Technology platform WP28

Functional genomics using marine fish cell lines and embryos - FICEL

Coordinator: M. Leonor Cancela, CCMAR, EDGE, Partner N°21

1. Objectives

The main objective of work package #28 was to develop molecular tools towards the identification of gene function in fish. The large pool of available genome sequence analysis, obtained through Marine Genomics Europe NoE requires functional genomics to assign a function to specific genes based upon experimental rather than *in silico* only evidences, thus requiring: (1) *in vitro* cellular models from various tissues to answer specific questions on gene function and regulation, (2) *in vivo* fish models suitable to verify the functional significance of selected genes, and (3) molecular tools for large scale analysis of gene function. *In vitro* studies on specific gene function and regulation were done using gilthead seabream [*Sparus aurata*] cell lines/cultures already available (bone, branchial arch and fin) or developed within the scope of this work package. *In vivo/ in vitro* analysis of specific gene function was performed by silencing splicing/translation using miRNA, RNA interference (short hairpin RNA technology) or morpholinos or by up-regulation using expression vectors functional in both fish cells. Embryos from Atlantic killifish [*Fundulus heteroclitus*] and zebrafish [*Danio rerio*] were used for *in vivo* studies. Global analysis of gene expression was performed using seabream microarrays developed within the scope of MGE. (Summarized in Figure 1)

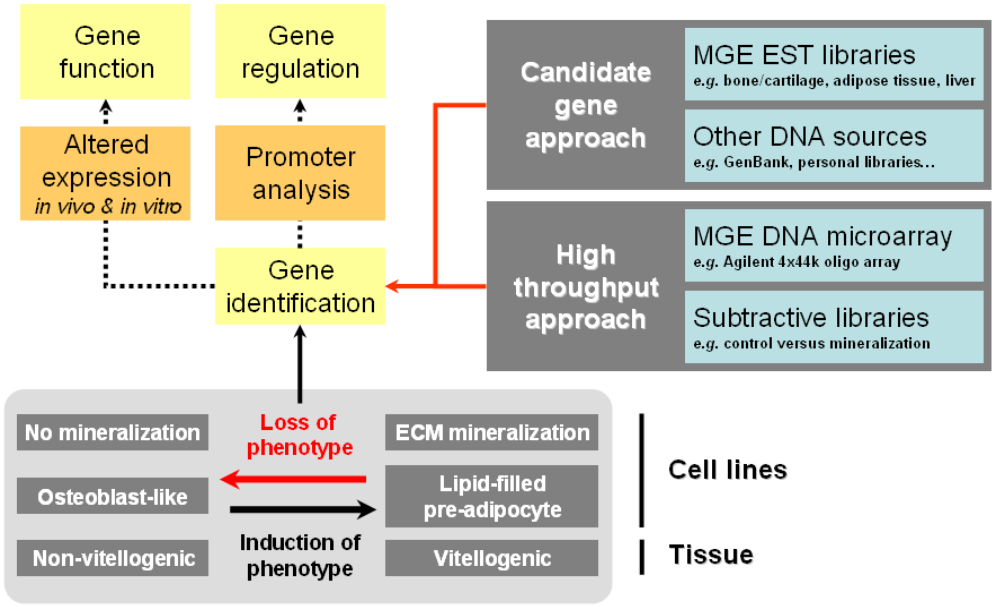

Figure 1: High throughput approach according to WP28. FICEL-WP28 is a pilot project to initiate development/testing of high throughput molecular tools towards the identification of gene function in fish.

2. Partners involved in WP28

#21 Centre of Marine Sciences (CCMAR), Faro, Portugal
#25 Israel Oceanographic and Limnological Research (IOLR), Haifa, Israel
#34 Institut for Food and Agricultural Research & Technology (IRTA), Barcelona, Spain
#35 University of Barcelona (UB), Barcelona, Spain

3. Most significant achievements of WP28-FICEL

3.1 Development/characterization of *S. aurata* cell cultures/lines from selected tissues

S. aurata cell lines derived from bone, branchial arch and fin tissues were already available in partner #21 laboratory (Figure 2). Additional ones were developed: (1) hepatocyte primary culture (partner #25) (2) adipocyte from transdifferentiation of osteoblast-like cell line (partner #21; Figure 2), (3) embryonic stem cell line (partner #21), (4) osteoclast primary cultures (partner #21), and (5) osteoclast progenitor cell culture (partner #21). Characterization included optimization of culture conditions and transfection methods, as well as expression and production of specific genes and proteins in control and specific treatment conditions.

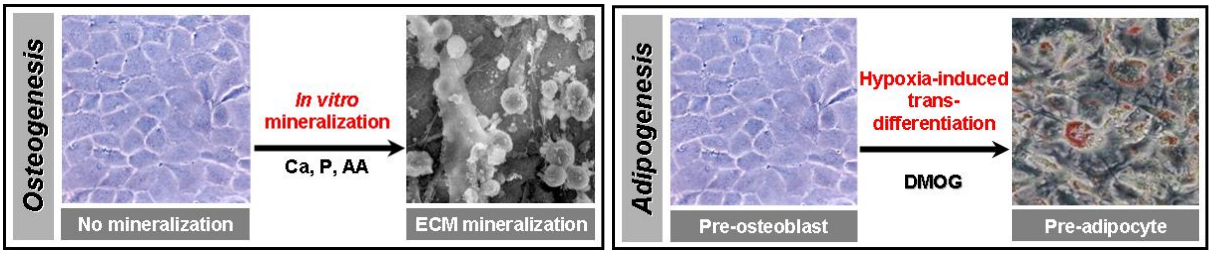

Figure 2: Seabream bone-derived cell undergoing mineralization (*left panel*) or adipocytic transdifferentiation (*right panel*)

3.2 Development of molecular tools and functional analysis of selected gene promoters

Identification of genes involved in a particular physiological process: Several strategies were used to identify genes involved in physiological processes studied within the scope of WP28-FICEL project (i.e. osteogenesis, adipogenesis and vitellogenesis). Genes were identified from (i) ESTs generated within the scope of MGE, annotated sequence databases or standard cDNA cloning, (ii) from subtractive libraries (e.g. mineralizing versus control cDNA), and (iii) from global expression analysis using DNA microarray. Seabream oligo-array developed within the scope of Fish & Shellfish node in collaboration with Agilent (where each unique sequence is represented by two non-overlapping probes (60mers) and each array is composed by 39,399 probes) has been made available to WP28-FICEL partners in September 2007. Preliminary results on genes differentially expressed during *in vitro* mineralization (partner #21), vitellogenesis (partner #25) and adipogenesis (partner #35) were obtained recently (Figure 3) and are being processed. Tissue distribution of a number of genes involved in osteogenesis, vitellogenesis and adipogenesis has been characterized by quantitative real-time PCR in adult fish and during development by partners #21, #25 and #35.

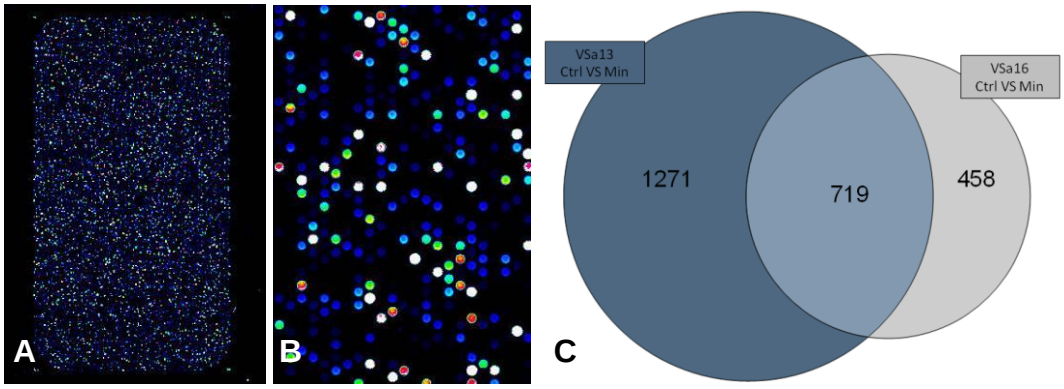

Figure 3: Seabream oligo-array hybridized (collaboration with L. Bargelloni & S. Ferrareso; partner #24) with RNA prepared from bone-derived cells grown under control or mineralizing conditions in order to identify genes differentially expressed and therefore likely to be involved in mineralization mechanisms. **A**, 44K array (each slide contains four 44K arrays) hybridized with control RNA; **B**, zoom in the array showing a selection of spots hybridized with labeled probes (differences in color intensity represent differences in gene expression level) ; **C**, Venn diagram illustrating the number of genes differentially expressed in VSa13 and VSa16 cells, as well as in both cell lines.

microRNAs involved in E2 regulation of vitellogenesis: Differentially expressed microRNA were identified by partner #25 upon estrogen exposure (vitellogenesis is an estrogen-regulated physiological process) using a zebrafish microRNA array. Results provided valuable information to analyze corresponding effects in seabream.

Altered expression of selected genes: Overexpression / silencing of mineralization-related genes was achieved by partner #21 and effect on growth performance & ability to mineralize extracellular matrix characterized using *in vitro* cell systems. These strategies appeared to be successful and thus suitable for high-throughput approaches.

Functional promoter analysis for mineralization related genes: A number of promoter sequences were identified and cloned into pGL2basic vectors by partner #21 to study regulation of mineralization-related genes. The molecular characterization and functional analysis of multiple promoters (e.g. MGP, BMP2, FHL2, SDR, IGF-1 and OP, Figure 4) were performed and many more promoters (of genes identified through microarray analysis) are in WP28-FICEL pipeline. Data allowed identification of key regulators/regulatory motifs. For example, MGP was shown to be controlled by µM concentrations of retinoic acid, a metabolite of vitamin A (Figure 5).

Functional promoter analysis for genes related to adipose tissue function: Partner #35: The promoters of glucose transporter GLUT4, considered a marker for adipogenesis, and interleukin-6 (IL-6), an adipokine (cytokine secreted by adipose cells), were cloned into pGL3basic vector, transfected into L6 cells (although not piscine in origin, this cells are appropriate for expression of various fish adipose or muscle-related promoters) and characterized in terms of regulation by hormones and cytokines.

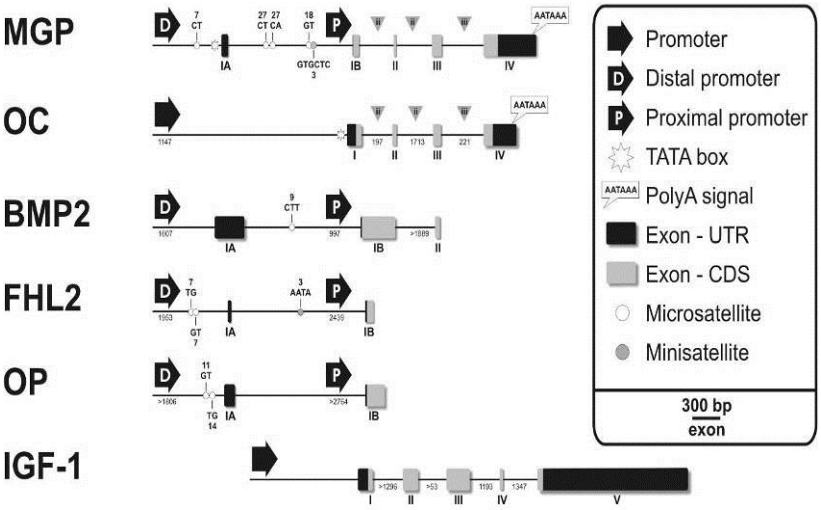

Figure 4: Organization of seabream mineralization-related genes for which promoter activity is analyzed. MGP, matrix Gla protein; OC, osteocalcin; BMP2, bone morphogenetic protein 2; OP, osteopontin; IGF-1, insulin-like growth factor 1.

3.3 *In vivo* studies of gene function using marine fish embryos

Methods for over-expression of specific genes, RNAi and dominant-negative mutation were developed for killifish embryos by partner #34 (Figure 5). The analysis of gene expression of different water channels, aquaporins (AQPs), during killifish embryo development revealed that aquaporin-3 (AQP3) was highly expressed during gastrulation. This expression appeared to be down-regulated during removal of water from embryos. Development of dominant-negative methods based on engineered AQP3 with altered permeability through site-directed mutagenesis to identify specific amino acids implicated in water and solute permeability showed that changes in one single amino acid significantly reduced AQP3 permeability at three different pH.

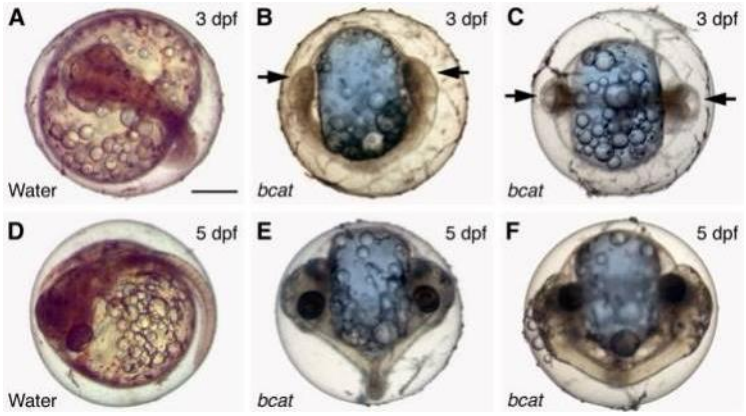

Figure 5: Overexpression of zebrafish *beta-catenin* (a subunit of the cadherin protein complex) in killifish embryos induces a secondary embryonic axis. *Beta-catenin* cDNA was cloned into pCS2+ expression vector (efficient in zebrafish) and cRNA (1-10 ng per embryo) was injected into the yolk of 2-4 cells embryo through the chorion. Zebrafish and killifish *beta-catenin* sequences are 95% identical

3.4 Development of informatic tools to disseminate achievements and provide new source of information

FICEL webpage, a webpage describing the WP28-FICEL project within the scope of the MGE, and *FICEL database* (FICELdb) were created and made available to the scientific community through the institutional webpage of partner #21 (fcma.ualg.pt/EDGE/Ficel/main.htm) with link in MGE site.

3.5 Dissemination and education/training

Results obtained within the scope of FICEL-WP28 resulted in 12 publications in peer-reviewed journals (more are expected, e.g. from microarray data analysis, cell line characterization and functional promoter analysis) and 14 presentations in meetings within and outside MGE (including 2 oral presentations). Nine students (including 3 PhD students partially financed through FICEL) have been trained within the scope of this project.

4. Conclusions

This pilot project, in addition to its obvious scientific achievements, contributed to develop important molecular and cellular tools and develop new information suitable to promote (i) use of state of the art technology, (ii) interactions among different partners/institutions from different countries, (ii) training of younger scientists/students, (iv)dissemination of information through web site and data base created within this project, and (v) contribution to establish a new European Platform recently funded by EU through FP7 (Project ASSEMBLE) where part of the knowledge and tools obtained during FICEL will be shared with scientific community through services and future collaborations.