THE EVOLUTIONARILY CONSERVED PROTEIN ANKEF1 CONFERS SPERM MIDPIECE FLEXIBILITY AND IS ESSENTIAL FOR MALE FERTILITY

Chelsea M. Canon, MD1, Erkan Buyuk, M.D.2, Alan B Copperman, M.D.2, Adolfo García-Sastre, PhD1 and Lisa Miorin, PhD1, (1)Icahn School of Medicine at Mount Sinai, New York, NY, (2)Icahn School of Medicine at Mount Sinai/Reproductive Medicine Associates of New York, New York, NY

Title:
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Submitter's E-mail Address:
cccanon@rmaofny.com

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Abstract Text:
OBJECTIVE: The ankyrin repeat and EF-hand domain containing protein 1 (Ankef1) has been found to be highly enriched in the testes, yet its function remains uncharacterized in mammals. An Ankef1 knockout (KO) mouse model was created by the CRISPR-cas9 system to help elucidate its function. The male mice homozygous for the Ankef1 knock out mutation were found to be sterile, however the sperm count and morphology was similar to wild type (WT) mice. The objective of this study is to characterize the function of Ankef1 and its role in male fertility.

MATERIALS AND METHODS: The sperm from Ankef1 deficient mice was examined by performing in vitro fertilization (IVF) studies using both heterozygous (Ankef1+/−) and homozygous knockout (Ankef1−/−) mouse sperm. IVF was performed using sperm that were collected from caudae epididymides into FHM medium and capacitated during incubation. Ovulation was induced by sequential intraperitoneal injection...
of Pregnant Mare Serum Gonadotropin (PMSG) and human chorionic gonadotropin (hCG). On the day of the IVF, the cumulus masses were recovered from the oviducts and incubated with sperm in Cooks medium for 4 hours. The oocytes/zygotes were rinsed in FHM media and incubated in KSOM + amino acids overnight at 37°C. The following day, the number of 2 cell embryos was recorded. Zona Free IVF was performed as before but WT cumulus masses were first incubated in hyaluronidase to remove cumulus cells and zona pellucida (ZP). A sperm-ZP binding assay was performed to observe if Ankef1 KO mouse sperm were able to bind the ZP of WT oocytes. Flagellar waveform analysis was performed using non-capacitated or capacitated spermatozoa from the cauda epididymis of Ankef1+/- and Ankef1-/- males. Spermatozoa were plated on fibronectin-coated coverslips and sperm motility was recorded for 2s with 200 fps on a Zeiss Axioimager Z2M.

**RESULTS:** The initial IVF experiments showed that while fertilization rates remained high for Ankef1+/- sperm, Ankef1-deficient sperm were not able to fertilize WT oocytes. The ZP binding assay showed that Ankef1+/- sperm were able to bind to the ZP, but they were unable to penetrate it, thus fertilization did not occur. Ankef1+/- sperm was not visualized in the perivitelline space in these experiments. However, when the ZP was removed and zona-free IVF performed, fertilization was then restored. Flagellar waveform analysis revealed that Ankef1+/- sperm had a rigid midpiece that rendered their flagella unable to perform the high amplitude bends that define hypermotility and were thus unable to penetrate the ZP.

**CONCLUSIONS:** These experiments demonstrate that Ankef1 is required for sperm midpiece flexibility which is required for sperm hyperactivation, and Ankef1 knockout blocks ZP penetration and prevents successful fertilization. Therefore, Ankef1 plays a critical role in sperm function and male fertility.

**IMPACT STATEMENT:** The Ankef1 protein is required for sperm midpiece motility and therefore hyperactivation and ZP penetration.

**Presenting Author**

Chelsea M. Canon, MD  
Email: ccanon@rmaofny.com -- Will not be published

Icahn School of Medicine at Mount Sinai  
Obstetrics, Gynecology, and Reproductive Science  
1176 Fifth Ave, Klingenstein Pavilion  
New York NY 10029  
USA

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No - disclosing all COI  
Signature: Chelsea M Canon  
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Second Author
Erkan Buyuk, M.D.
Email: ebuyuk@rmaofny.com -- Will not be published

Icahn School of Medicine at Mount Sinai/Reproductive Medicine Associates of New York
Department of Obstetrics, Gynecology, and Reproductive Science
New York NY
USA

Within the past 2 years, have you or your spouse/partner had any potential COI?
No - disclosing all COI
Signature: Erkan Buyuk, M.D.
- 2022-02-15 19:09:44

Third Author
Alan B Copperman, M.D.
Email: acopperman@rmany.com -- Will not be published

Icahn School of Medicine at Mount Sinai/Reproductive Medicine Associates of New York
Department of Obstetrics, Gynecology, and Reproductive Science
New York NY
USA
Within the past 2 years, have you or your spouse/partner had any potential COI?
No - disclosing all COI
Signature: Alan B Copperman
- 2022-02-15 19:09:44

Fourth Author

Adolfo García-Sastre, PhD
Email: adolfo.garcia-sastre@mssm.edu -- Will not be published

Icahn School of Medicine at Mount Sinai
Division of Infectious Diseases, Department of Medicine
1468 Madison Avenue
Annenberg Building Floor 16
New York NY 10029
USA

Within the past 2 years, have you or your spouse/partner had any potential COI?
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Signature: Adolfo Garcia-Sastre
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Fifth Author

Lisa Miorin, PhD
Email: lisa.miorin@mssm.edu -- Will not be published
Within the past 2 years, have you or your spouse/partner had any potential COI?
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Signature: lisa miorin
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