Identification by systematic *in situ* hybridization screen on medaka embryos of new genes as targets for anti-cancer therapy

Guillaume Serin (a), Violette Thermes (b), Frédéric Vincent (a), Jochen Wittbrodt (c), Jean-Stéphane Joly (b), Philippe Genne (a)

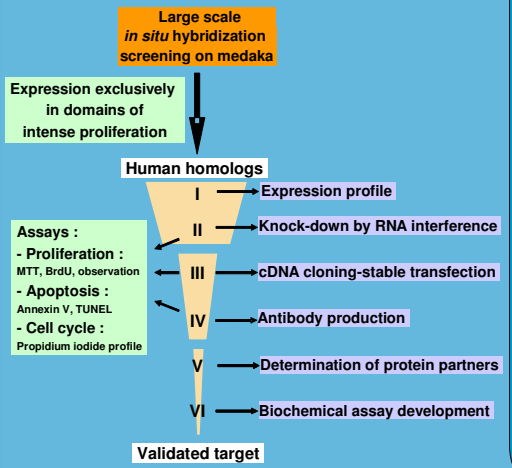
(a) Oncodesign, Dijon, France; (b) INRA/CNRS, Gif-sur-Yvette, France; (c) EMBL, Heidelberg, Germany

 # 4112

Embryogenesis is the period of life that shows the most intense and regulated cell proliferation and apoptosis. A lost of balance between cell proliferation and apoptosis results in cancer. Aims of the 5th PCRD European Project EAC - Embryos Against Cancer - were i) to identify new unknown medaka (orizias lapizes) proteins implicated in these two cellular mechanisms during embryo morphogenesis and ii) to estimate the interest of their human homolog as targets for anti-cancer therapy.

High throughput analysis of an hybridization pattern collection, constructed with Medaka (Oryzia latipes) cDNA library, enabled characterization of several new genes highly expressed in dedicated domains of intense cell proliferation, localized in the optic tectum or the retina under development. The human homologues of these genes were analyzed through a validation process for their role in proliferation of cancer cells. First, their mRNA expression was evaluated from a panel of primary tumors and tumor cell lines. Also, we have tested if the reduction of their expression by RNA interference changed the cell proliferation rate or induced apoptosis. If positive, we isolated human cDNA, observed over-expression effects on proliferation and characterized protein partners.

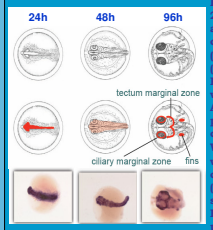
One of these genes, Simplet, is directly involved into cell proliferation mechanisms: injection of morpholino antisens into medaka egg provoked a delay in cell division, gastrulation defects, and apoptotic phenotype, finally conducing to embryonic death. hSimplet (FAB53) is expressed in tumoral cells. Y2H screening and co-immuno-precipitation assays showed that hSimplet interacts with 14-3-3 protein, an important tumor suppressor gene (Development, 2006, 133: 1881-90).



Identification of genes over-expressed in embryo proliferative domains

• Large-scale *in situ* hybridization to identify sequences expressed in proliferation domains

The high fecundity of medaka and the external development of the completely transparent embryos made this small teleost a particularly suited organism for large-scale *in situ* screen.



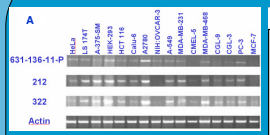
Illustrations represent embryo development 24, 48 and 96 hours post fertilization. In red are indicated the domains of highly intense cell proliferation localized in the retina (ciliary marginal zone), in the optic tectum (tectum marginal zone) and fins.
Pictures represent ISH patterns obtained with PCNA (Proliferating Cell Nuclear Antigen) probe, revealing expression of a protein exclusively expressed in proliferating cells.
Whole mount *in situ* hybridization (WMISH) was performed by a robot with a cDNA library constructed with a stage 30 medaka embryon (anterior brain) from INRA/CNRS and with a stage 20 medaka embryo (whole body) from EMBL. Only probes that showed interesting patterns of expression were sequenced.

• Selected sequences provided by EAC partners

Clone ID	Human homolog	Similarity score	Function
Ok4p	p27	4 e-22	CDK inactivation
smp	FAM53B	1 e-54	None
PAI-RBP1	PAI-RBP1	6 e-74	PAI mRNA binding protein
Insulinoma A1	Insulinoma A1	4 e-76	Pancreas expression, nuclear protein
XP 116 508	XP 116 508	5 e-3	Yeast homolog : RPA43
KIAA 0731	KIAA 0731	7 e-6	La protein similarity
53	BC015116	e-102	Uracil Phosphoribosyl-Transferase
123	KIAA1233	e-54	Unknown
182	KIAA 0268	1 e-47	Colon antigen 16
231	AK001248	7E-31	Similar to gastrulation specific protein G12
243	NM 003413	5 e-45	Neurogenesis
309	BC032542	4 e-59	L-fucose Kinase
724	NM 005348	4 e-41	HSP90
737	KIAA1891	5 e-31	Unknown
62	MGC2655	e-48	Unknown
212	BAA05063	> 0,13	Unknown
277	FLJ32915	1 e-15	Unknown
322	AY157622	6E-89	PHF6 - neurogenesis
186	AY177201	3 e-20	Ubiquitin-2 (de-ubiquitinylation)
264	KIAA 0173	4E-76	Unknown
317	NM 014306	0	Unknown
320	AK022680	e-109	Unknown
464	MGC24160	1.00E-48	Unknown
510	AW094697	5.00E-52	Unknown
631-134-22-C	AF143723	1.00E-37	HSP 60
631-135-17-K	BC000196	4.00E-71	Cyclin G1
631-136-10-C	X60287.1	1.00E-03	cMax
631-136-20K	AF349314	5.00E-47	Ribosome and proteasome biogenesis
631-136-11-P	NM_02788	e-142	Proteasome sub-unit HC8 (alpha-3)
631-135-16-H	BC000606	2.00E-43	Ribosomal protein L14
631-136-23-H	NM_152295	e-144	Threonyl tRNA transferase (TARS)
631-136-23-P	BC020679	e-75	Ribosomal protein L6

Several thousands of expression patterns enabled the identification of 32 genes showing an expression specifically localized in proliferation zones. Sequencing of these cDNAs revealed that half of the proteins were involved in gene transcription, protein synthesis and general metabolism of highly proliferating cells. Notably, four proteins corresponded to proteins already characterized as key factors for tumor growth (p27, Cyclin G1, cMax, HSP90). The other half of sequences corresponded to poorly or totally unknown genes.

Expression in tumors



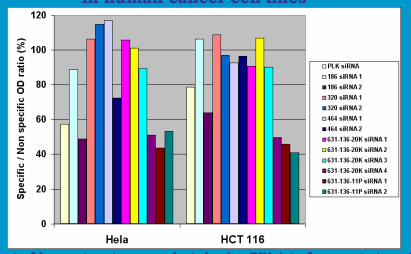
(A) mRNA expression was determined by RT-PCR (semi-quantification) on human cell lines of different origins. Expressions of three targets is shown, with actin as internal standard. Expression profiles show an ubiquitous expression within this tumor cell line panel.

(B) FAM53B (human smp) mRNAexpression was analyzed by RT-QPCR in a panel of cell lines and primary tumors. Quantitative amplification was performed with Sbyr green dye. ΔCT indicates the QPCR quantitative value normalized with β2-microglobulin expression. The expression increase corresponds to the ratio of mRNA expression within each cell line compared to the respective mean expression.

B

	Origin	Cell line ID	Δ CT	Expression increase compared to mean
Cell lines	Breast	MCF-7	-0.5	1.07
		BT-474	+2.0	0.04
	Ovary	IGROV-1	1.4	0.39
		NIH:OVCAR-3	2.3	0.75
	Uterus	HeLa	3.0	2.22
	Prostate	PC-3	1.8	0.54
		DU 145	1.8	0.52
	Bladder	RT4	3.3	1.54
	Kidney	HEK-293	0.3	0.19
		A-498	3.7	1.95
	Pancreas	Mia PaCa-2	1.7	0.51
	Liver	SK-HEP-1	3.4	1.61
	Lung	Calu-6	3.8	2.10
		NCI-H460	3.3	1.54
	Head and neck	KB	3.4	1.57
	Colon	HCT116	1.6	0.47
		HT-29	2.6	0.92
		LoVo	-2.7	0.02
	Skin	A-375	3.1	1.33
		B16	2.2	0.70
		CMEL-5	0.9	0.28
	Brain	COL-3	2.6	0.84
		SK-N-AS	1.3	0.39
		U87-MG	4.4	3.32
	Blood	ARH-77	3.3	1.56
		NAMALWA	1.8	0.55
Primary tumors	Breast	A002147	2.0	0.47
		A012361	2.0	0.48
		A97000602	1.7	0.38
	Ovary	A03000947	3.0	0.92
		A98001747	2.0	0.45
		A98000328	2.2	0.52
	Testis	A023497	2.2	0.52
		A981824	3.1	0.96
		A91002084	1.0	0.24
	Kidney	A043520	2.7	0.74
		A931766	4.1	1.92
		A97002018	2.3	0.58
	Stomach	A021265	3.4	1.21
	Colon	A981581	2.8	0.80
		A051345	1.1	0.25
	Rectum	A021025	3.1	0.96
		A921659	4.8	3.44
	Melanoma	A0102628	2.0	0.48
		A013024	2.3	0.58
	Brain	TOL0024	-0.4	0.09
		TOL0027	5.4	4.99

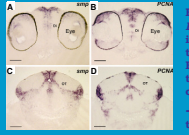
Validation by loss-of-function using RNA interference in human cancer cell lines



The interest of human targets was evaluated using RNA interference strategy. Two to four pre-designed siRNAs (Qiagen) were used per protein. They were transfected on different types of tumor cell lines using RNAiFect (Qiagen) as transfection reagent. The proliferation status of transfected cells was determined by BrdU assay 72 h post-transfection, using non specific siRNA transfected cells as control. The histogram represents the ratio of the OD obtained with specific transfections versus non specific transfection. PLK (Polo-like kinase I), a well known kinase necessary for mitosis, was chosen as positive control.
→ SiRNA induced loss-of-function of three genes (186, 631-136-20K and 631-136-11P) shows a reduction of cell proliferation.

Simplet : a new protein highly expressed in proliferating domains, important for development and interacting with 14-3-3 protein

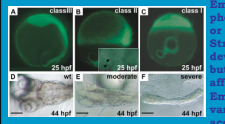
• *smp* and *PCNA* transcript distributions by WMISH



Embryos (34/35 somite stage) were sectioned after WMISH with a *smp* (A,C) or a *PCNA* (B,D) probe. smp and *PCNA* expression domains in the CNS were compared in the diencephalon (A, B) and mesencephalon (C,D). Diencephalic sections show that the dorsal and peri-ventricural domains are positive for *smp* and *PCNA* (Di). Eyes also show ciliary marginal zones positive for both *smp* and *PCNA*. Sections through the mesencephalon show *smp* and *PCNA* staining at the margin of the optic tectum (OT). Scale bars : 60 µm.

→ smp is expressed in highly proliferative domains of the retina and optic tectum

• *smp* loss of function in medaka using morpholino antisens

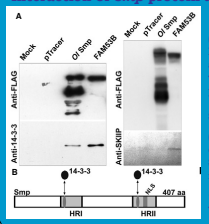


Embryos were injected with smp morpholino antisens. Dose dependent phenotypes were observed by lateral view at the late gastrula stage (25 hpf, A-C) or dorsal views at the 16 somites stage (44 hpf, D-F). (A,D) Control embryos. (B) Strong delay in epiboly and abnormaly large blastomere, (C) Arrest of development at the beginning of epiboly. (E) Mildly affected embryo (16 somites but small eyes and poorly developed midbrain-hindbrain regions). (F) Severely affected embryo (lacks any diagnostic feature of the developmental stage). (G) Embryos injected with control morpholino antisens (smpMOCI, 8 mg/ml) or various doses of simplet morpholino (smpMOI, 2-8 mg/ml) were scored according to the severity of developmental defects. Scale bars: 100 µm.
→ smp morpholino lead to dose-dependant developmental defects.

G

Morpholino	Concentration (mg/ml)	Number of embryos	Phenotypic response at 25 hpf (%) Wild type (Class II)	Weak (Class I)	Strong (Class I)	Phenotypic response at 44 hpf (%) Wild type	Moderate	Severe	Dead or not gastrulated
*smp*MOCI	8	60 (2)	100.0	0.0	0.0	100.0	0.0	0.0	0.0
*smp*MOI	2	48 (1)	91.0	8.0	0.0	49.0	45.0	6.0	0.0
	4	221 (4)	49.7	45.6	4.7	10.0	62.0	22.6	5.4
	6-8	485 (15)	31.6	41.2	27.2	15.5	41.5	33.0	10.0

• Interaction of *smp* protein and its human homolog (FAM53B) with 14-3-3 and SKIIP



(A) pTracer containing FLAG-smp or FLAG-FAM53B cDNA (human homolog of smp) was transfected in HCT 116 colon tumor cells. 72 hours after transfection, cells were lyzed by RIPA buffer and immunoprepitated with anti-FLAG antibody. Western-blot shows the presence of FLAG protein and its co-immunoprecipitated partners (14-3-3 and SKIIP). 14-3-3 interacts with both medaka and human Simplet protein while SKIIP only interacts with human form.

(B) Computational analysis of smp protein revealed two highly conserved domains (HRI and HR II). HRII contains a putative Nuclear Localization Signal. Both domains contain a conserved consensus 14-3-3 recognition motif (RSXpSXP).

→ FAM53B interacts with two proteins involved in cell cycle control and with a role in signaling pathways involved in oncogenesis.

Conclusions

The strategy defined during EAC project enabled the selection of several original gene candidates for anti-cancer therapy. The interest of these proteins to become targets for drug screening is under evaluation at Oncodesign, following the defined strategy.
Furthermore, our results have provided a robust support for the new promising 6th PCRD "Plurigenes" program, stem cells oriented, thus more dedicated to the differentiation process.

This work was supported by EAC key action QLK3-CT-2001-01890, Plurigenes STREP project LSHG-CT-2005-018673 and Oncodesign.