How the *FMR1* gene and the AKT/mTOR signaling pathways potentially control folliculogenesis

The AKT/mTOR signaling pathway is generally active in cell proliferation, differentiation and growth. It is expressed in granulosa cells in women´s ovary. Granulosa cells are essential for proper oocyte maturation and growth. There mTOR is supposed to regulate early folliculogenesis by maintaining the dormancy of primordial follicles. The *FMR1* (Fragile X Mental Retardation 1) gene is also predominantly expressed in GCs in human ovary and is assumed to affect women´s ovarian reserve and response by the level of its gene and protein expression. So elevated mRNA expression level with reduced protein expression levels have been reported in case of premutation of the gene (54-200 CGGs), that is associated with Premature Ovarian Failure. But also CGGs repeats <24 or >34 have been described to affect female fertility by leading to Diminished Ovarian Reserve .

We investigated the expression profile of *FMR1*/FMRP and the mTOR/AKTsignaling pathway under proliferative and inhibiting conditions in human GCs. We were able to demonstrate in the human granulosa cell model (COV434) a significant increase of *AKT*- and *mTOR*-expression levels after stimulation with recFSH, while *S6K*- and *FMR1*-expression decreased. After specifically inhibiting mTOR and AKT, the *FMR1*- and *S6K*-expression significantly increased. Due to the suggested *FMR1*/FMRP-negative-feedback-loop a decrease in FMRP levels was expected, but interestingly this was only found for AKT inhibitor treatment, while mTOR-inhibition, as well as FSH-stimulation led to elevated FMRP level. These results indicate a decoupling of this *FMR1/*FMRP-negative-feedback-loop in our model system. A mouse model carrying a human *FMR1*-CGG-premutation-allele was also reported to show reduced mTOR-protein-levels. Based on these data it can be supposed that both regulators

of folliculogenesis (*FMR1* and the mTOR-signalling pathway) are putatively linked to each other, hence influencing proper follicular development and ovarian reserve.

E2F1-mediated epigenetic mechanism in ovarian response controls FMR1 expression in human granulosa cells

*FMR1/*FMRP is expressed predominantly in brain and in male and female germline. It is involved in translational control of gene transcripts. In human granulosa cells and in leukocytes, *FMR1/*FMRP levels seem to be quantitatively tightly controlled and are supposed to impact women´s ovarian reserve. It has been described that increased cellular transcript levels cause reduction of protein level due to a negative feedback loop. However putative underlying molecular control mechanisms are still under investigation. Some epigenetic-elements, FREE1 and FREE2, were recently identified in human female leukocytes wherein a differential pattern of CpG site methylation was demonstrated to influence the rate of *FMR1*/FMRP expression. *FMR1*/FMRP is predominantly expressed in granulosa cells in human ovary. We analyzed the presence of Differentially Methylated Regions (DMRs) like FREE1 and FREE2, as well as screened for new ones in the human female germline as putative part of a functional epigenetic *FMR1* expression-control mechanism.

We could confirm the presence of FREE1 and FREE2 in human granulosa cells and revealed additionally a novel epigenetic control element located in *FMR1* intron1 and named it *FMR1*-DMR3. Interestingly this new DMR includes a conserved binding site for E2F1, a transcriptional activator binding only to unmethylated CpG sites. We could demonstrate that E2F1 binds to the consensus sequence of *FMR1*-DMR3 if it contains an unmethylated CpG on position 94. In primary granulosa cells of women with different ovarian response, we found a statistically significant difference (p=0.01) in the rate of CpG 94 site methylation depending on their response profile. In women with poor ovarian reserve (POR) a stronger E2F1 binding

in *FMR1*-DMR3 due to increased unmethylated CpG 94 was indicated and higher *FMR1* expression levels in their granulosa cells compared with those of women with normal ovarian reserve (NOR) could be detected. Our data suggest a variable rate of *FMR1* expression in human granulosa cells from patients with different ovarian reserve that may be due to distinct CpG 94 methylation rates in the consensus sequence of E2F1 in *FMR1* DMR3.