Single Cell RNA Sequencing of Testicular Tissues from Infertile Men with Loss of Function Variants

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Cellular and molecular alterations underlying male infertility remain largely unknown. As a result, about 70% of men are not receiving a causal diagnosis for their condition. While an increasing number of genetic causes has been associated with male infertility in recent years, functional importance of identified candidate genes remains to be assessed. Prominent candidate genes include those involved in meiotic recombination. In line with this, impairment of meiotic recombination often leads to an arrest of spermatogenesis at meiosis and thus, male infertility. During meiotic recombination, heterodimers of MSH4 and MSH5 bind to the DNA and stabilize double Holliday junctions. Moreover, proteins forming part of the mammalian ZZS complex, namely SHOC1, TEX11, and SPO16, interact with M1AP and support stabilization of early recombination intermediates, enabling crossover formation.

Here, we aimed to assess the cellular and molecular alterations caused by homozygous loss-of-function (LOF) variants in spermatogenesis-relevant genes involved in meiotic recombination. To this end, we generated single cell RNA sequencing (scRNA-seq) datasets of testicular tissues obtained from men with full spermatogenesis (controls; n=3) and men with germ cell differentiation failure associated with LOF variants in *MSH5*, *SHOC1,* and *M1AP* (n=3). Patients underwent testicular biopsy at the Centre of Reproductive Medicine and Andrology in Münster for evaluation/treatment of azoospermia. Control group data was supplemented with two previously published datasets generated elsewhere.

Following preprocessing and an established bioinformatic workflow, we obtained RNA expression data for controls (~24,000 cells) and the cases (~15,000 cells). According to respective marker gene expression, these cells were assigned to germ cell (spermatogonia, spermatocytes, and spermatids) or somatic cell types (Sertoli, Leydig, perivascular, endothelial, peritubular, and immune cells). Interestingly, in the patient with a LOF variant in *MSH5* histological analyses showed that 41% tubules displayed spermatogonial arrest, 23% showed a spermatocyte arrest, and no tubules contained spermatids. In line with these histological results, majority of germ cells captured by scRNA-seq (>85%) were assigned to spermatogonial stage. In contrast, testicular tissues from patients with LOF variants in *M1AP* and *SHOC1* showed a prominent spermatocyte arrest pattern, with 76% and 93% of seminiferous tubules displaying this phenotype, respectively. Consistently, scRNA-seq data of these patients showed decreasing cell numbers from pachytene-stage onwards, as 90% and 98% of their germ cells were assigned to spermatogonia or early spermatocytes. Furthermore, we investigated cell-specific expression of the mutated genes in all samples. Expression of *MSH5 and M1AP* was detected in the controls but not in the patients carrying the respective LOF variant, while the patient with *SHOC1* LOF variant showed expression of the mutated gene, aligning with the prediction that this variant only leads to slight shortening of the resulting protein and thus, likely does not cause a complete loss of function. In addition, the expression

of ZZS members *TEX11* and *SPO16* was analyzed. As expected, *TEX11* was specifically expressed by spermatocytes and early spermatids whereas *SPO16* expression was observed in spermatogonia and spermatocytes in all patients.
In conclusion, LOF mutations in *MSH5* and *M1AP*, but not *SHOC1*, seem to ablate the expression of these genes in the germline. Moreover, these datasets reveal transcriptomic changes caused by disturbance of meiotic recombination genes in human testis and elucidate their role in male infertility, providing the basis to uncover the cellular and molecular alterations caused by the LOF variants via differential gene expression and trajectory analyses.