Title: Towards the development of technologies for cryopreservation of fish oocytes

Proposal Acronym: CRYOCYTE
Sept 2002- Nov 2005

Website: http://cryocyte.ocean.org.il

Specific program: Quality of Life and Management of Living Resources

Key action 5: Sustainable Agriculture, Fisheries and Forestry, and Integrated Development of Rural Areas Including Mountain Areas

Research objectives: 5.1.2: Sustainable fisheries and aquaculture; Improvement of Aquatic production

Type of Proposal: RTD
Why should we test fish oocytes?

* No consistent results with cryopreservation of fish embryos

* Xeno-transplantation of primordial stem cells (Takeuchi et al., 2004) requires time to maturation of host and is currently relevant to sibling/related species
Cryopreservation of fish embryos

• The main obstacles are:

1. Relatively large eggs
2. Contain yolk
3. Impermeable to cryoprotective (CPAs) agents - especially the syncytial layer
4. High water content- especially in hydrated marine pelagic eggs
5. Early embryonic developmental stages are sensitive to chilling
• Successful results in cryopreservation of oocytes from Mammalian species

• Why not test fish oocytes?
Objectives

1. Studies on the main biological barriers for cryopreservation of fish oocytes.

2. Identification of markers (including genomic, proteomic and functional markers) to monitor oocyte viability after manipulation and/or cryopreservation.


# Cryopreservation of Oocytes

## Biological Barriers

### Oocyte envelopes
- **WP 1**

### Hydration
- **WP 2**

## Cryopreservation methods
- **WP 7**
- **WP 8**

### Zebrafish (ZF)

### Gilthead seabream (GSB)

## Biological markers for Viability

### Staining methods
- **WP 7**

### Physiological markers
- **WP 3**, **WP 5**, **WP 6**
- GVBD, Cathepsins, hydration

### Genomic markers
- **WP 3**

### Proteomic markers
- **WP 4**
Fish models

- Zebrafish
  *Danio (Brachydanio) rerio*

- Gilthead seabream
  *Sparus aurata*
Model fish species

Zebrafish (ZF) – easy to raise
reproducing females are always available
model for freshwater fish
full genome published in 2003

Gilthead seabream (GSB) – cultured in the Mediterranean basin
short reproductive season
pelagic (floating eggs)
model for marine fish

* Share the same ovarian reproductive strategy
  Daily spawners
  group synchronous oocyte development
Differ in the culture temperature: ZF- 25-28°C; GSB- 18-20°C
Oocyte developmental stages

Gilthead seabream

Pelagophil

Benthophil

500µm

Zebrafish

I  II  III  IV  Egg
Germinal vesicle breakdown (GVBD) after *in vitro* incubation

Zebrafish oocytes

Gilthead seabream oocytes

500 µm
• What developmental stage should be selected for cryopreservation?
  - End of vitellogenin uptake
  - Before hydration in pellagic floating eggs \((GBS)\)
  - Egg envelop permeability
Achievements

• A complete sequence data-set of maternal mRNA stored in zebrafish germ cells at the end of oogenesis; Identification of 11,399 unique transcripts and the development of an oocyte viability molecular signature assay (OVMS) for determining oocyte viability - Patrick Babin, University of Bordeaux, France

• 210 identified proteins from zebrafish oocytes and 113-135 proteins from Gilthead seabream oocytes – Arie Admon, Technion, Israel

• Molecular characterization and protein structure of egg envelope proteins (zona pellucida proteins) in zebrafish and the gilthead seabream – Per-Erik Olsson, Örebro University, Sweden

• Newly characterized ovarian specific aquaporins associated with oocyte hydration in pelagic eggs – Joan Cerdà, IRTA, Spain (Science, 2005)

• Molecular characterization and functional studies on cathepsins associated with processing of yolk proteins during oocyte development in zebrafish and the gilthead seabream – Oliana Carnevali, Ancona, Italy

• A recombinant GSB-LH for promoting in vitro final oocyte maturation was successfully produced by Hanna Rosenfeld and Iris Meiri, IOLR-NCM, Israel

• Studies on cryopreservation of mature –yolk containing oocytes of zebrafish and gilthead seabream – Tiantian Zhang, David Rawson (UK) and Esther Lubzens (IOLR-Haifa)
Achievements- continued

International Workshop on Oocyte Cryopreservation – Bordeaux, France (Sept 2005)

The Fish Oocyte – Springer (end 2006-2007)
Transcriptome of zebrafish oocyte:

ZEbraOV SAGE-tag library

647,065 bases sequenced (576 clones)
27,486 total tags
11,399 different tags (variety)

Frequency of each SAGE tag
= Relative mRNA abundance

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Whole-mount *in situ* hybridization screening on oocytes using the identified most abundant maternal transcripts

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Oocyte Viability Molecular Signature (OVMS) assay
(Multicoloured *in situ* hybridization for monitoring oocyte viability and cryopreservation procedures)
Cyclin B

DAZL

Me-II Lectomedin

Whole-mount in situ hybridization screening on oocytes using the identified most abundant maternal transcripts
2D-PAGE of staged Zebrafish oocytes

pH 3-10

Stages 1+2 (140 µm - 0.3mm)

Stage 3 (0.34 – 0.73 mm)

loaded with increasing amounts of proteins to compensate for the vitellogenin uptake

Stage 4 (0.73 – 0.75 mm)
2D-PAGE of staged GSB oocytes with differential loading

pH 3-10

Stages 1+2 (140 µm - 0.3mm)  Stage 3 (0.34 – 0.73 mm)

Stage 4 (0.73 – 0.75 mm)  Stage 5 (1.2- 1.4 mm)
Protein spots

- 210 Zebrafish oocyte proteins
- 113 GSB oocyte proteins by two methods
- 22 proteins by one method of analyses - require confirmation
- Several others waiting for release of GSB ESTs from MGE and other EC funded projects (??)
A. HEPATIC ZONA PELLUCIDA SYNTHESIS

B. OVARIAN ZONA PELLUCIDA SYNTHESIS

Partner 2- Per-Erik Olsson, Sweden
Fig. 23: Schematic representation of a preliminary model for the assembly and composition of the zebrafish eggshell. The model is based on the calculated abundance of different isoforms as determined by MassPT. For each ZPX molecule there are approximately 4 ZPC and 8 ZPB molecules.
Identification of a novel aquaporin, SaAQP1o, in *Sparus aurata* oocytes (Fabra et al., 2005)
CATHEPSINS: INTRACELLULAR ENZYMES with endopeptidase activity

ASPARTIC PROTEASE: CATHEPSIN D

CYSTEINE PROTEINASE: CAT B AND CAT L
Activity of cathepsins B, D and L during GSB oocytes maturation

2: early vitellogenesis
3: middle vitellogenesis
4: late vitellogenesis
5: hydrated eggs
Effects of hCG & rLH on final oocyte maturation (FOM)
Assessment of oocyte viability

- Staining – CFDA, Propidium iodide, Trypan blue, MTT
- Genomic markers
- Proteomic markers
- Functional – GVBD, Cathepsin activity
Evaluation of viability in oocytes

- GVBD – Large oocytes < 550 µm – variable results – 20-80%
- Staining with MTT* – suitable for all sizes

* MTT- thiazolyl blue tetrazolium bromide
GVBD

Significant differences between years: ANOVA, p=0.011
Freezing of GSB oocytes

Survival of GSB oocytes after freezing with EG 3.0 M (in 75% L-15)

- MTT percent of control
- Visual - percent of control

≤300µm  400 µm  ≥ 600µm
Survival of GSB oocytes

(≥ 600 µm)  
Non-frozen, 24° C  
15 min  
Thawed from LN₂

0 hr

1 hr

2 hr

(≥ 250-300 µm)  
Non-frozen, 24° C  
15 min  
Thawed from LN₂
Conclusions and prospects

- Oocyte envelopes are relatively permeable to CPAs.
- Oocytes tolerate relatively high concentrations of CPAs.
- Small size (but not mature) oocytes are viable after cryopreservation (MTT staining).
- Main obstacle: Maintenance of the native structure of VITELLOGENIN.
- Molecular and functional tools were developed for assessing in depth viability of oocytes and contributing to our knowledge on oocyte development and maturation.
- In vitro maturation (Ovulation? Fertilization?) seems feasible.
IDEAS FOR FP7 BY CRYOCYTE PARTNERS

• The yolk proteins and structural functional changes occurring during oocyte maturation.

• Development of oocytes; a molecular evolutionary approach to the process of oocytes development with an emphasis on maternal factors, contributions to differentiation of stem-cells and de-differentiation associated with cancer.

• Factors inducing atresia/apoptosis in fish oocytes and the effect on spawning biomass.

• Vitamins in oocytes: their role on the subsequent embryonic development of fertilized eggs and survival of larvae.

• In vivo and in vitro maturation of oocytes in fish: processes associated with germinal vesicle breakdown, ovulation and fertilization (morphological, protein profiles, cathepsins, aquaporins, hormones, receptors, competence etc.)

• Cryobanks for fish - an infrastructure project- important for QTL programs