovaries. The oviduct was separated from the horn of the uterus and the ovary with a single incision each. Oviducts were then transferred to the IVF dish (35 mm Petri dish, Nunc™ 150255) containing a decentral 90 µl drop of HTF + GSH (see 2.6) under mineral oil (preincubated 30 min at 37 °C under 5 % CO_2). In the oil phase of the IVF dish, we pierced the oviduct ampulla and retrieved the cumulus oocyte complexes (COCs) by dragging them into the HTF+GSH drop with a needle. Oviducts were then removed from the IVF dish. The COCs were resting in the fertilization drop for at least 30 minutes at 37 °C under 5 % CO_2 before the sperm suspension was added.

2.3.2 Sperm preparation

Frozen sperm samples were thawed by removing them from liquid nitrogen, holding in the air for 10 seconds, and then placing them in a 37 °C water bath in the incubator for 10 minutes. Sperm straws were then dried with a paper towel, cut open and a 10 µl aliquot of each sperm sample was pushed out with the help of a metal rod and placed into a 90 µl drop of TYH covered

with mineral oil in a pre-incubation dish (PI dish; 35 mm Petri dish, 353001, Falcon®). PI dishes were then incubated for 30 min at 37 °C under 5 % CO_2 to allow capacitation.

After incubation 10-20 µl of the sperm suspension were collected from the peripheral part of the PI drop and added to the fertilization drop in the IVF dish. IVF dishes were then incubated for 3½ hours at 37 °C under 5 % CO_2 to allow fertilization to occur. After this period, remaining cumulus cells and attached sperm were removed by washing the oocytes in four 90 µl drops of HTF. Therefore, all oocytes were collected from the IVF dish with a glass capillary connected to a mouth pipette and transferred to the washing drops. The washed oocytes (see Figure 1) were then transferred to a culture dish (50 mm Center Well dish, 353037, Falcon®), filled with 800 µl of HTF in the center well and 5 ml of NaCl (0.9 %, 3570350, Braun, Germany) in the periphery and cultured overnight at 37 °C under 5 % CO_2.

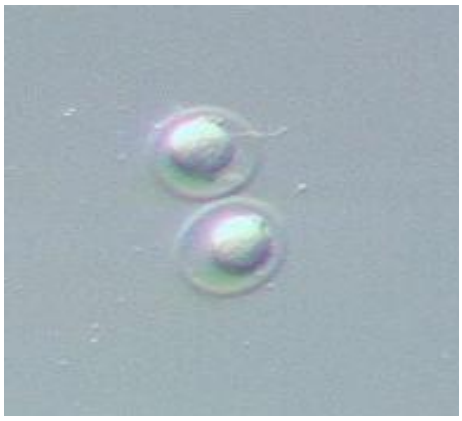

Figure 1: Washed oocytes with dilated zona pellucida

2.3.3 IVF outcome

After overnight incubation in culture dishes, the fertilization success in each IVF assay was determined by visually inspecting cells. Two-cell rates were calculated as the percentage of oocytes that developed into two-cells out of the number of cells that were incubated. In addition, we also assessed blastocyst rates. Therefore, two-cell embryos (see Figure 2) were separated from unfertilized or degenerated oocytes, then washed through two drops of M16 and transferred to a new culture dish (50 mm Center Well culture dish, 353037, Falcon®) filled with 800 µl M16 in the center well and 5 ml NaCl (0.9 %, 3570350, Braun, Germany) in the periphery.

After three additional days of incubation at 37 °C under 5 % CO_2 the number of blastocysts (see Figure 3) were counted and the blastocyst rate was calculated as the percentage of two-cells that developed into blastocysts.

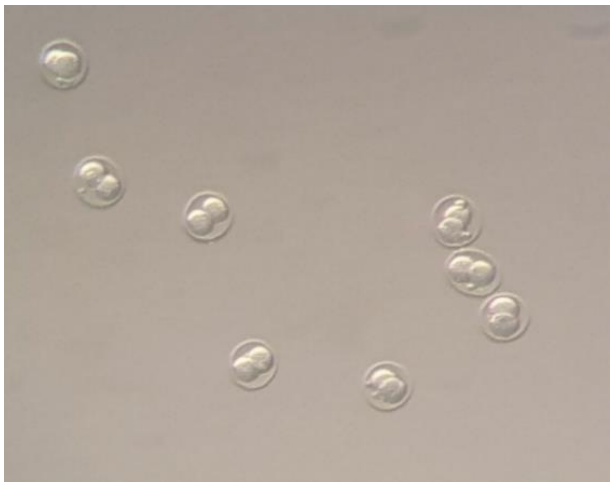

Figure 2: Two-cell embryos after IVF

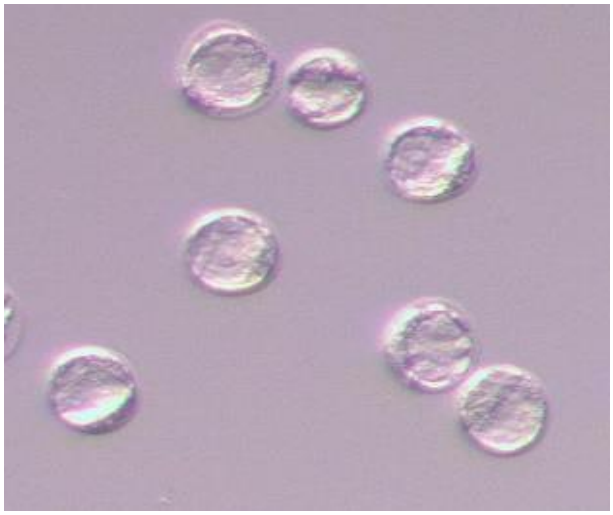

Figure 3: Blastocysts after IVF

2.4 Reagents and media

All media were prepared in-house using ingredients purchased from Sigma-Aldrich, Austria, unless otherwise stated.

To dilute the sperm for IVF and to allow capacitation, TYH medium was used, a modified Krebs-Ringer bicarbonate solution. TYH consists of 119.37 mM sodium chloride, 4.78 mM potassium chloride, 1.71 mM calcium chloride, 1.19 mM magnesium sulfate heptahydrate, 1.19 mM potassium dihydrogen phosphate, 25.07 mM sodium bicarbonate, 5.56 mM D+ glucose, 0.5 mM sodium pyruvate, Penicillin G (7.5 mg/100 ml), Streptomycin (5.0 mg/100 ml), and polyvinyl alcohol (100 mg/100 mL) and was supplemented with 0.75 mM methyl-beta-cyclodextrin.

HTF was used as fertilization medium and to culture the fertilized oocytes overnight. HTF consists of sodium chloride (593.8 mg/100 ml), potassium chloride (35 mg/100 ml), magnesium sulfate heptahydrate (4.9 mg/100 ml), potassium dihydrogen phosphate (5.4 mg/100 ml), sodium bicarbonate (210 mg/100 ml), sodium pyruvate (3.7 mg/100 ml), D+ glucose (50 mg/100 ml), calcium chloride (75.5 mg/100 ml), sodium lactate 60 % (340 µl/100 ml), Penicillin G (7.5 mg/100 ml), Streptomycin (5.0 mg/100 ml) and bovine serum albumin (Merck, 400 mg/100 ml).

The fertilization medium (HTF+GSH) containing reduced glutathione (GSH, Sigma® G4251, Sigma-Aldrich, Austria) was prepared by adding 1 ml human tubal fluid (HTF) medium to a tube containing 30.7 mg GSH. It was mixed until the GSH was dissolved. From this GSH + HTF stock 25 µl were taken, added to 2.5 ml HTF medium and mixed gently (final concentration 1 mM GSH). Before the fertilization medium was used it was filtered into a new tube through a 0.22 µm syringe end filter.

M16 medium (M7292, Sigma®) with added Penicillin G (6.3 mg/100 ml) and Streptomycin sulfate (5 mg/100 ml) was used to culture two-cells after IVF until blastocysts.

Mineral Oil (BP2629-1, Fisher Bioreagents, USA) was used to cover all media.

2.5 Statistical analysis

As previously determined in Boersma et al. (2022), proAKAP4 concentrations ranged between 13.4 to 65.4 ng/ml (37.0 ± 13.4 ng/ml) in frozen-thawed samples. We classified sperm based on their proAKAP4 concentration as having either a low (< 25 ng/ml), medium (> 25 ng/ml < 50 ng/ml) or high (> 50 ng/ml) level to investigate whether proAKAP4 level is predictive for sperm fertilization ability.

Due to a contamination in IVF media the first 12 samples, including all IVF assays with SWISS and 2 C57BL/6N sperm samples, had to be excluded from the statistical evaluation. Thus, we report results on 12 B6D2F1 hybrid, 12 BALB/c and 10 C57BL/6N sperm samples.

All statistical analyses were performed with SPSS 28 (SPSS Statistics, IBM, USA). To assess which factors determine fertilization success we run linear mixed effects models where we used two-cell or blastocyst rates as dependent variables and proAKAP4 class (high, medium or low), male strain and female strain as fixed factors. We included IVF day and number of oocytes within each IVF dish as random factors in models to control for variation caused by these variables. We confirmed that model assumptions were fulfilled and used Fischer LSD as posthoc test.

3 Results

3.1 ProAKAP4 class and two-cell rate

We found a significant effect of proAKAP4 class on the two-cell rate after IVF (F=4.166, p=0.029, Figure 4). Posthoc tests revealed that two-cell rates of sperm from the low class, which ranged between 13 and 59 % (mean 33 %), were significantly reduced compared to sperm from the medium (p=0.009) or high (p=0.029) class. Two-cell rates of sperm from the medium (5-100 %, mean 57 %) and high class (15-88 %, mean 50 %) did not differ between each other (p=0.859).

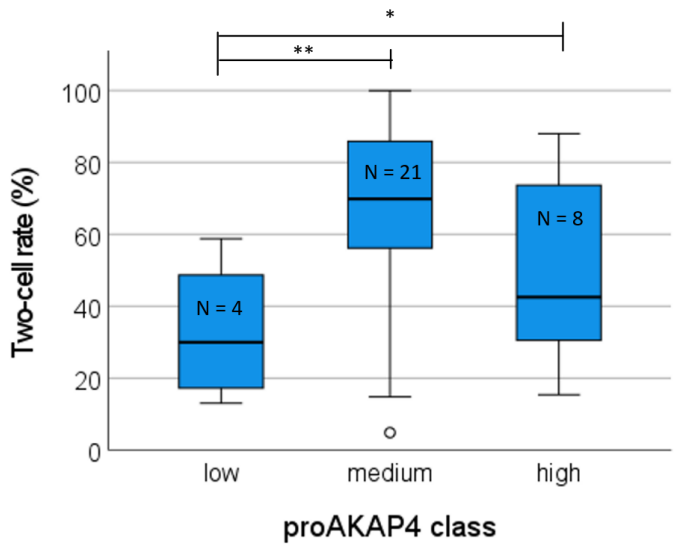

*Figure 4: Percentage of oocytes that developed into two-cell embryos after IVF using frozen-thawed epididymal sperm that were classified as having either a low (< 25 ng/ml; N=4), medium (>25 ng/ml < 50 ng/ml; N=21) or high (> 50 ng/ml; N=8) proAKAP4 concentration. Circles represent mild outliers (Q1-1.5*IQ, or Q3+1.5*IQ).*

In addition to sperm proAKAP4 concentration class, we also found that both female (F=3.868, p=0.026, Figure 5) as well as male strain (F=16.193, p<0.001, Figure 6) significantly affected two-cell rates.

SWISS oocyte donors showed significantly lower two-cell rates (mean 30 %) compared to BALB/c (mean 60 %) (p=0.005) and tended to have lower two-cell rates than B6D2F1 hybrids (mean 69 %) (p=0.058). There was no significant difference in the two-cell rate between SWISS and C57BL/6N (mean 64 %) oocyte donors (p=0.315) and we did not find any other strain specific differences in oocyte donors (all p>0.065).

Sperm of B6D2F1 hybrids showed highest two-cell rates on average (mean 81 %) and were significantly better in fertilizing oocytes compared to C57BL/6N ($p<0.001$) and BALB/c ($p=0.001$). Two-cell rates of C57BL/6N (mean 34 %) sperm showed the lowest two-cell rates and were significantly beneath BALB/c (mean 51 %) ($p=0.045$).

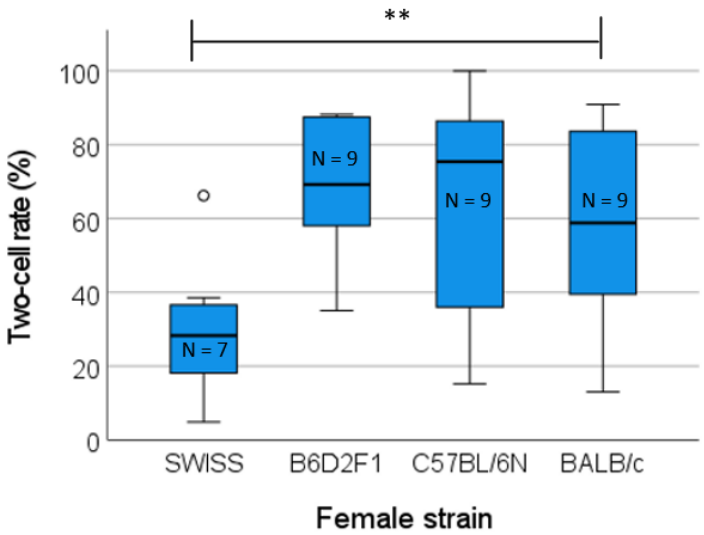

*Figure 5: Percentage of oocytes that developed into two-cell embryos after IVF with frozen-thawed sperm samples depending on the strain of oocyte donors. Circles represent mild outliers (Q1-1.5*IQ, or Q3+1.5*IQ).*

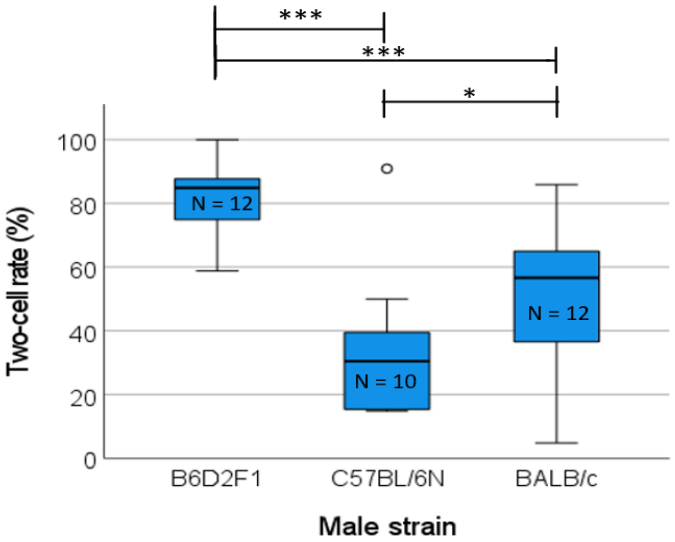

*Figure 6: Percentage of oocytes that developed into two-cell embryos after IVF depending on the strain of the sperm donor. Circles represent mild outliers (Q1-1.5*IQ, or Q3+1.5*IQ).*

3.2 ProAKAP4 class and blastocyst rate

We found that proAKAP4 class tended to have an effect on the blastocyst rate after IVF (F=3.308, p=0.057, Figure 7). However, as this effect was marginally non-significant, we did not perform any posthoc tests and only show the distribution of blastocyst rates in relation to proAKAP4 class in Figure 7.

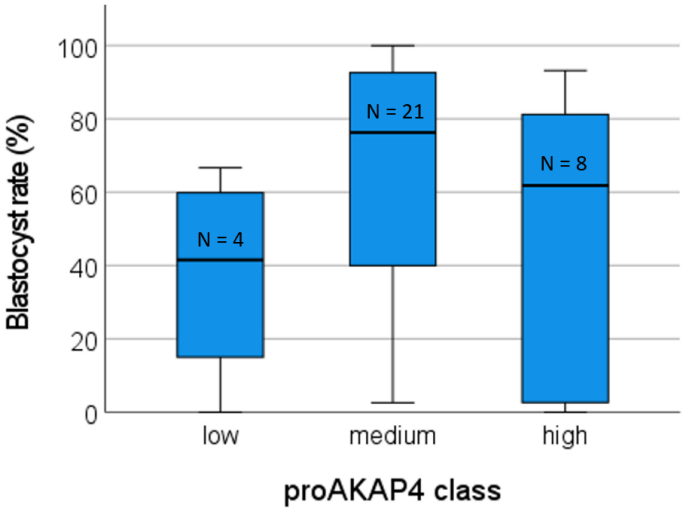

Figure 7: Percentage of two-cell embryos that developed into blastocysts after IVF using frozen-thawed sperm that were classified as having either a low (< 25 ng/ml), medium (> 25 ng/ml < 50 ng/ml) or high (> 50 ng/ml) proAKAP4 concentration.

We did not find any effect of male strain on blastocyst rate (F=1.048, p=0.370, Figure 8). However, female strain significantly affected blastocyst rates after IVF (F=25.528, p=<0.001, Figure 9) and posthoc tests revealed that all strains differed significantly between each other (all p<0.044).

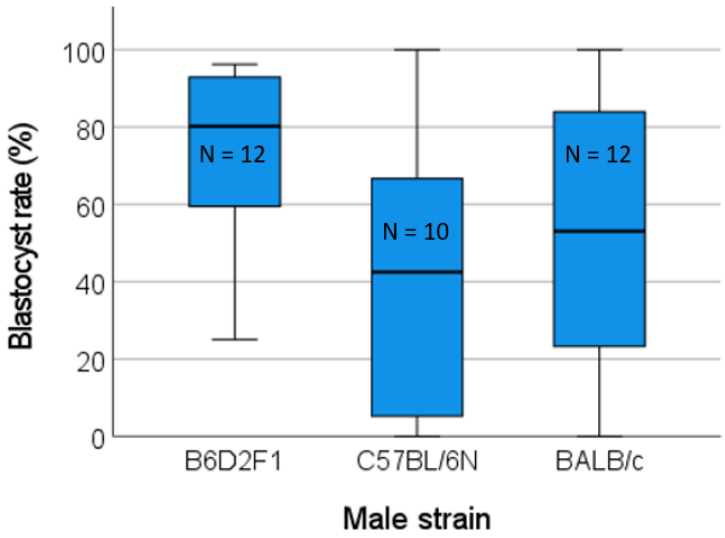

Figure 8: Percentage of two-cell embryos that developed into blastocysts after IVF depending on the strain of the sperm donor.

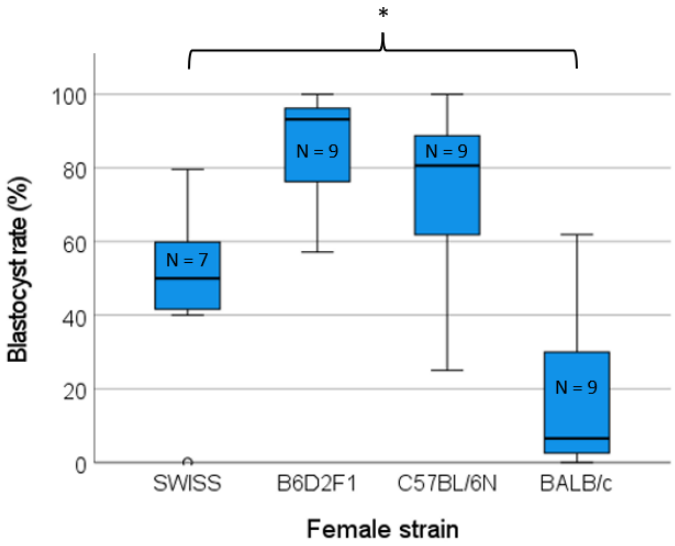

*Figure 9: Percentage of two-cell embryos that developed into blastocysts after IVF depending on the strain of oocyte donors. Circles represent mild outliers (Q1-1.5*IQ, or Q3+1.5*IQ).*

B6D2F1 hybrids showed highest rates with 86 % on average (57-100 %), followed by C57BL/6N with 73 % on average (25-100 %) and SWISS with 48 % on average. BALB/c oocyte donors showed the lowest blastocyst rates with 19 % on average (0-62 %).

4 Discussion

In this study, we describe to our knowledge for the first time the relationship between proAKAP4 concentration and fertilization ability in epididymal mouse sperm. Even though we did not find an overall positive relationship between proAKAP4 concentration and IVF outcome, we were still able to confirm our hypothesis that proAKAP4 concentration could be a possible marker for male fertility in mice. We found that proAKAP4 class could be a good predictor of male fertilization ability in *in vitro* fertilization assays, as sperm from the low proAKAP4 class (< 25 ng/ml) showed a significantly lower two-cell rate, compared to sperm from the medium or high proAKAP4 class. Dordas-Perpinyà et al. performed a similar study using post-thawed bull sperm to artificially inseminate cows (24). They classified sperm samples based on their proAKAP4 concentration, which showed a similar range than found in mice, as either having a high or low concentration and found that samples from the low concentration group had a lower Non-Return-Rate (the proportion of cows that are not subsequently re-bred within a specified period of time after an insemination) (24). Similarly, in bulls, the concentration of AKAP4 in high-fertile animals was higher than in low-fertile animals (22). A low proAKAP4 concentration has been related to an impairment in the process of sperm capacitation, which gives spermatozoa the ability to fertilize the egg and could explain the decrease in fertility (28).

There seems to be a threshold level that has to be reached in order to get good IVF rates. Sperm with a proAKAP4 concentration beneath 25 ng/ml will not bring high fertilization rates. When this threshold is exceeded, we no longer observe differences between IVF results, whether the concentration is between 25 ng/ml and 50 ng/ml or above 50 ng/ml. Surprisingly, we even observe a decline in fertilization rates in the high proAKAP4 class; however, this observation was not significant. Interestingly we also find this distribution in blastocyst rates, where the effect seems to be even more pronounced. In summary, our results support the hypothesis that proAKAP4 could be a marker for fertility, but one should consider whether there is a direct correlation or a threshold that could classify the fertilization ability of sperm. Future studies could focus on determining the exact threshold and investigate potential morphological and physiological differences in sperm below and above this threshold to better understand how proAKAP4 is affecting sperm performance. In addition, future studies should address whether

there is a linear relationship in proAKAP4 concentration and fertilization ability, or whether there is a ceiling effect, or even a reverse effect when proAKAP4 levels are extremely high.

In addition to proAKAP4 concentration class, we also found a significant effect of both female and male strain on IVF outcome. This result was expected as strain specific differences in fertility are well known in mice (39). We found highest two-cell rates in B6D2F1 and C57BL/6N oocyte donors with 69 and 64 % respectively on average. Two-cell rates of BALB/c oocyte donors were slightly lower with 60 % on average. Surprisingly though, SWISS females had the lowest two-cell rates (30 %) of all strains. As an outbred strain they should express *hybrid vigor* (40), and thus show high two-cell rates. We observed anatomical changes in uteri and ovaries in some of the SWISS females we used as oocyte donors, and overall, SWISS females responded less well to superovulation compared to females of the other strains. Thus, it could be that the SWISS females we used in our study derived from an unfit population, which could explain their lower than expected two-cell rates.

In males, we found clear significant differences between strains, and we could confirm that B6D2F1 hybrids, achieve significantly higher two-cell rates compared to the inbred strains C57BL/6N and BALB/c. We cannot exclude that there were strain specific interaction between males and females affecting IVF outcomes (41). However, given that the combination of male and female strain in IVF assays was randomized, our study design might have added addition variation in results but unlikely biased our results and gave us the possibility of seeing the effects of female as well as male strain on the two-cell rate and blastocyst rate.

Assessing two-cell rates is a good means of determining sperm fertility as it reflects immediate effects of sperm traits on fertility. We further determined blastocyst rate, which is on the other hand, a delayed point in time for determining fertility. We found the same effect of proAKAP4 class on blastocyst rate as on two-cell rate. However, the observed effects were weaker. Strain specific effects were similar, though we did not observe any significant effect of male strain on blastocyst rate anymore. Since female strain remained to have a significant effect on the blastocyst rate and male did not, we assume that the constitution of the oocyte is of more importance for the further development of the embryo (42).

In summary, by measuring proAKAP4 levels in frozen-thawed mouse sperm, an estimation of sperm fertilization ability can be made and proAKAP4 could be used as a marker of fertility to avoid poor IVF outcomes. Thus, screening proAKAP4 levels in sperm allows detaining sperm with low fertilization abilities, thereby contributing to the reduction of laboratory animals in accordance with the 3 R's.

5 Conclusion

We were able to analyze for the first time the relationship between proAKAP4 concentration and fertilization ability in mice. We found that sperm with low proAKAP4 concentration had reduced fertilization ability, measured in the percentage of oocytes developing into two-cells and blastocysts after performing IVF. Our results are consistent with other studies examining the relationship between proAKAP4 level and fertility in other animal species. But in contrast to those, we state that there could be a specific threshold in proAKAP4 concentration, which will yield high fertilization rates when exceeded. Not surprisingly, we also found that both the female strain used as oocyte donor and the male strain used as sperm donor have an effect on the two-cell rate. In conclusion, the data from our study suggest that the sperm protein proAKAP4 could be used as a marker for the fertilization ability in epididymal frozen-thawed mouse sperm.

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7 Declaration of independence

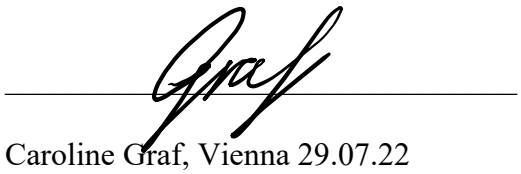
I, Caroline Graf, hereby certify that:

- no other resources and literature sources than those mentioned have been included,

- the decisive work has been carried out by myself and all collaborators have been indicated with their contribution to the work,

- the bachelor thesis submitted for assessment has been written independently, and

- the bachelor thesis has not been submitted or published elsewhere.



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