

Project Final Summary

Section 1: PROJECT IDENTIFICATION Information to be provided for project identification	NOT CONFIDENTIAL
---	-------------------------

Title of the project DEVELOPMENT AND IMPLEMENTATION OF NEW 'IN VIVO' AND 'IN VITRO' SYSTEMS FOR THE CHARACTERIZATION OF ENDOCRINE DISRUPTORS

Acronym of the project EDERA

RTD	Total project cost (in euro) 879 000 €
------------	--

Contract number	Duration (in months)	EU contribution (in euro)
QLK4-CT-2002-02221	42 Months	700 000 €

Commencement date 01-01-2003	Period covered by the final report (e.g. 1 February 2000 – 31 January 2001) 1 Gen 2003 – 30 Jun 2006
-------------------------------------	---

PROJECT COORDINATOR

Name Adriana Maggi	Title Full Professor	Address Via Balzaretti n°9, 20133 Milan, Italy
Telephone 0039-02-58358375	Telefax 0039-02-58358290	E-mail address Adriana.Maggi@unimi.it

Key words (5 maximum - Please include specific keywords that best describe the project.). Estrogen Receptor, Endocrine Disruptors, SERMs, Mouse ERE-luc

World wide web address (the project's www address) www.edera.unimi.it

List of participants Provide all partners' details including their legal status in the contract i.e., contractor, assistant contractor (to which contractor?).

(1) Adriana Maggi Coordinator (UNIMI)	(2) Diego Di Lorenzo Contractor (BRHSP)	(3) Sandro Rusconi Contractor (UNIFR)	(4) Jan-Ake Gustafsson Contractor (UNIHU)
(5) Paul Tomkins SubContractor of (1)			

Objectives:

The aims of the EDERA project were:

- to set up protocols based on bioluminescence techniques enabling to measure the activity of estrogenic compounds in living animals and to establish their efficiency;
- to validate the ERE-Luc model system for the study of compounds with estrogenic activity present in diet and environment by measuring the effects of selected estrogenic compounds on ER transcriptional activity *in vitro* and *in vivo*;
- to improve the original ERE-Luc reporter mouse generating a model with which to discriminate between compounds acting on each of the two receptor subtypes (ER α or ER β);
- to set up methodologies for the preparation of 3d cell cultures from animal tissues;
- to generate vectors for the preparation of novel reporter mice.

Results and Milestones:

Milestones at 12 months of partner (1): - Setting up methodologies for *in vivo* analysis of luciferase by optical imaging (**M3a**). -Protocols for *in vitro* and *in vivo* studies of luciferase activity (**M4a**). - Validation of luciferase as a marker of estrogen activity *in vivo* (**M4b**). -Pattern of luciferase expression in mouse 1 after estradiol administration (**M5a**).

- (**M3a**). The instrumentation necessary for the *in vivo* imaging by optic systems was acquired and set up (Night Owl Berthold Technologies equipped with WinLight 32 imaging software). Using the ERE-Luc mouse already available in the lab, imaging analysis was perfected: signal intensity and signal localization, were optimised for optical imaging detection of photon emitting mice. Several parameters had to be set up for imaging the photon emitting mouse, none of those is trivial: anaesthesia was a major obstacle to overcome, we now can anaesthetize the mouse repetitively within a day allowing for image analysis in a time course of hours; we selected luciferin doses necessary to have a significant signal in all organs without toxicity, the acquisition time was selected to ensure that the signal is measured when luciferin has reached all organs and is not yet metabolised; mouse positioning was optimised in order to have a standard assay for photon imaging.

(**M4a; M4b**).-Mouse 1 was exposed to acute and chronic administration of estradiol. Protein extract from different tissues was set up in order to evaluate *in vitro* luciferase activity. Primary bone marrow cell culture were successfully derived and challenged with estradiol, SERMs and antagonist to have an *in vitro* system to detect estrogen receptor-dependency of the luciferase expression. Acute treatment with a single injection of increasing doses of estrogen as well as estrogen antagonist ICI 182,780 definitively prove that luciferase expression is estrogen-dependent in mouse 1. A protocol for chronic administration using pellet with a continuous a long term release of Estrogen action was further analysed demonstrating the estrogen receptor activation in mouse 1 and established a method to compare chronic exposition to estrogenic compounds.

(**M5a**) The exact pattern of luciferase expression in the ERE-Luc mouse challenged with estradiol for short (time course and dose-response) and long time was generated

Milestones at 24 months of partner (1):

(**M3b**) The ERE-Luc mouse has been fully characterized by analysing its response to agonists and antagonists of the estrogen receptor and by verifying that the response of luciferase is not linked to activation of ERRs (by the use of ICI 182,780 which is agonist of ERR and antagonists of ERs).

(**M5b**) By using the ERE-Luc mouse we have generated the pattern of response to estradiol treatment of adult female and male mice. Part of the information has been published. The other parts will appear in publications in preparation.

(**M5c**) The ERE-Luc mouse was fully validated for studies of ligands active through the ERs. The methodologies used to quantitate the ER state of activation are *in vivo* imaging and quantitation of photons emission by bioluminescence and enzymatic analysis of luciferase activity in tissue extracts.

Milestones at 36 months of partner (1):

(**M3c**) Protocols for innovative methodologies for combined pharmacodynamics and pharmacokinetics. Protocols were generated and pharmacodynamics and pharmacokinetics of raloxifene and of tamoxifen were studied.

(**M7a**) Protocols of quantitative measurements of ED action *in vivo*. Protocols were generated and quantitative measurements were performed for genistein, soya milk, resveratrol and DDT.

(**M7b**) Pattern of tissue distribution and accumulation of at least 4 EDs *in vivo*. Pattern of tissue distribution and accumulation of genistein, soya milk, resveratrol and DDT were obtained.

(M7c) Persistence of the activity on ER of at least 4 EDs in vivo by photon imaging analysis. Persistence of the activity on ER of genistein, soya milk, resveratrol and DDT was evaluated in vivo by CCD camera analysis.

Milestones at 42 months of partner (1):

(M9a) Protocols of luciferase expression in vivo after administration of oestradiol and EDs to ERE-Luc/ErbetaKO.

Milestones at 12 months of partner (2):

- No milestone was planned for partner 2 at 12 month, however to expedite future experiments with the mice generated within the present programme we further validated **Mouse 1** with respect to the response to different classes of EDs; therefore we established protocols that will be used to characterize and validate **Mouse 2** as well (**D4a**). In particular, we have studied: **Organochlorines** (p,p'DDT, p,p'DDE, o,p'DDT, β BHC) by dose-response and time-course studies we have assessed that different ER-ligands have different time-dependent and dose-dependent actions. We described their activity in 10 tissues and found that they have a compound specific and tissue specific effect. The most interesting finding relates to the discovered inhibition of reporter activity by the pesticide β BHC. **Phytoestrogens** (genistein). We produced a body map of ER activation in Mouse 1. Genistein was able to activate the reporter in all the tissues analysed, although with a different dose response and time course action. **Heavy metals** (cadmium). Preliminary experiments with cadmium indicate that Mouse 1 is sensitive to detect the estrogenic action of this heavy metal. A time course study showed peak activity of a single injection of cadmium is recorded at 24-48 hours. Activity is still detectable at 14 days.

Milestones at 24 months of partner (2):

(M6a) The pattern of DDT activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

(M6b) The pattern of DDE activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

(M6c) The pattern of BHC activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

(M6d) The pattern of genistein activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

Milestones at 36 months of partner (2):

(M6e) Dynamics of cadmium on luciferase activity on ERE-Luc. The pattern of cadmium activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

(M6f) Dynamics of tributyltin chloride on luciferase activity on ERE-Luc. The pattern of tributyltin chloride activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

(M6g) Dynamics of 2,4,5-trichlorophenoxyacetic acid on luciferase activity on ERE-Luc. The pattern of 2,4,5-trichlorophenoxyacetic acid activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

Milestones at 42 months of partner (2):

(M6h) Dynamics of nonylphenol on luciferase activity on ERE-Luc. The pattern of hydroxylated polychlorobiphenyl activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

(M6i) Dynamics of an hydroxylated polychlorobiphenyl (4(OH)-2',3,3',4',5'-penta-chlorobiphenyl, OH-PCB) on luciferase activity on ERE-Luc. The pattern of OH-PCB activity in ERE-Luc mouse was generated in ERE-Luc mouse by enzymatic analysis of the luciferase activity in a minimum of 4 organs (liver, brain, lung, testis)

Milestones at 12 months of partner (3):

(M1a) Generation of mouse 2: We have assembled and validated double-reporter cassettes (ER-responsive Luciferase + ubiquitously expressible LacZ control gene). - We have chosen the most suitable combination and integrated it into the vector for HPRT targeting locus. - The responsiveness of the finalised targeting vector named HPRT (E4LucUbiqLacZ) was validated. - The targeting vector was sent in November to the company Nucleis Inc (Lyon). - Chimeric transgenic founders were born in December 2003 and January 2004. - The mating for F1 has started in January 2004 and continues in February 2004. F1 will be genotyped by Nucleis Inc. As an alternative to be used in case mouse 2 did not prove as useful as predicted an analogous double reporter cassette (E4Luc + LacZ) was assembled in a way to be inserted via cre-lox recombination into the specially engineered ES cell line (hold by Polygene AG Zuerich). The integration occurs into the beta actin locus. - Two versions of the double cassette have been constructed and tested.

Very much in advance with the predicted time we have generated reporter and expression recombinant adenovirus (**M11a**). - We have assembled and validated in cell line systems a recombinant adenovirus containing an estrogen-responsive Luc reporter identical to the one used for transgenic mouse 2. - We have assembled and validated in cell lines two recombinant adenoviruses with expression cassettes for ERalpha and ERbeta. - These vectors add up to a growing panel of vectors of our laboratory that include response reporters to other steroid receptors and ubiquitous expression cassettes to be used as references genes.

Milestone at 24 months of partner (3):

(M11a) The program has been significantly altered due to the discovery the estrogenic activity of tetracyclins on ERE-reporters genes. The project is following the due course.

Milestones at 36 months of partner (3):

(M11b) Functionality of adeno-reporters in living animals. This milestone could not be achieved as the adeno-reporter vectors prepared demonstrated inefficacious in eliciting satisfactory infections in neither mice nor guinea pigs.

Milestones at 42 months of partner (3):

(M10a) Measurement of tetracycline activity on ERalpha and ERbeta *in vitro* and *in vivo*. *In vitro* studies were completed and complemented with the *in vivo* study which was repeated twice at two different times. Both set of experiments demonstrated that tetracycline may regulate the transactivation of ER target gene: the *in vivo* study shows that this action is limited to selected organs (liver). The mechanism underlying this effect remains to be established.

Milestones partner (4), none at 24 month however:

To expedite the breeding of the novel model system generated within the EDERA group, the unit has been working on the BERKO mouse to improve its fertility using ART. In particular: different hormonal treatments were tested to improve ovulation (superovulation); an *in vitro* fertilization system has been set up; a system for *in vitro* maturation of folliculi was set up; embryo transfer technology was initiated and cryopreservation methodologies were tested; applications were made for approval from the Animal Ethical Committee at Karolinska; BERKO colonies were selected; appropriate procedures for breeding were set up.

Milestones at 36 months of partner (4):

(M8a) Protocols on the generation of double mutant mice (ERE-Luc/ERbetaKO). The protocol was completed and double mutant ERE-Luc/ERbetaKO mice were generated.

(M8b) Protocols for breeding a novel mouse line (ERE-Luc/ERbetaKO). The new mouse line ERE-Luc/ERbetaKO is being bred to amplify the colony in view of future experiments.

(M8c) A mouse line (ERE-Luc/ERbetaKO) expressing the estrogen responsive reporters in ERbetaKO background. The ERE-Luc/ERbetaKO mouse was obtained and the line is underway to be delivered.

Milestones at 42 months of partner (4):

(M8d) Preliminary reports on the pattern of ER inducibility in the ERE-Luc/ERbetaKO. Luciferase production was successfully quantitated by western blot. The effect of estrogen treatment of ERE-Luc/ERbetaKO mice was tested in selected organs. ERbeta ablation resulted in decreased capability of estradiol to induce luciferase synthesis in brain and mammary gland.

Milestones partner (5), none at 12 month however:

An organotypic seminiferous tubule model supporting Sertoli-germ cells interactions and fluid flow with integrated ER activation expression reporting (GFP) was developed. a way to be inserted via cre-lox recombination into the specially engineered ES cell line (hold by Polygene AG Zuerich). The integration occurs into the beta actin locus. - Two versions of the double cassette have been constructed and tested.

Very much in advance with the predicted time we have generated reporter and expression recombinant adenovirus (M11a). - We have assembled and validated in cell line systems a recombinant adenovirus containing an estrogen-responsive Luc reporter identical to the one used for transgenic mouse 2. - We have assembled and validated in cell lines two recombinant adenoviruses with expression cassettes for ERalpha and ERbeta. - These vectors add up to a growing panel of vectors of our laboratory that include response reporters to other steroid receptors and ubiquitous expression cassettes to be used as references genes.

Milestones partner (5), none at 24 month however:

A significant progress was made in the evaluation of estrogenic compounds in *in vitro* systems and in the generation of *in vitro* systems.

Milestones at 36 months of partner (5):

(M12a) Protocols for *in vitro* and *in vivo* testing of compounds active through the ER. Protocols for *in vitro* testing of compounds active through ER in a model of seminiferous tubuli were prepared.

(M13a) A patentable product. No patentable product was delivered, due to the failure of obtaining MOUSE2 model.

Milestones at 42 months of partner (5):

(M12a) Protocols for in vitro and in vivo testing of compounds active through the ER. Protocols for in vitro testing of compounds active through ER in a model of seminiferous tubuli were prepared. During the final phase of the project the effects of 13 estrogenic compounds were tested in full co-culture system and single Sertoli cell cultures and comparative compound analysis was done using YES assay.

Benefits and Beneficiaries:

Public research: The reporter mouse methodology is at its infancy: our group, having shown its applicability to in vivo imaging to reporter animals has greatly helped public research providing a novel, very powerful tool for the in vivo analysis of gene activity.

All Environmental Agencies: All of the in vivo tests currently available for the identification of endocrine disrupters and for the analysis of their effects in vivo are extremely limited. Our model is providing a very innovative system allowing to visualize in real time the action of xenobiotics active through the ERs. The model is a paradigm for other models to be developed which will be of tremendous impact in the toxicological field and for the protection of European citizens.

Future Actions (if applicable):

Validate officially the mouse system for toxicological analysis