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

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**FMR1 (Fragile X Mental Retardation 1) gene**

Localization: POI 1 Locus (OMIM database)
POI Loci: Candidate genes for POI (Premature Ovarian Insufficiency) development → reported gene mutations, chromosomal deletions and X/autosomal balanced translocation in those regions associated with POI
→ X chromosomal: important genes for proper female development/phenotype, double dose effect vs X inactivated genes

**OMIM - Online Mendelian Inheritance in Man. https://www.omim.org ; picture from Rehnitz et al. 2018**
Expression of gene and protein (FMRP in many tissues: brain, gonads, leukocytes, placenta)

Brain: in neurons (cellular growth, polarity, organization, synaptic plasticity)

Ovary: mainly in granulosa cells (follicular maturation, differentiation, growth)

Picture from Schuettler et al. 2011
**FMR1 gene function**

- **FMRF**: Protein in translational control → transport of preRiboNucleoProtein (RNP)-complexes from nucleus to cytoplasm
  → tissue specific mRNA expression

- Phospho-FMRP binds spec. RNA-structures (G-quadruplex RNA)
  → translational regulation via RISC (RNA-induced silencing complex) building (Melko et al. 2010)

- dephosphorylation of FMRP → dissociation of RISC → translation of PSD95 (postsynaptic density mRNA) (Blice-Baum et al. 2014)
**FMR1** and associated diseases

- **gene structure**
- **CGG repeat**
- **mRNA-amount**
- **protein level**
- **phenotype**

*Picture adapted from Willemsen et al. 2011*
**FMR1**: hypotheses for ovarian damage

- **RAN-translation**: ATG independent translation including the CGG repeat → toxic proteins / inclusions → decreased „normal“ FMRP amount (Sellier et al. 2014)

- PM-mRNA association with DNA → RNA:DNA-hybrid → loops on bound DNA (R-Loops) secondary DNA-damage

- Repeat length depending binding of different RNA-binding proteins → sequestration and intracellular inclusions (Oostra and Willemsen 2003)

- Increased expression of antisense FMR1 transcripts as the cause of PM-associated ovarian pathologies (Sellier et al. 2014)

- Unknown impact of epigenetic effects
General epigenetic mechanisms

• **Chromosomal level**: X-Inactivation

• **Chromatin level**: Histone-
  • Acetylation (activation) /
  • Methylation (inactivation)

• **DNA level**: CpG-Methylation
Known epigenetic control elements in *FMR1*

- **MR=Methylated Region / SMB= Stable Methylation Boundary**
  - binds specifically to nuclear proteins. SMB is supposed to carry a specific chromatin structure that keeps a hypermethylated area (MR) in the genome apart from the unmethylated *FMR1* promoter
  - Protection from the spreading of DNA methylation
  - Missing in FRAXA (Naumann et al. 2009)

- **FREE1, FREE2 = Fragile X Epigenetic Element 1 and 2**
  - Methylation of both regions was correlated negatively with lymphocyte’s FMRP expression and with methylation status using Southern Blot in FM males. In male and females probes including premutation, grey zone and FM alleles, methylation status of FREE 1 could be used to differentiate between FXS males and females from controls, as well as from carriers of GZ/PM alleles, but not between GZ and PM alleles and controls (Godler at al. 2010, 2012)
E2F1

• **Background:** Transcription factors bind to distinct DNA regions (CIS-elements: enhancer or silencer) up- or downstream of a gene promoter region and help to form the transcription-initiation-complex

  → TF-binding regulated via concentration or epigenetically via CpG methylation.

**E2F1:** TF from the E2F-family: consisting of 3 activators (E2F1-3a) and 6 repressors (E2F3b-8)

• Binding capacity epigenetically modulated and is highly expressed in female ovary in oocytes and granulosa cells (Yin et al., 2014, Douville and Sirard, 2014).

• Expression in GCs is associated with PCO in human (Yin et al. 2014).

• Knockdown of the retinoblastoma protein (known to bind and inactivate E2F1) in transgene mice → POI like phenotype (Andreu-Vieyra et al. 2008).
Screening for epigenetic control elements in the FMR1 promotor region in female germline

- **Model system COV 434**
  - Granulosa cell tumor cell line; from 27yrs old woman; nearly normal karyotype (46 XX +5) (van den Berg-Bakker et al. 1993)
  - *FMR1/FMRP*-expressing and CGG triplet repeat number (n: 23/40 +/-1) below the PM range (Schuettler et al. 2011)
  - proliferating GCs characteristics: FSH-dependent, 17β-estradiol production, the formation of intercellular connections also in co-cultures with cumulus cells and immature oocytes but not without oocytes (Zhang et al. 2000)
  - high accordance with fresh granulosa cells from healthy IVF patientts (Haltia et al. 2017)

- **Ex vivo fresh GCs:**
  - Patients either normal (N:60) or poor responding (N:40) to controlled ovarian stimulation for IVF
Workflow

- gDNA isolation
- Bisulfite conversion
- MSP or BSP
- Cloning
- Sequencing

Allele 1 (methylated)

\[\text{ACTCGAGG} \rightarrow \text{TGGATGGCT} \rightarrow \text{TGGATGGGA} \rightarrow \text{TGGATGGGA}\]

Bisulfite treatment
Alkylation
Spontaneous denaturation

Allele 2 (unmethylated)

\[\text{ACTCGAGG} \rightarrow \text{TGGATGGCT} \rightarrow \text{TGGATGGGA} \rightarrow \text{TGGATGGGA}\]

Non-methylation-specific PCR
Methylation-specific PCR

Differentiation of bisulfite-generated polymorphisms
Results in cell line

- **COV 434:**

  Picture from B. Youness thesis
• *FMR1*-DMR3 present in ovary, female kidney and leukocytes (different CpG-methylation pattern):

*:* significant differences

Picture from B. Youness thesis
Methylation depend. TF binding site screening

- Screening on all DMRs → identification of:

→ E2F1 binding site exclusively on DMR3

Picture from B. Youness thesis
EMSA /electronic mobility shift assay

- Principle:
Result of EMSA

**E2F1** binds to *FMR1*-DMR3 in case of **unmutated CpG 94**
**FMR1-mRNA-expression in fresh GCs**

- *FMR1-gene expression NOR vs. POR* → **significant higher gene expression in POR**

![Graph showing FMR1 gene expression comparison between NOR and POR](p: 0.0002)
CpG methylation of DMR3 in fresh GCs

- CpG Methylation within *FMR1*-DMR3 of NOR vs. POR
  → significant lower methylation rate of CpG94 in POR patients

![Bar chart showing CpG methylation rates for NOR and POR patients](Picture from B. Youness thesis)
Assumption

*FMR1* gene expression is **epigenetically modulated** by different CpG-Methylation and subsequent variable **E2F1 binding** on *FMR1-DMR3* that impacts women’s ovarian response.
Future Prospects

• Verification of the assumed interaction of E2F1 with FMR1-DMR3 direktly on *ex vivo* probes patients and evaluation of results on *larger total sample sizes*
Future Clinical Implication

- Utilization of the specific E2F1 binding site’s methylation pattern within *FMR1*-DMR3 as a novel diagnostic molecular marker for women’s ovarian reserve
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