Hormones stéroïdes et perturbateurs endocriniens dans le développement gonadique et la cancérogenèse

Hélène Dumond

UNIVERSITÉ DE LORRAINE

Ecole Doctorale BioSE (Biologie, Santé, Environnement)

Mémoire

présenté pour la candidature au diplôme

D'HABILITATION À DIRIGER DES RECHERCHES

par

Hélène DUMOND

Hormones stéroïdes et perturbateurs endocriniens dans le développement gonadique et la cancérogenèse

(Section CNU 65)

Parrain scientifique : Mr Stéphane FLAMENT
Professeur, Université de Lorraine, CNRS UMR 7039, Centre de Recherche en Automatique de Nancy, Vandoeuvre les Nancy.

Université de Lorraine - Centre de Recherche en Automatique de Nancy - CNRS UMR 7039 - BP 70239 - 54506 VANDOEUVRE CEDEX - FRANCE

UNIVERSITÉ DE LORRAINE

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Hormones stéroïdes et perturbateurs endocriniens dans le développement gonadique et la cancérogenèse

(Section CNU 65)

Rapporteurs Marie-Christine RIO, DR1 CNRS, Strasbourg.
Gabriel LIVERA, Professeur, PARIS VII.
Eric PAILHOUX, DR INRA, Jouy en Josas.

Examinatrices Denyse BAGREL, Professeur, Université de Lorraine.
Valérie SCHEIBER, DR2 CNRS, Strasbourg.

Invitée Monique BOLOTIN-FUKUHARA, DR émérite CNRS, Orsay.

Parrain scientifique Stéphane FLAMENT, Professeur, Université de Lorraine.

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SOMMAIRE

REMERCIEMENTS

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Je remercie chaleureusement Stéphane Flament de m'avoir accueillie dans son laboratoire en tant que maître de conférences depuis 2003 puis d'avoir été mon tuteur pour cette HDR.

Mille mercis à mes encadrant(e)s de DEA, thèse et post-docs : Eric Petrochilo, Monique Bolotin-Fukuhara ; Philippe Vernier, Josiane et Gilbert de Murcia, Jean-Yves Jouzeau, Bernard Terlain.

Une pensée nostalgique pour les compagnons de route à qui je dois d'avoir gardé moral et bonne humeur dans les moments difficiles et qui m'ont appris (dans le désordre) la critique, l'autonomie, la culture cellulaire, la génomique, la biologie cellulaire, la physiopathologie de l'arthrose, la physiologie de la reproduction, les modèles animaux, la rédaction d'articles, pleins de détails qu'on oublie mais et qui permettent de construire ensuite ses propres projets :

Michèle Valens, Hélène Rohou, Aline Huber, Nathalie Presle, Pascale Pottie …

les membres du laboratoire et en particulier Amand Chesnel pour son soutien et sa grande contribution aux projets en cours et à venir.

les étudiants que j'ai eu le plaisir d'encadrer, en particulier Angelina Wallacides et Hussein Ajj.

Enfin, je dédie ce mémoire d'HDR à mon grand-père qui, toute petite, m'a ouvert les yeux sur la nature et m'a lancée sur la piste de la biologie.

CURRICULUM VITAE

IDENTIFICATION

Nom patronymique	**DUMOND**
Prénom	**HELENE**
Née le	17/10/1972
Situation	Pacsée – 2 enfants
Grade	Maître de conférences classe normale
Etablissement	Université de Lorraine- Faculté des Sciences et Techniques - Nancy
Section CNU	**65**

DEROULEMENT DE CARRIERE

1995 : Paris XI : DEA de Génétique cellulaire et moléculaire

1996-1999: Thèse préparée sous la direction de M. Bolotin-Fukuhara dans le laboratoire de Génétique Moléculaire à l'Institut de génétique et microbiologie, Paris XI.

"Approches génomiques pour l'étude des réseaux de régulations impliquant l'activateur transcriptionnel yAP-1 chez *Saccharomyces cerevisiae* : nouvelles classes de gènes et interactions entre réseaux de régulation. "

Thèse obtenue le **06 juillet 1999** à l'Université Paris XI avec mention très honorable et les félicitations orales du jury. **Composition du jury:** C. Jacq, Président; J. Verdière et M. Labouesse, Rapporteurs; B. Dujon et M. Bolotin-Fukuhara, examinateurs.

1999-2000: **Poste ATER** à temps complet à l'Université Paris XI.

Stage post-doctoral dans le laboratoire de Phylogenèse et Signalisation neuronale dirigé par P. Vernier au CNRS de Gif-sur Yvette.

Septembre 2000- Avril 2001: Stage post-doctoral dans le laboratoire de poly-ADP-ribosylation et réponses cellulaires aux dommages dans l'ADN dirigé par G. De Murcia à l'ESBS de Strasbourg.

Avril 2001-septembre 2003: Stage post-doctoral dans le laboratoire de Physiopathologie et Pharmacologie articulaire dirigé par le Professeur P. Netter ; Faculté de Médecine de Nancy.

Septembre 2003 : Nomination en tant que **Maître de Conférences** stagiaire à l'Université Nancy I ; 68^{ième} section

Septembre 2004 : Titularisation en tant que **Maître de Conférences** à l'Université Nancy I .

Septembre 2009 : Changement de section CNU (68->65) et reconversion thématique.

2011 : Obtention d'une **Prime d'Excellence Scientifique**.

ACTIVITES D'ENSEIGNEMENT

1 – Vacations en génétique et génomique

1996-2000 ; 86h eq TD, DEUG, Licence, Maitrise, Université Paris XI Orsay

2- Attachée temporaire d'enseignement et de recherche (temps plein)

En 1999-2000, j'ai obtenu un poste d'**A**ttachée **T**emporaire d'**E**nseignement et de **R**echerche à **temps complet** à l'Université Paris XI. L'enseignement consistait à **concevoir, préparer et assurer les TP et les TD** du module de génomique qui était créé à l'Université Paris XI dans le cadre des maîtrises de génétique moléculaire et biochimie.

3- Maître de conférences à Nancy-Université

Enseignements de biologie animale, biologie cellulaire et biologie de la reproduction en licence et master.

2005-2007 : membre permanente du **jury des stages** du parcours M1 Biologie Cellulaire Intégrée.

Depuis 2005, **responsabilité de l'UE 2.03** « Biologie cellulaire des Eucaryotes » en 1^{ière} année de licence SV.

Depuis 2010 : **responsabilité de l'UE 1.01** « De la cellule aux écosystèmes » en 1^{ière} année de licence SV.

Depuis 2010, enseignant référent d'un groupe de TP de L1 SV.

2010-2011, participation à 2 **comités de sélection** à Nancy, section 65.

Tableau récapitulatif : service d'enseignement 2003-2012.

	Licence			Master			
	CM	**TD**	**TP**	**CM**	**TD**	**TP**	Total eq TD
2003-2004 **Congé maternité**	0	29	108	0	5	14	114.5
2004-2005	0	103	102	0	7	22	192
2005-2006	0	68	122	2	21	32	193
2006-2007 **Congé maternité**	52	16	17	4	20	0	132
2007-2008	52	58	46	2	22	0	197
2008-2009	52	72	74	4	8	0	208
2009-2010	60	51	46	3	8	0	199.5
2010-2011	82	38	22	4	8	0	197
2011-2012 **CRCT (1 sem)**	39	14	22	2	0	0	97.5

ACTIVITES D'ENCADREMENT

1- Stagiaires

1996 et 1997 une étudiante de **BTS Biotechnologies** (Nolwenn Danielou)

1998, un stagiaire de **maîtrise** et une étudiante de **DEUST** pendant 4 mois.

1999 une étudiante du **magistère** de Génétique de l'université Paris VII.

2000, un étudiant du **DEA** "Interface Physique-Biologie" de l'Université ParisVII.

2002/2003, co-encadrement d'Yshen Yousseff, **DEA de Pharmacologie** de Nancy

2004-2005, Emilie Entringer, **maîtrise** BCI à Nancy

Agathe Entringer, **Master** Sciences de la vie et de la santé

2005-2006, Sarah Benedic, **maîtrise** BCI à Nancy

Catherine Bourdon, 2ième année de **DUT** ABB à Nancy

2006-2007, Clémence Foltz, 2ième année de **DUT** ABB à Nancy

2007-2008, Charlotte Sencier, 2ième année de **DUT** ABB à Nancy

2008-2009, Alicia Château, 2ième année de **DUT** ABB à Nancy

Hussein Ajj, **DU** à l'UHP-Nancy 1

2009-2010, Damien Sdao, **Master1** de l'Université de Tours

Aurélie Mougel, **L3 BC** à Nancy

Justine Flayac, 2ième année de **DUT** ABB à Nancy

Katie Barkus, 3ième année de **l'Université de Chicago** USA

2010-2011, Justine Flayac, **Licence professionnelle GMC** IUT Nancy

2011-2012 Clémence Chamard, **Master 1 SVS, Nancy**

2- DEA / Master 2

2002/2003, co-encadrement d'Yshen Yousseff, **DEA de Pharmacologie** de Nancy

2006-2007, co-encadrement Angelina Wallacides, **Master 2 BAAN** à Nancy

2009-2010, co-encadrement Hussein Ajj, **Master 2 BAAN** à Nancy

2012-2013, -encadrement Clémence Chamard, **M2R SVS** à Nancy

3- Co-encadrements de thèse (50% avec S. Flament)

2008-2011 Angelina Wallacides, ED **BioSE ; Bourse du Gouvernement Syrien**

Soutenance thèse le 18-10-2011

2010- Hussein Ajj, ED **BioSE ; Bourse du Gouvernement Syrien**

Soutenance thèse en novembre 2013

ACTIVITES DE RECHERCHE

1- Stages pré-doctoraux :

- laboratoire du Professeur A. Adoutte : morphogenèse chez les Ciliés ; interactions membrane-cytosquelette chez la Paramécie ; culture de cellules pour la purification la caractérisation d'anticorps monoclonaux.

- laboratoire du Docteur M. Bolotin-Fukuhara : étude de la régulation des gènes régulés par l'activateur transcriptionnel Cyplp.

- sous la direction de E. Petrochilo : régulation du gène *CYP3* chez *S. cerevisiae ;*

- laboratoire du Docteur A. Bucheton : génétique de *Drosophila melanogaster* ; clonage du gène Flamenco qui contrôle la transmission du rétrotransposon Gipsy ; analyse des conséquences de mutations dans ce gène sur l'expression et la mobilité du transposon.

2- Doctorat (06/07/99): Approches génomiques pour l'identification des gènes cibles de l'activateur transcriptionnel Yap1p chez *S. cerevisiae* : nouvelles classes fonctionnelles et interconnections entre réseaux de régulation **(UMR CNRS Univ. Paris XI 8621).**

Génétique/ biologie moléculaire

Génomique fonctionnelle (banque de fusions, membranes à haute densité)

→ Yap1 joue un rôle crucial dans l'adaptation de la vitesse de division cellulaire à la quantité d'oxygène disponible dans le milieu.

Publications n° 1-2

3- Stage post-doctoral : 1999-2000: Conséquences de l'expansion de la famille des gènes des récepteurs à la dopamine sur la physiologie des vertébrés. **(UPR2197 CNRS - Gif sur Yvette)**

Physiologie des vertébrés

Génomique comparative et bioinformatique

Hybridation *in situ*.

→ Bases moléculaires pour comprendre l'évolution du système nerveux des vertébrés.

Publication n° 3

4- Stage post-doctoral : 2000-2001 : Caractérisation de la protéine PARP-3 de mammifère, appartenant à une famille de protéines impliquées dans le maintien de l'intégrité du génome : les poly-ADP-ribose-polymérase (PARP). **(UPR 9003 CNRS - Strasbourg)**

Biologie cellulaire

Culture cellulaire (transfection, immunocytologie, expression hétérologue…)

Interaction protéines/protéines (immunoprécipitation, double-hybride)

→ Relation entre duplication des centrosomes, réplication de l'ADN et initiation du cycle cellulaire.

Publication n° 5

5- Stage post-doctoral : 2001–2003 : rôle des cytokines et plus particulièrement des adipocytokines dans les lésions du cartilage au cours de l'arthrose. **(UMR CNRS UHP 7561 ; P. Netter)**

Expérimentation animale (modèles expérimentaux de pathologie, électroporation *in vivo*…)

Culture cellulaire (lignées, cultures primaires, transfection…)

Biologie moléculaire, génomique, immunohistochimie…

→ Contribution de la leptine dans la pathologie arthrosique.

Publications n° 4-6-7-8-10

6- Activités de recherche dans l'EA3442 en tant que MCF : 2003-2008 : rôle des hormones stéroïdes dans la différenciation sexuelle d'un amphibien urodèle, le Pleurodèle.

Biologie cellulaire et moléculaire, immunohistochimie

Expérimentation animale, Culture organotypique.

Co-encadrement du M2R et du doctorat de Melle A. Wallacides

Contrôle de l'entrée en méiose par l'acide rétinoique et les stéroïdes chez P. waltl.

Publications n° 9-11-12-13-14-16 + revue + chapitre d'ouvrage

7- Activités de recherche dans l'EA4421 Sigreto et reconversion thématique : 2009-2010 : rôle des stéroïdes naturels et de perturbateurs endocriniens sur la prolifération des tumeurs testiculaires d'origine germinale

Co-encadrement du doctorat de Melle A. Wallacides, du M2R et du doctorat de Mr H. Ajj

Sensibilité des cellules séminomateuses TCam-2 aux stéroïdes

Rôle clé d'une forme tronquée du récepteur ERalpha aux oestrogènes

Analyse des effets de perturbateurs endocriniens oestrogéno-mimétiques

Publication n° 15

ACTIVITES D'ADMINISTRATION

1- Responsabilités Collectives

Membre élue (Cadres B) du Collégium Sciences et Technologies de l'Université de Lorraine depuis 2012

2- Contrats - Collaborations nationales et internationales

Participation à des contrats

1999- Contrat Européen QLK6-1999-02072- 3 ans - 200 000 € - 8 partenaires européens

Au cours de mon stage post-doctoral dans le laboratoire du Pr Netter à la faculté de médecine de Nancy j'ai **représenté le laboratoire** aux réunions bisannuelles et communiqué le compte rendu des travaux effectués aux autres partenaires du contrat. Mon travail sur les cytokines et la caractérisation du modèle d'arthrose expérimentale induite par l'acide monoiodoacétique s'est en effet inscrit dans le cadre d'un contrat européen (QLRT impliquant des laboratoires anglais (C. Westacott, M. Nicklin), irlandais (B. Bresnihan), danois (K. Bendtzen), suédois (T. Saxne), néerlandais (W. Van den Berg), allemand (M. Martin) et israélien (E. Livne). J'ai plus particulièrement collaboré avec le laboratoire dirigé par E. Livne pour la mise au point et l'échange de techniques d'analyse du cartilage et avec le laboratoire de W. Van den Berg pour la génomique du cartilage.

2002- Contrat de Programme de Recherche Clinique – 1 an - 16 000 € - Hôpitaux de Nancy

Le projet sur l'implication de la leptine dans la pathologie arthrosique a été mené dans le cadre d'un Contrat de Programme de Recherche Clinique en collaboration avec les services de Rhumatologie (D. Loeuille) et de Chirurgie Orthopédique (D. Mainard) des hôpitaux de Nancy.

Responsabilité de contrats

2008 - Financement par l'Université Nancy 1 dans le cadre du BQR d'un projet « Jeunes Chercheurs » - 1an- 10 000 euros

Interactions entre acide rétinoïque et hormones stéroïdes dans la différenciation de la lignée germinale.

2009- Contrat avec l'AFSSET (Agence Francaise de Sécurité Santé Environnement Travail) – 2 ans- 78 000 euros- 2 partenaires nancéiens.

Influence des micropolluants en mélange sur la croissance de tumeurs testiculaires d'origine germinale.

PUBLICATIONS

16 - Al-Asaad I, **Dumond H**, Chardard D, Chesnel A, Flament S. (2012) Busulfan-mediated germ cell depletion does not alter gonad differentiation in the urodele amphibian Pleurodeles waltl.Sex Dev. 6(4):188-200.

15 - Wallacides A, Chesnel A, Hussein Ajj, Martine Chillet, Flament S, **Dumond H**. (2012) Estrogens promote proliferation of the seminoma-like TCam-2 cell line through a GPER-dependent ERα36 induction. Mol Cell Endocrinol. 350(1):61-71.

14 - Dumond H, Al-Asaad I, Chesnel A, Chardard D, Boizet-Bonhoure B, Flament S, Kuntz S. (2011) Temporal and spatial SOX9 expression patterns in the course of gonad development of the caudate amphibian Pleurodeles waltl. J Exp Zool B Mol Dev Evol. 15;316B(3):199-211.

13 - Wallacides A, Chesnel A, Chardard D, Flament S, **Dumond H**. (2009) Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. Dev Dyn. 238(6):1389-98.

12 - Flament S, **Dumond H**, Chardard D, Chesnel A. (2009) Lifelong testicular differentiation in Pleurodeles waltl (Amphibia, Caudata). Reprod Biol Endocrinol. 5;7:21.

11 - Dumond H, Maufroid JP, Ko CI, Chardard D, Chesnel A, Flament S. (2008) Freemartin in the amphibian Pleurodeles waltl: parabiosis between individuals from opposite sex triggers both germ and somatic cells alterations during female gonad development. Mol Reprod Dev. 75(3):439-449.

10 - Presle N, Pottie P, **Dumond H**, Guillaume C, Lapicque F, Pallu S, Mainard D, Netter P, Terlain B. (2006) Differential distribution of adipokines between serum and synovial fluid in patients with osteoarthritis. Contribution of joint tissues to their articular production. Osteoarthritis Cartilage. 14(7):690-695.

9 - Kuntz S, Chardard D, Ko CI, **Dumond H**, Ducatez M, Callier M, Flament S, Chesnel A. (2006) Female-enriched and thermosensitive expression of steroidogenic factor-1 during gonadal differentiation in Pleurodeles waltl. J Mol Endocrinol. 36(1):175-86. Erratum in: J Mol Endocrinol. (2006) 37(2):375.

8- Terlain B, **Dumond H**, Presle N, Mainard D, Bianchi A, Loeuille D, Netter P, Pottie P. (2005) Is leptin the missing link between osteoarthritis and obesity? Ann Pharm Fr. 63(3):186-93.

7 - Dumond H, Presle N, Pottie P, Pacquelet S, Terlain B, Netter P, Gepstein A, Livne E, Jouzeau JY. (2004) Site specific changes in gene expression and cartilage metabolism during early experimental osteoarthritis. Osteoarthritis Cartilage. 12(4):284-95.

6 - **Dumond H**, Presle N, Terlain B, Mainard D, Loeuille D, Netter P, Pottie P. **(**2003**)** Evidence for a key role of leptin in osteoarthritis. Arthritis Rheum. 48(11):3118-29.

5 - Augustin A, Spenlehauer C, **Dumond H**, Ménissier-De Murcia J, Piel M, Schmit AC, Apiou F, Vonesch JL, Kock M, Bornens M, De Murcia G. (2003) PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression. J Cell Sci. 116(Pt 8):1551-62.

4 - Pacquelet S, Presle N, Boileau C, **Dumond H**, Netter P, Martel-Pelletier J, Pelletier JP, Terlain B, Jouzeau JY. (2002) Interleukin 17, a nitric oxide-producing cytokine with a peroxynitrite-independent inhibitory effect on proteoglycan synthesis. J Rheumatol. 29(12):2602-10.

3 - Kapsimali M**, Dumond H**, Le Crom S, Coudouel S, Vincent JD, Vernier P. (2000) Evolution and development of dopaminergic neurotransmitter systems in vertebrates. J Soc Biol. 194(2):87-93.

2 - Dumond H, Danielou N, Pinto M, Bolotin-Fukuhara M. (2000) A large-scale study of Yap1p-dependent genes in normal aerobic and H2O2-stress conditions: the role of Yap1p in cell proliferation control in yeast. Mol Microbiol. 36(4):830-845.

1 - Billard P, **Dumond H**, Bolotin-Fukuhara M. (1997) Characterization of an AP-1-like transcription factor that mediates an oxidative stress response in Kluyveromyces lactis. Mol Gen Genet. 257(1):62-70.

Article de revue

Dumond H, Kuntz S, Chesnel A, Ko CI, Wallacides A, Chardard D, Flament S. (2008) Sexual development of the urodele amphibian Pleurodeles waltl. Sex Dev.;2(2):104-14.

Chapitre d'ouvrage

Sex determination and sexual differentiation in amphibians.
Stéphane Flament, Dominique Chardard, Amand Chesnel, **Hélène Dumond**
Hormones and Reproduction of Vertebrates, edited by Drs David O. Norris and Kristin H. Lopez. 2010.

CONGRES

Communications orales

Paris, août 2008: XXth International Congress of Zoology. Sex differentiation of the urodele amphibian *Pleurodeles waltl*. Chardard D., Chesnel A., **Dumond H**., Gelhaye M., Ko C.I., Kuntz S., Wallacides A., Flament S.

Hawaï, juin 2007: **Fourth international symposium on vertebrate sex differentiation**. Advances in the understanding of sex differentiation of the newt *Pleurodeles waltl*. Ko CI., Chesnel A., Chardard D., **Dumond H**., Flament S.

Nancy, septembre 2004 : 5ème atelier déterminisme et différenciation du sexe. Les expressions gonadiques et cérébrales de l'aromatase et de Sf1 sont différenciellement affectées par la température chez le Pleurodèle**.** Kuntz S., Chesnel A., **Dumond H.,** Flament S., Chardard D.

Orsay, mai 2004: Rencontres du club amphibien. Expression de l'aromatase et de SF1 lors de l'inversion sexuelle par la température chez le Pleurodèle. S. Kuntz, A. Chesnel, **H. Dumond**, S. Flament, D. Chardard.

Rennes, avril 2002 : Congrès de la Société Francaise de Pharmacologie : Effect of a selective COX-2 inhibitor on cartilage lesions and chondrocyte apoptosis during experimental osteoarthritis in rat. C. Boileau, **H. Dumond**, N. Presle, S. Etienne, P. Pottie, B. Terlain, P. Netter and J.Y. Jouzeau.

Strasbourg, novembre 2001 : SBCF meeting 2001 : Poly (ADP-ribose) polymerase-3 localized to the daughter centriole plays a role in centrosome cycle control. C. Spenlehauer A. Augustin, **H. Dumond**, J. Ménissier-de Murcia, M. Kock, F. Apiou, JL. Vonesh, M. Bornens, T. Höger and G. de Murcia.

New-York, juin 2001 : ADPR 2001 meeting : The PARP family : maintaining the genome stability by interplay with many biological process. G. de Murcia, V. Schreiber, JC. Amé, **H. Dumond**, A. Augustin, C. Niedergang, A. Huber, T. Höger and J. Ménissier- de Murcia.

Arcachon, septembre 1998 : Levure, Modèle et Outil IV. "L'activateur transciptionnel Yap1p régule partiellement l'expression de gènes impliqués dans le contrôle de la prolifération cellulaire chez *Saccharomyces cerevisiae* " **H. Dumond**, M. Bolotin-Fukuhara.

Grenade, septembre 1998: European research conferences: Gene transcription in yeast. "The *Saccharomyces cerevisiae* transciption factor yAP- 1 can control the expression of several genes involved in cell proliferation" **H. Dumond**, M. Bolotin-Fukuhara.

Lyon, septembre 1997: IXth meeting on the biology of Kluyveromyces. "Oxydative stress response in *Kluyveromyces lactis*: characterization of an AP-1-like transcription factor" P. Billard, **H. Dumond**, K. Jamali and M. Bolotin-Fukuhara.

Communications affichées

Barcelone, juillet 2012 : 22th Meeting of the European Association for Cancer Research. Estrogens and alkylphenols promote proliferation of the seminoma-like TCam-2 cell line trough ERA36-dependent pathways. H. Ajj, A. Wallacides, S. Flament, A. Chesnel, **H. Dumond**. European Journal of Cancer (2012) 48S5:286.

Congrès de l'IFSBM, 16 mai 2012 : Influence des alkylphénols sur la croissance de tumeurs testiculaires d'origine germinale. H. Ajj, A. Chesnel, S. Pinel, J. Flayac, S. Flament**, H. Dumond**.

Journée de la recherche biomédicale, 3 mars 2012 : Influence des alkylphénols sur la croissance de tumeurs testiculaires d'origine germinale. H. Ajj, A. Chesnel, S. Pinel, J. Flayac, S. Flament**, H. Dumond**.

Strasbourg, octobre 2011: 5^{ième} Forum du Canceropôle Grand-est. Influence d'un mélange d'alkyphénols sur la croissance de tumeurs testiculaires d'origine germinale. H. Ajj, A. Chesnel, S. Pinel, J. Flayac, S. Flament**, H. Dumond**.

Strasbourg, octobre 2010: 4^{ième} Forum du Canceropôle Grand-est. Influence de micropolluants en mélange sur la croissance de tumeurs testiculaires d'origine germinale. H. Ajj, A. Wallacides, S. Pinel, F. Plénat, S. Flament, A. Chesnel, **H. Dumond**.

Oslo, juin 2010 : 21th Meeting of the European Association for Cancer Research Crosstalk between retinoid and steroid regulation pathways in the control of seminoma cells proliferation A. Wallacides, A. Chesnel, S. Flament, **H. Dumond.**

Strasbourg, novembre 2009: 3^{ième} Forum du Canceropôle Grand-est. Crosstalk between retinoid and steroid regulation pathways controls proliferation and differentiation of seminoma cells. A. Wallacides, A. Chesnel, H. Ajj, S. Flament, **H. Dumond.**

Paris, août 2008: XXth International Congress of Zoology. Retinoic acid triggers meiosis entry in the urodele amphibian *Pleurodeles waltl*. Wallacides A., Chesnel A., Chardard D., Flament S., **Dumond H**.

Luxembourg, janvier 2000: Signal transduction pathways and regulation of gene expression as therapeutic targets. "Large-scale study of yAP-1 dependent transcriptome in *Saccharomyces cerevisiae*: yeast AP-1 homolog can regulate expression of genes involved in cell proliferation control " **H. Dumond**, M. Bolotin-Fukuhara.

Lisbonne, septembre 1997: EMBO Workshop, **Gene expression under stress conditions:** "The *Saccharomyces cerevisiae* transciption factor yAP- 1 seems to control expression of different gene families in response to oxydative stress" **H. Dumond**, C. Rodrigues-Pousada and M. Bolotin- Fukuhara.

Strasbourg, juin 1996: Levure, Modèle et Outil III. "Recherche et analyse des gènes régulés par l'activateur transcriptionnel yAP- 1 chez *Saccharomyces cerevisiae"* **H. Dumond**, M. Henriques, C. Rodrigues-Pousada et M. Bolotin-Fukuhara.

Séminaires sans comité de lecture

Paris, juillet 2005: Réunion du groupe transversal « Différenciation des gonades chez les vertébrés »: Gonadogenèse dans un modèle de parabioses chez le pleurodèle. **Dumond H,** Maufroid JP, Ko CI, Chardard D, Chesnel A, Flament S**.**

Figeac, octobre 2001: Troisièmes rencontres : Intéraction Acides Nucléiques-Protéines et expression du génome : Les nouvelles poly (ADP-ribose) polymerases et la surveillance de l'intégrité du génome. G. de Murcia, V. Schreiber, JC. Amé, **H. Dumond**, A. Augustin, C. Niedergang, A. Huber, L. Tartier, C. Spenlehauer et J. Ménissier- de Murcia.

Paris, juin 1998: XVIIIème Forum de Cancérologie. "Existe-t-il une conservation du rôle des protéines de la famille AP-1 entre la levure et les mammifères?" **H. Dumond**, M. Bolotin- Fukuhara.

ACTIVITES DE RECHERCHE
1996 – 2011

A - Présentation synthétique

En 1996 a été publiée la séquence complète du premier génome eucaryote, celui de *Saccharomyces cerevisiae* : 16 chromosomes, 13 millions de paires de bases et 6 275 gènes. L'accès à cette masse de données gigantesque et nouvelle a marqué un tournant méthodologique dans la recherche en biologie et a ouvert l'ère des « Omics », d'abord génomique et transcriptomique puis protéomique, métabolomique…

C'est dans ce contexte et avec les balbutiements des analyses transcriptomiques que j'ai débuté mon travail de doctorat en génétique cellulaire et moléculaire. J'ai en effet développé des approches génomiques pour identifier les réseaux de régulations impliqués dans le contrôle de la prolifération de *S. cerevisiae* en réponse à la quantité d'oxygène disponible : hypoxie, normoxie et stress oxydant induit par le peroxyde d'hydrogène. Dans un second temps, l'identification de ces mêmes médiateurs de la réponse au stress oxydant chez une autre espèce de levure, *Kluyveromyces lactis*, a permis de poser les bases d'une étude de génomique comparative chez les eucaryotes.

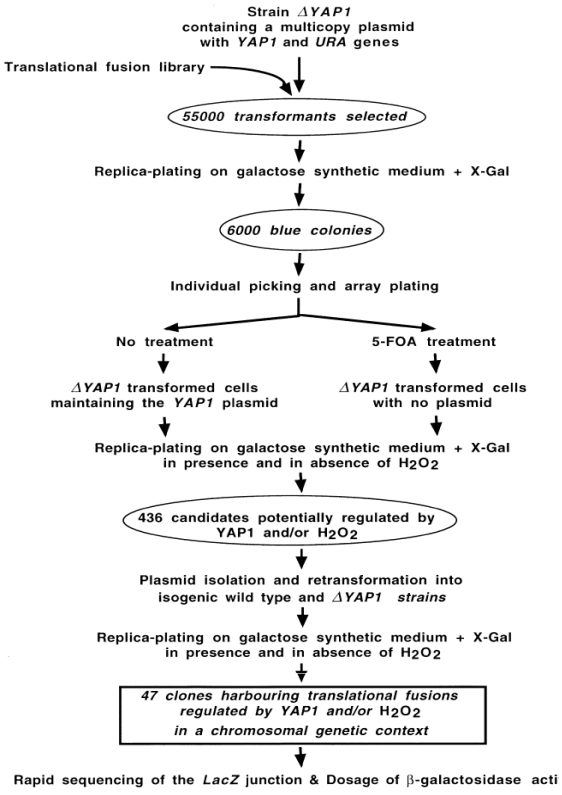
Malgré la grande diversité des modèles et des problématiques sur lesquelles j'ai ensuite travaillé, j'ai tenté de conserver cette approche de physiologie et génomique comparatives pour identifier et caractériser des **médiateurs moléculaires impliqués dans le contrôle de la prolifération des cellules eucaryotes en réponse aux variations de l'environnement.** Je me suis également attachée à replacer les mécanismes moléculaires et cellulaires étudiés dans un contexte plus général de physiologie de l'organisme.

Lors de mon premier stage post-doctoral, j'ai étudié les conséquences de l'expansion de la famille des gènes des récepteurs à la dopamine sur la physiologie des vertébrés. J'ai caractérisé la diversité des récepteurs, la structure des gènes et les mécanismes de leur expression par une approche de génomique comparative et bioinformatique. Nous avons ainsi posé des bases moléculaires pour l'étude de l'évolution du système nerveux des vertébrés.

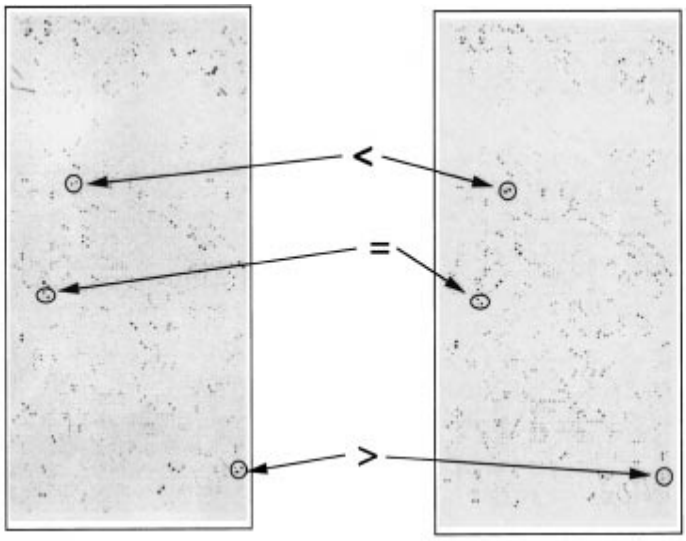
Mon deuxième stage postdoctoral a été consacré à l'étude d'une famille de protéines impliquées dans le maintien de l'intégrité du génome et le contrôle de la division cellulaire: les poly-ADP-ribose-polymérase (PARP). J'ai précisé la localisation de la PARP-3 au centrosome, caractérisé sa fonction dans le cycle cellulaire et identifié ses partenaires par double-hybride. Nous avons ainsi relié la duplication des centrosomes, la réplication de l'ADN et l'initiation du cycle cellulaire.

Mon troisième stage post-doctoral m'a permis de caractériser les cibles pharmacologiques qui se situent à l'interface entre la rupture de l'homéostasie du cartilage et

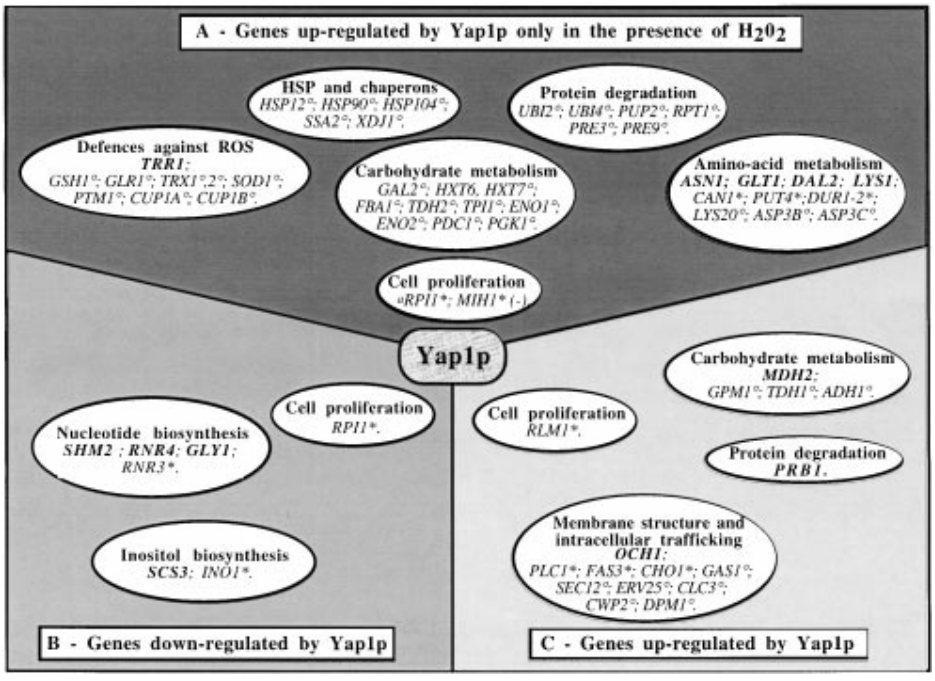
Figure 1: Approches pré-génomiques permettant de dresser une image globale des gènes cibles du facteur de transcription Yap1 en réponse aux variations de quantités de ROS chez la levure *Saccharomyces cerevisiae*



Criblage d'une banque de fusions traductionnelles



Hybridation de membranes à haute densité



Analyse fonctionnelle des gènes cibles de Yap1 D'après Dumond et al., 2000.

le métabolisme lipidique. Dans le cadre d'un Contrat de Programme de Recherche Clinique, j'ai notamment évalué la contribution de la leptine et plus généralement des adipokines dans la régulation du métabolisme chondrocytaire.

Depuis ma nomination en tant que Maître de Conférences à l'Université Nancy 1, je me suis focalisée sur l'influence des hormones stéroïdes sur la prolifération et la différenciation normale ou pathologique des cellules germinales. C'est sur cette thématique qu'ont porté les 2 co-encadrements de thèse et c'est à partir des résultats déjà obtenus que je compte développer mon projet de recherches.

Ces nombreuses expériences de recherche sur des thématiques et des modèles variés, dans des environnements professionnels divers, m'ont permis de découvrir le mode de fonctionnement de plusieurs laboratoires, de développer une grande capacité d'intégration et d'adaptation et de transférer les méthodologies scientifiques et techniques d'un modèle à l'autre. Cette expérience multiple, enrichie de nombreux contacts et collaborations, me permet maintenant de porter un projet de recherche original au sein du CRAN (2013-2018).

<u>B - Doctorat (06/07/99) :</u> Approches génomiques pour l'identification des gènes cibles de l'activateur transcriptionnel Yap1p chez *Saccharomyces cerevisiae* : nouvelles classes fonctionnelles et interconnections entre réseaux de régulation (UMR CNRS Univ. Paris XI 8621).

La régulation de l'expression génique joue un rôle critique dans le contrôle de la croissance et de la différenciation des cellules eucaryotes. Des mécanismes de régulation complexes permettent, (i) de répondre à des stimuli divers, (ii) d'adapter la synthèse des constituants cellulaires aux disponibilités du milieu externe, (iii) ou encore d'engager des programmes de développement. En 1996, les travaux de nombreux laboratoires suggéraient que l'expression des gènes en réponse aux variations de l'environnement était principalement régulée au niveau de la transcription, après la fixation de plusieurs facteurs de transcription sur leur promoteur.

Dans ce contexte, j'ai souhaité aborder les relations entre disponibilité en oxygène et transition énergétique en développant des outils d'analyse génomique, chez le premier organisme eucaryote dont le génome avait été séquencé. Même si ce travail était réalisé chez un organisme unicellulaire, il s'agissait ici d'une tentative de relier l'expression des gènes, le métabolisme énergétique, la physiologie cellulaire et les conditions environnementales.

Mon travail de thèse a consisté à caractériser, chez *Saccharomyces cerevisiae*, l'ensemble des gènes cibles du facteur de transcription de levure Yap1p, puis à mesurer les variations de leur

niveau d'expression en fonction de la présence de Yap1p et en réponse aux modifications de la quantité d'oxygène disponible.

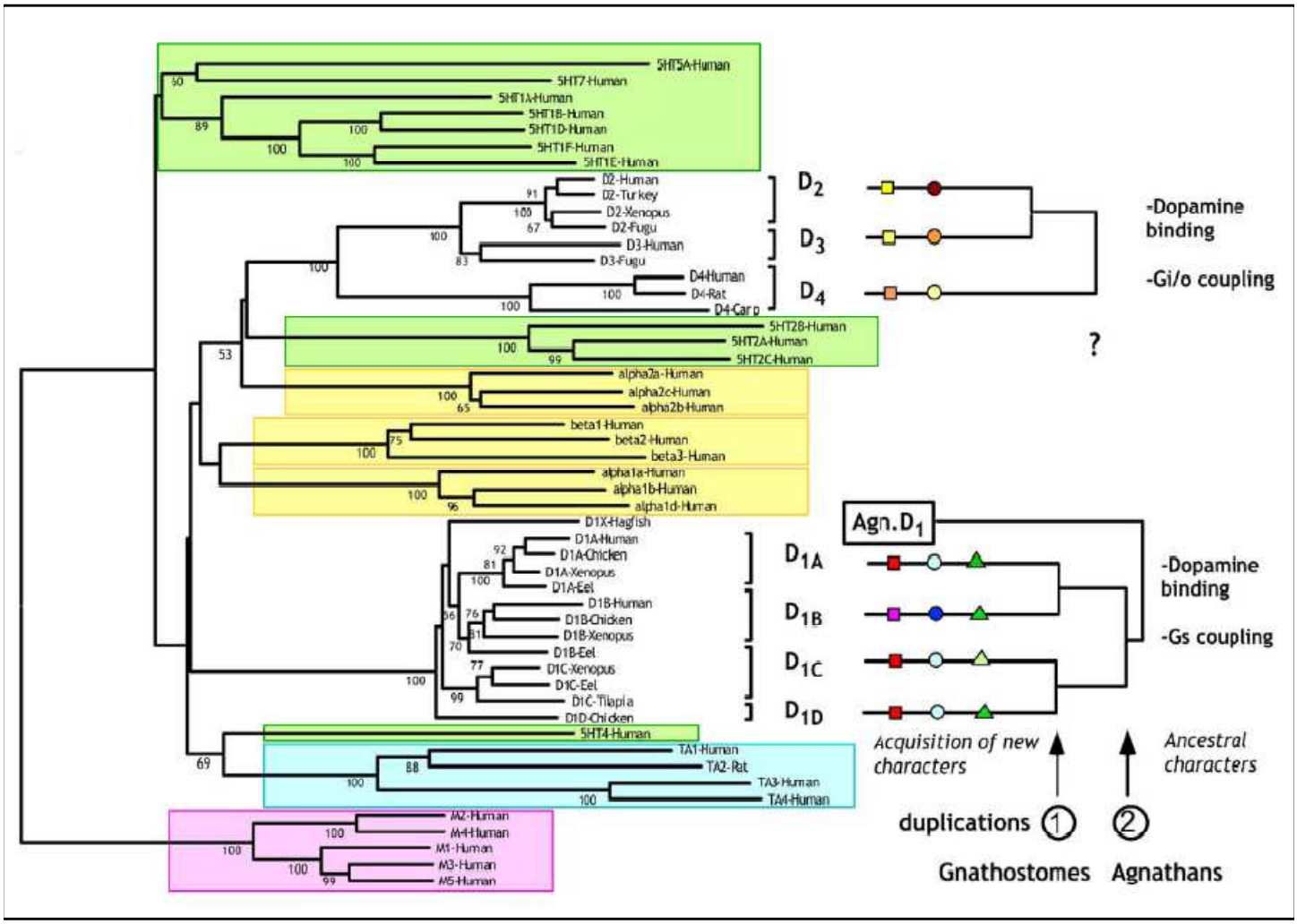
Au début de ce travail, nous savions que l'activateur transcriptionnel Yap1p était un homologue des oncogènes de la famille AP-1 de mammifères (Moye-Rowley, 1989), membre d'une famille de facteurs de transcription (Fernandes, 1997) qui régulent la réponse à différents agents de stress (radicaux oxygénés, métaux lourds) chez les levures (Toone, 1999). J'ai notamment contribué à la caractérisation de l'orthologue de Yap1p chez *Kluyveromyces lactis* (Billard, 1997). La structure commune à Yap1p et aux facteurs AP-1 et la similarité de leurs séquences cibles suggéraient que Yap1p joue un rôle plus large chez *Saccharomyces cerevisiae*. En particulier, nous avons fait l'hypothèse qu'il relie la réponse au stress et le contrôle de la prolifération cellulaire. Nous avons donc cherché, grâce à l'utilisation d'un mutant *yap1*, à identifier les fonctions cellulaires nécessaires à la coordination entre l'adaptation à la présence de radicaux oxygénés dans le milieu et la vitesse de division cellulaire.

Cette hypothèse a été testée chez la levure *Saccharomyces cerevisiae* qui possède un métabolisme aérobie facultatif, en étudiant de manière globale le rôle de Yap1p dans la réponse au stress induit par le peroxyde d'hydrogène, en conditions standard de croissance et en hypoxie. Les techniques classiques de génétique moléculaire permettaient d'appréhender seulement partiellement l'ensemble des modifications du transcriptome dépendantes de la présence de Yap1p et/ou de la disponibilité en oxygène.

Le début de ma thèse étant contemporain de la publication du génome de levure, et des débuts de la génomique, j'ai donc mis en œuvre différentes approches génomiques : crible génétique d'une banque d'expression (Dang, 1994), développement du protocole d'hybridation de membranes à haute densité fournies par le laboratoire de J. Hoheisel (Heidelberg, Allemagne). Ce travail a été réalisé dans le cadre d'un projet européen de mise au point des outils d'analyse transcriptomique chez *S.cerevisiae* (Figure 1).

J'ai mis en évidence un rôle de régulateur à la fois positif et négatif pour Yap1p et caractérisé des gènes cibles impliqués dans les voies de contrôle du métabolisme carboné et de la prolifération cellulaire. Il apparaissait donc que Yap1p joue un rôle crucial dans la coordination entre la consommation de l'oxygène pour la production d'énergie et la prolifération cellulaire, en conditions normales ou en présence d'un excès de radicaux oxygénés. (Dumond, 2000). En étudiant ces réseaux de régulation chez une autre levure, *Kluyveromyc es lactis*, et grâce aux données bibliographiques, nous avons également pu

Figure 2: Arbre phylogénétique des récepteurs aux monoamines chez les vertébrés - Hypothèse sur l'évolution des récepteurs à la dopamine



A gauche, les relations phylogénétiques entre les différentes classes de récepteurs aux monoamines ont été étudiées en analysant les séquences protéiques correspondantes. L'arbre a été raciné avec la classe des récepteurs muscariniques (violet) et la longueur des branches est proportionnelle à la similarité globale entre groupes. L'arbre indique clairement une divergence ancienne entre chaque classe de récepteurs, y compris pour les classes D1 et D2. 5HT (vert): récepteurs à la sérotonine; alpha et beta (jaune): récepteurs adrénergiques; M (violet) récepteurs muscariniques; TA (bleu): récepteurs aux amines traces; D1 et D2: récepteurs à la dopamine.

A droite est proposée une histoire évolutive des récepteurs D1 (et D2) basée sur des analyses structure/fonction: 2 étapes de duplications géniques auraient présidé à l'apparition de 4 sous-types D1 et 3 sous-types D2 depuis l'émergence des gnathostomes, donnant naissance à 4 paralogues D1. Après les duplications géniques, les nouveaux sous-types auraient acquis des propriétés (affinité pour la dopamine: triangle), des fonctionnalités (activité intrinsèque: carré) et des localisations spécifiques (rond), permettant l'acquisition de fonctions physiologiques particulières et donc leur conservation. En revanche, le type de couplage aux protéines G serait un caractère ancestral puisqu'il est retrouvé dans les sous-types de lamproies, myxines et amphioxus.

D'après Callier et al., 2003.

estimer la conservation fonctionnelle qui existe entre les réseaux de régulation impliquant les protéines de la famille AP-1 chez les levures et chez les mammifères. Ces résultats ont été confirmés par la suite dans des études de génomique comparative. C'est ce type d'approche que j'ai appliquée, dans un autre contexte biologique au cours de mon premier stage post-doctoral.

C - Stage post-doctoral (1999-2000) : Evolution des récepteurs à la dopamine chez les vertébrés (UPR2197 - Gif-sur-Yvette)

Lors de mon premier stage post-doctoral, j'ai étudié la diversité et les relations localisation/fonction des sous-types de récepteurs à la dopamine chez les vertébrés, en utilisant des approches de génétique comparative et de physiologie comparée. Parallèlement, j'ai profité de mon expérience en génomique pour effectuer un service d'ATER à temps complet dans le module de génomique à l'Université Paris XI.

Chez les vertébrés comme chez les levures, la transmission de l'information est une condition indispensable à l'adaptation du comportement et à la survie cellulaire. L'équipe de P. Vernier s'intéressait plus particulièrement à la famille des récepteurs aux monoamines. Si de nombreuses séquences des régions codantes de ces récepteurs étaient déjà disponibles chez les mammifères placentaires (Cardinaud, 1997, 1998), les données demeuraient encore insuffisantes chez d'autres groupes tels que les reptiles ou les monotrèmes pour établir une phylogénie de cette famille multigénique chez les vertébrés, en déduire les implications fonctionnelles, et résoudre les incertitudes sur les relations évolutives entre les différents groupes.

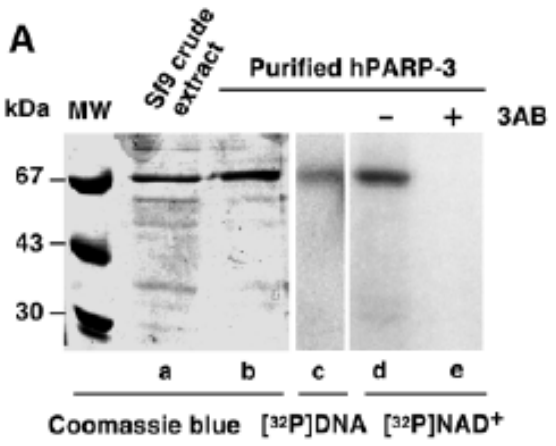
Mon projet de recherche a consisté à étudier, à grande échelle, la diversification des récepteurs catécholaminergiques chez les vertébrés, à décrire l'organisation des gènes codant ces récepteurs et à analyser les mécanismes de leur expression spécifique. Pour le mener à bien, j'ai utilisé une approche de génétique comparative et confronté les résultats avec ceux obtenus par des techniques de bioinformatique et d'hybridation *in situ*.

Ce travail s'est déroulé en plusieurs étapes :

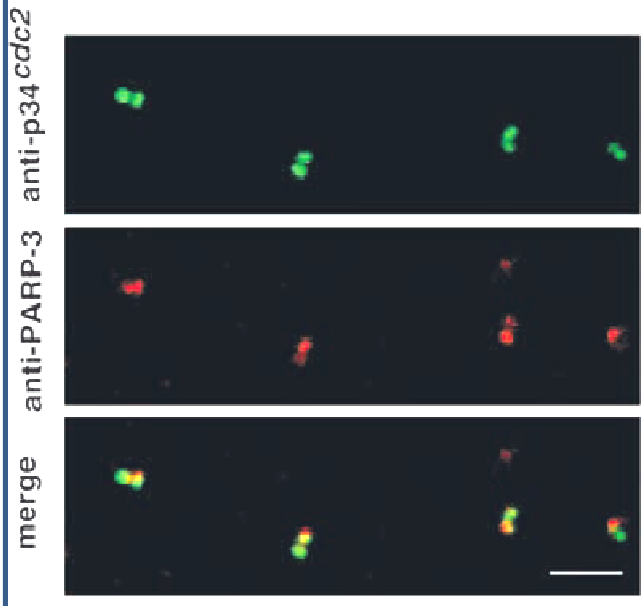
- identifier les gènes des récepteurs aux monoamines chez des espèces diverses, dont le génome n'était pas -ou très partiellement- séquencé, représentatives des grandes étapes de l'évolution des vertébrés ;

- cloner par PCR dégénérée, séquencer et analyser *in silico* des fragments des gènes codant une des classes (D1) de récepteurs à la dopamine chez une espèce de tortue, de lézard et de serpent, chez l'ornithorynque et l'échidné. Cette étude nous a permis de dresser une

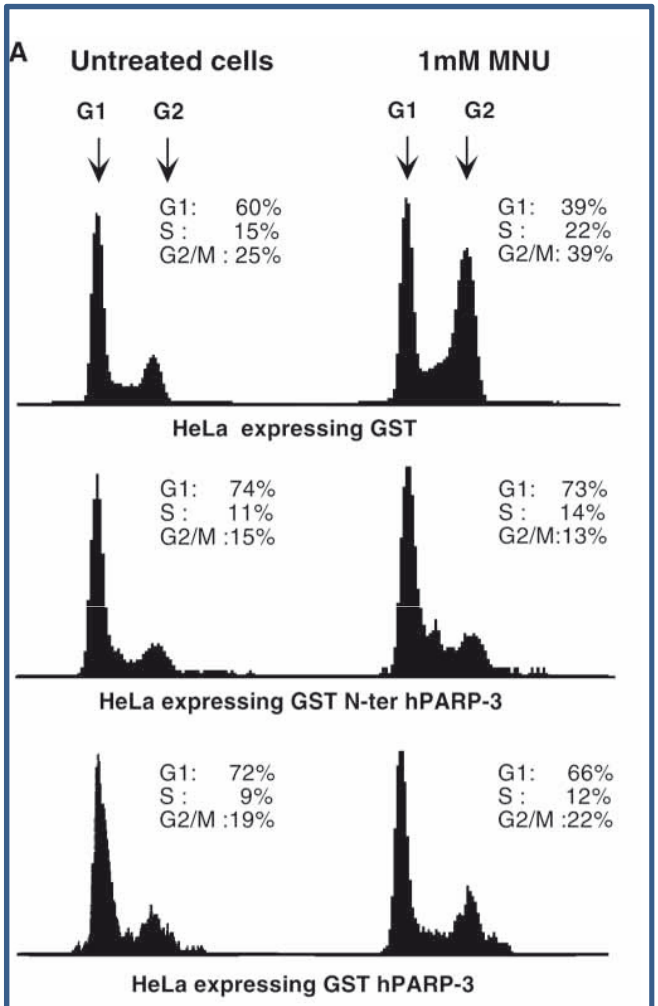
Figure 3: Détection, purification, localisation et analyse fonctionnelle de la PARP-3 dans des cellules humaines



Purification et caractérisation de PARP-3 recombinante dans des cellules Sf9 infectées par baculovirus avant (a) ou après(b-e) purification. Activité de liaison détectée par south-western blot (c). Autopoly(ADP-rybosyl)ation de PARP3(d,e).



Préparation de centrosomes marqués (vert) indiquant la **localisation de PARP-3 au centriole fils** (rouge).



La **surexpression de PARP-3** humaine recombinante induit un blocage du cycle cellulaire en G1, y compris après traitement à la MNU (methyl-nitroso-urée).

D'après Augustin et al., 2003.

image plus complète mais aussi plus complexe de la phylogénie moléculaire des récepteurs D1 chez les vertébrés ;

- comparer entre elles les séquences codantes des gènes orthologues et réaliser une étude approfondie de la structure des régions régulatrices des gènes codant les récepteurs D1 chez la lamproie et le médaka (une espèce proche du poisson-zèbre) ;

- localiser, par hybridation *in situ*, les régions cérébrales qui expriment les différents sous-types identifiés chez le médaka.

L'approche génomique, couplée à des techniques plus classiques de biologie moléculaire d'imagerie, et la mise en relation avec des données anatomiques et physiologiques, nous ont permis de mieux positionner les différents groupes taxonomiques les uns par rapport aux autres (Figure 2). Dans ce contexte d'organismes complexes et non modèles, j'ai eu à nouveau l'occasion de mettre en relation des données moléculaires (expansion d'une famille de gènes) et physiologiques (complexification du système dopaminergique et acquisition de nouvelles fonctions cérébrales au cours de l'évolution) (Kapsimali, 2000).

D - Stage post-doctoral (2000-2001) : Caractérisation de la protéine PARP-3 de mammifères (UPR 9003 CNRS- Strasbourg)

Mon deuxième stage post-doctoral m'a donné l'opportunité de me focaliser sur la dissection fine de mécanismes cellulaires impliquant une protéine, PARP-3, située au carrefour des mécanismes de maintien de l'intégrité du génome et des réseaux de régulation de la prolifération cellulaire chez les eucaryotes supérieurs. Il s'agissait ici non plus d'utiliser des approches globales et sans *a priori*, mais de positionner précisément cette protéine dans des voies de signalisation connues par des techniques de biologie cellulaire que j'ai appris à maîtriser.

Le laboratoire de G. de Murcia s'intéressait plus particulièrement à une famille de protéines intervenant dans la détection des lésions de l'ADN et dans les étapes primaires de la signalisation intracellulaire : les poly-ADP-ribose-polymérases (ou PARP). La caractérisation biochimique et fonctionnelle de la PARP-3 a été entreprise en collaboration avec la société BASF. Les premiers résultats obtenus montraient que cette protéine est associée à l'appareil mitotique et qu'elle est plus particulièrement localisée au niveau du centrosome tout au long du cycle cellulaire. Des travaux antérieurs indiquaient qu'une dérégulation de la duplication du centrosome, elle-même couplée à la réplication de l'ADN, entraîne une ségrégation anormale des chromosomes et donc une forte instabilité du génome en réponse à l'irradiation

gamma. La localisation particulière de la PARP-3 suggérait donc fortement un rôle de cette protéine dans la dynamique du centrosome lors de la division cellulaire (Amé, 1999 ; d'Adda di Fagagna, 1999 ; Johansson, 1999 ; de Murcia, 2000).

Dans ce contexte, j'ai poursuivi la caractérisation fonctionnelle de la PARP-3 grâce à des techniques de biologie cellulaire et moléculaire, en me focalisant sur trois grands axes :

- préciser la localisation de PARP-3. J'ai confirmé la présence de PARP-3 au niveau du centriole fils en réalisant des expériences d'immunofluorescence sur des préparations de centrosomes isolés (Figure 3),

- caractériser la fonction de PARP-3. J'ai observé, après transfection dans des cellules Hela en culture, l'effet de la surexpression de la protéine et/ou de son domaine *N*-terminal sur la cinétique du cycle cellulaire avant ou après exposition de la lignée aux radiations ionisantes (Figure 3),

-identifier les partenaires de PARP-3 : par un crible double-hybride dans la levure puis par immunoprécipitation à partir d'extraits de cellules Hela.

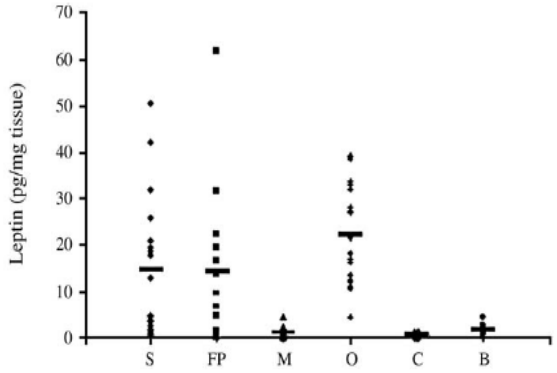
La caractérisation du rôle physiologique de PARP-3 et son intégration dans les réseaux de régulation de la division mitotique en relation avec les dommages de l'ADN a permis de mieux comprendre les relations entre la cinétique de duplication des centrosomes, la réplication et la réparation de l'ADN et les différentes phases du cycle cellulaire (Augustin, 2003).

<u>E - Stage post-doctoral (2001 –2003) :</u> Caractérisation de modèles d'arthropathie chez le rat (UMR CNRS UHP 7561 ; P. Netter)

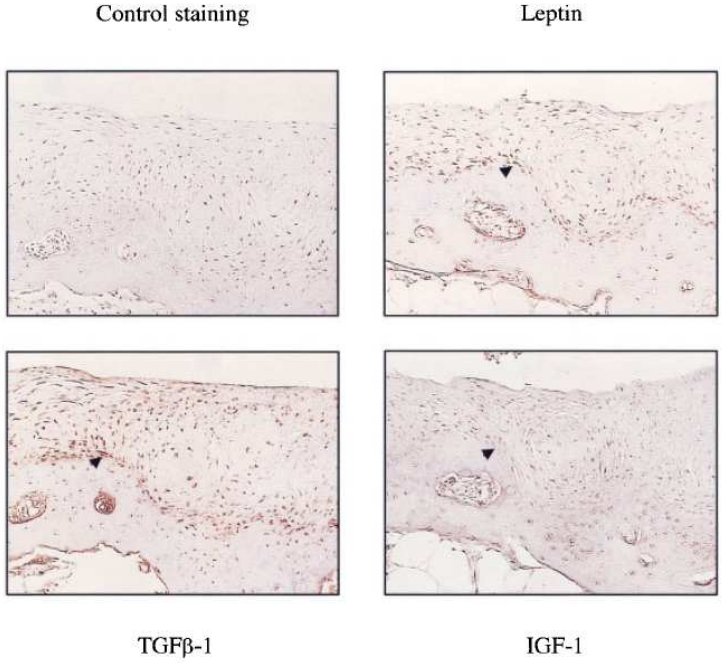
En avril 2001, j'ai intégré l'équipe de Physiopathologie et Pharmacologie Articulaires du Professeur Netter (Faculté de Médecine de Nancy) où j'ai appliqué mes connaissances en biologie cellulaire et moléculaire à la compréhension d'une pathologie humaine, l'arthrose. La complexité et le caractère multifactoriel de l'étiologie de cette maladie m'ont amenés à travailler sur des chrondrocytes en culture primaire, à développer un modèle de pathologie arthrosique chez le rat et également à analyser des prélèvements de cartilage humain, sain ou pathologique.

L'arthrose est la plus fréquente des pathologies articulaires et la deuxième cause d'invalidité en France, après les maladies cardiovasculaires. Cette pathologie chronique et multifactorielle apparaît comme une des manifestations inéluctables du vieillissement même s'il semble clair que l'âge ne suffit pas à expliquer toutes les modifications anatomiques et biochimiques qui conduisent à la destruction du cartilage. Son développement dépend au

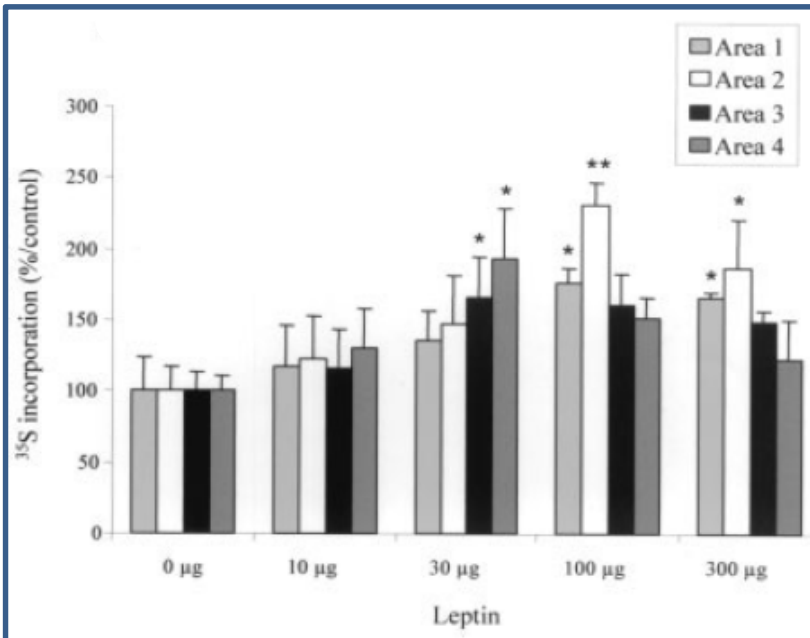
Figure 4: Stimulation de la réparation du cartilage arthrosique par la leptine présente dans le liquide synovial

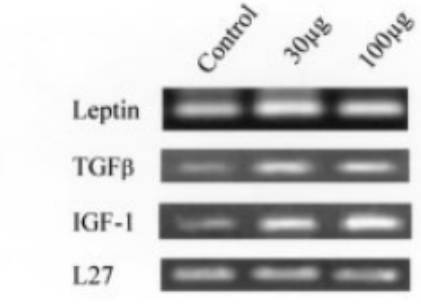


Contribution des différents tissus articulaires du genou à la production de leptine. S: membrane synoviale; FP: fatpad, M: ménisque; O: ostéophytes; C: cartilage; B: os. La synoviale, le fat pad et les ostéophytes sont les principaux producteurs locaux de leptine.



Immunolocalisation de leptine, TGFβ-1 et IGL-1 sur des coupes de cartilage de patients arthrosiques. La leptine et les facteurs de croissance sont exprimés dans les mêmes cellules au niveau des ostéophytes.





Une **injection intra articulaire de leptine** (10-100µg) induit la synthèse des protéoglycanes (à gauche, mesurée par incorporation de ^{35}S dans différentes zones des plateaux tibiaux) et l'expression des facteurs de croissance IGF-1 et TGFβ-1 (à droite, mesurée par RT-PCR).

D'après Dumond et al., 2003; Presle et al., 2006.

contraire de nombreux facteurs d'ordre génétique, physiologique, biochimique et biomécanique.

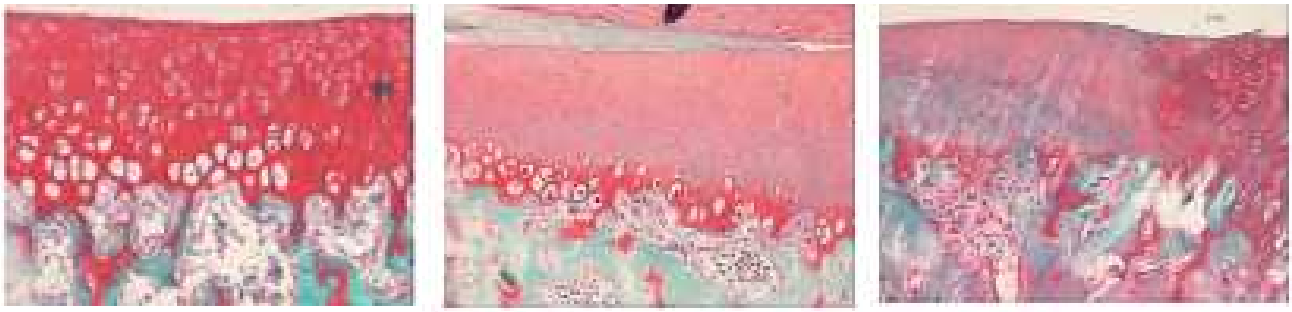
Les lésions du cartilage sont généralement associées à des contraintes de charge trop élevées ou anormalement réparties. L'articulation du genou est plus spécialement concernée (Hochberg, 1995, Gelber, 1999 ; Sharma, 2000, Coggon, 2001), mais des études montrent aussi une association positive entre la surcharge pondérale et l'arthrose de la main, notamment chez les sujets obèses (Carman, 1994 ; Oliveria, 1999). Par conséquent, des facteurs systémiques semblent se superposer aux contraintes mécaniques : en particulier, des facteurs métaboliques pourraient expliquer l'augmentation de la prévalence de l'arthrose digitale observée chez les sujets obèses.

Plusieurs études ont également permis de relier l'apport en acides gras à l'incidence et la progression des pathologies articulaires au cours du vieillissement (Geusens, 1994, Shapiro, 1996 ; Busso, 2002). L'association positive entre hypercholestérolémie et arthrose poly-articulaire chez les personnes âgées indique que des facteurs systémiques impliqués dans l'homéostasie lipidique participeraient aux changements physiopathologiques observés au cours de l'arthrose (Aspden, 2001).

Plusieurs laboratoires s'efforcent de définir et de préciser les phénomènes et les facteurs conduisant à l'érosion du cartilage puisqu'aucun traitement chondroprotecteur réellement efficace n'est actuellement disponible.

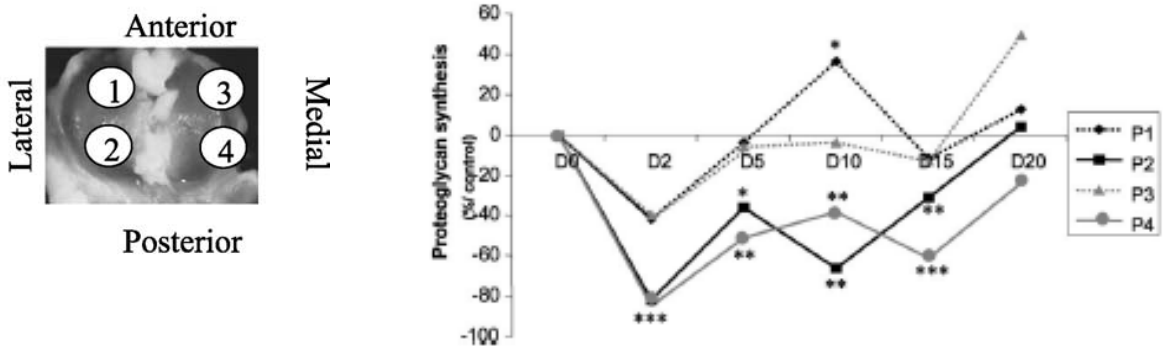
Dans ce contexte, nous avons cherché à caractériser les cibles pharmacologiques qui se situent à l'interface entre la rupture de l'homéostasie du cartilage et le métabolisme lipidique. L'augmentation de l'incidence de l'arthrose chez les sujets obèses suggère que le contrôle de l'homéostasie du cartilage et de la masse grasse pourrait mettre en jeu le (les) même(s) médiateur(s). Les adipocytokines participent au maintien de l'homéostasie lipidique et glucidique. Dans les conditions pathologiques, leur synthèse est modulée par les cytokines pro-inflammatoires (Coppack, 2001 ; Funk, 2001) comme l'IL1beta et le TNFalpha, leur production est altérée par l'obésité ou le diabète de type II. Parmi les adipocytokines, la leptine constituait un bon candidat pour relier les changements métaboliques liés à l'obésité et la physiopathologie de l'arthrose ; en effet, sa production est assurée par le tissu adipeux (intra- ou extra-articulaire), modulée au cours de la réaction inflammatoire, et augmentée chez les individus obèses et/ou âgés qui présentent un fort risque d'arthrose (Wang, 2001). La leptine est capable d'agir sur les tissus périphériques : de nombreux auteurs ont mis ainsi en évidence son implication dans la croissance et la différenciation osseuses (Cornish, 2002). Etant donné que des récepteurs fonctionnels de la leptine ont été identifiés dans les

Figure 5: Caractérisation d'un modèle d'arthrose induite par l'acide mono-iodoacétique

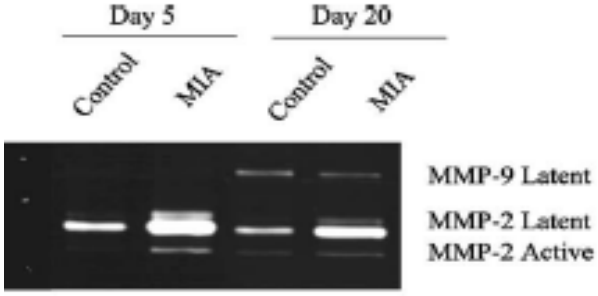


Solution saline J20 | MIA J5 | MIA J20

Coupes de cartilage de plateaux tibiaux colorées par un mélange de Safranin-O Fast green.
Les rats ont reçu une injection intra-articulaire de 0,03mg de MIA puis ont été sacrifiés à différents temps post-injection. Après 5 jours (J5), on observe dans les zones postérieures une perte de coloration correspondant à la détérioration de la matrice cartilagineuse; à 20 jours (J20), la surface du cartilage est irrégulière, la dégradation des protéoglycanes est importante (plus de coloration rouge) et on note la présence de chondrocytes hypertrophiés, reproduisant ainsi les différents caractères histologiques observés chez les patients arthrosiques.



A gauche, localisation des différentes zones des plateaux tibiaux où ont été effectuées les mesures de synthèse et de catabolisme de la matrice cartilagineuse. **A droite**, on observe la **cinétique de synthèse des protéoglycanes** dans ces zones (P1-P4) après injection de MIA: la perte d'anabolisme est variable selon la localisation et elle est surtout observable entre J2 et J15 post-injection. A J20, on observe un début de réparation des zones affectées.



Activité des métalloprotéases MMP-2 et MMP-9 mesurée par zymographie dans la zone P4. Le MIA induit principalement l'activité MMP-2.

D'après Dumond et al., 2004.

chondrocytes humains (Fingenshau, 2001), on pouvait faire l'hypothèse qu'elle agisse également sur ce tissu articulaire, et tout particulièrement au cours des changements métaboliques observés dans l'arthrose.

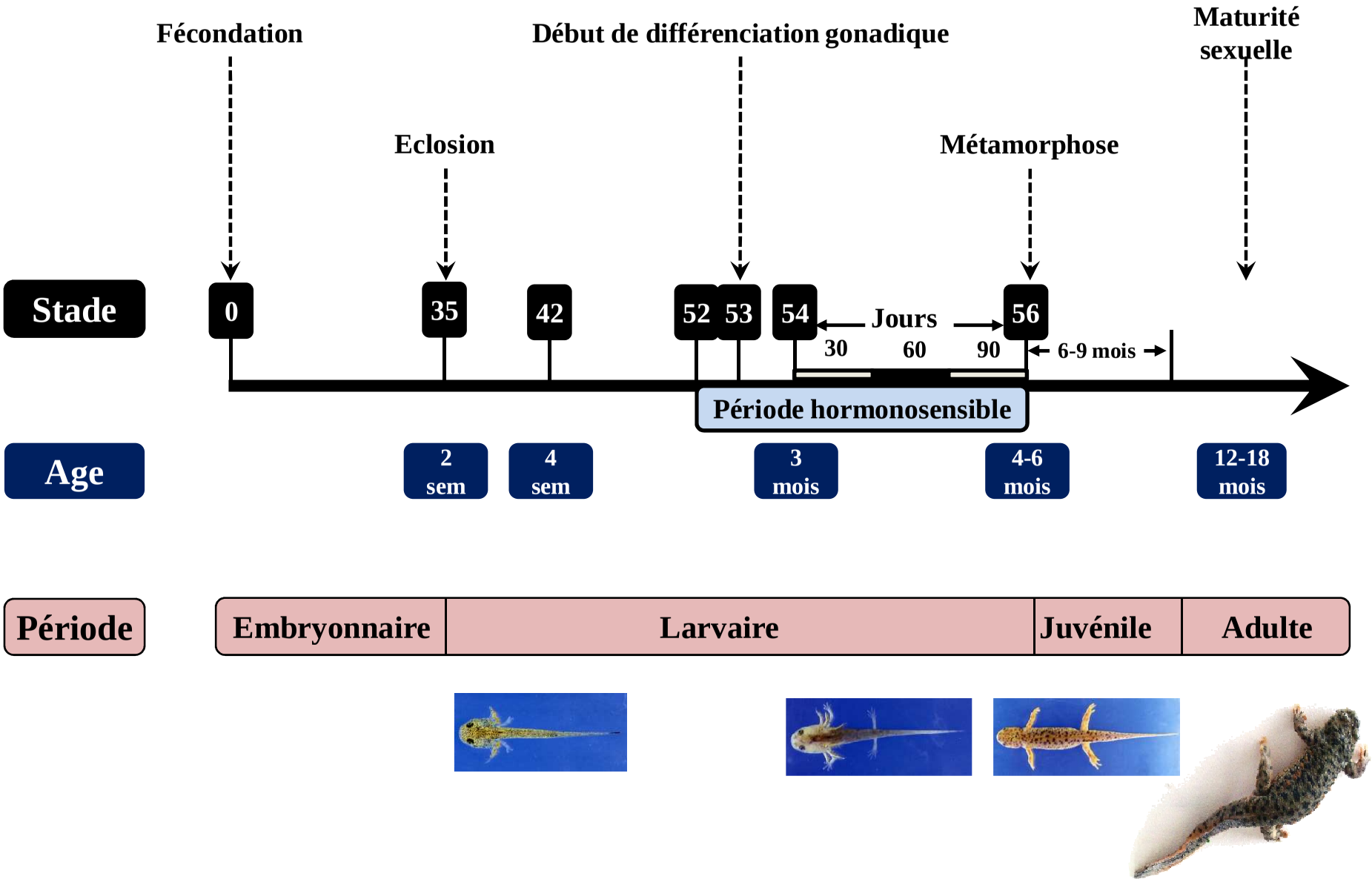
Dans le cadre d'un Contrat de Programme de Recherche Clinique, nous avons évalué la contribution de la leptine dans la pathologie arthrosique. J'ai ainsi collaboré avec l'équipe chirurgicale du Pr Mainard à l'hôpital central de Nancy pour la réception, la préparation et l'analyse de cartilage arthrosique curé lors de la pause de prothèse du genou. Pour la première fois, nous avons mis en évidence la présence de leptine dans le liquide synovial et le cartilage de patients arthrosiques. De façon intéressante, le niveau d'expression de la leptine dans le cartilage est relié à la sévérité des lésions arthrosiques et apparaît parallèle à celui des facteurs de croissance. Notre étude chez le rat a ensuite permis de mesurer les variations d'expression de la leptine dans un modèle d'arthrose (voir plus loin). De plus, une injection unique de leptine dans l'articulation stimule fortement les fonctions anaboliques des chondrocytes et induit la synthèse d'IGF1 et de TGFbeta1. L'ensemble de ces résultats confère une nouvelle fonction périphérique à la leptine comme régulateur clef du métabolisme chondrocytaire et montre qu'elle pourrait jouer un rôle important dans la physiopathologie de l'arthrose (Figure 4 ; Terlain, 2005).

Parallèlement, j'ai eu l'opportunité d'animer, au sein de l'UMR, un projet financé dans le cadre d'un contrat européen, et visant à comprendre le rôle des cytokines dans la destruction du cartilage au cours des phases précoces de l'arthrose. Mon rôle consistait précisément à mener le travail expérimental imparti à l'équipe mais également à collaborer avec les autres partenaires du projet et à rendre compte de l'avancée des travaux et des perspectives envisagées lors des réunions annuelles.

D'une part, nous avons étudié l'influence de l'IL-17 sur la synthèse des protéoglycanes et sur la production de NO (monoxyde d'azote) par les chondrocytes *in vitro* (Pacquelet, 2002). Ces effets ont été comparés à ceux obtenus avec l'IL1beta seule ou combinée à l'IL-17. Nous avons ainsi montré que le NO est un médiateur important mais non exclusif d'une inhibition de la synthèse des protéoglycanes induite par les cytokines pro-inflammatoires et que l'anion superoxyde constitue un régulateur clef des effets du NO sur le cartilage. D'autre part, j'ai eu l'occasion de développer un modèle animal mimant les symptômes de l'arthrose humaine en caractérisant les aspects moléculaires du modèle d'arthrose induite par injection de monoiodacétate (MIA) chez le rat (Dumond, 2004).

L'injection intra-articulaire de MIA (0,03mg) provoque une expression d'IL1 dans tous les tissus articulaires, concomitante à une baisse d'anabolisme des protéoglycanes

Figure 6: Table de développement de *Pleurodeles waltl*



D'après Gallien et Durocher, 1957.

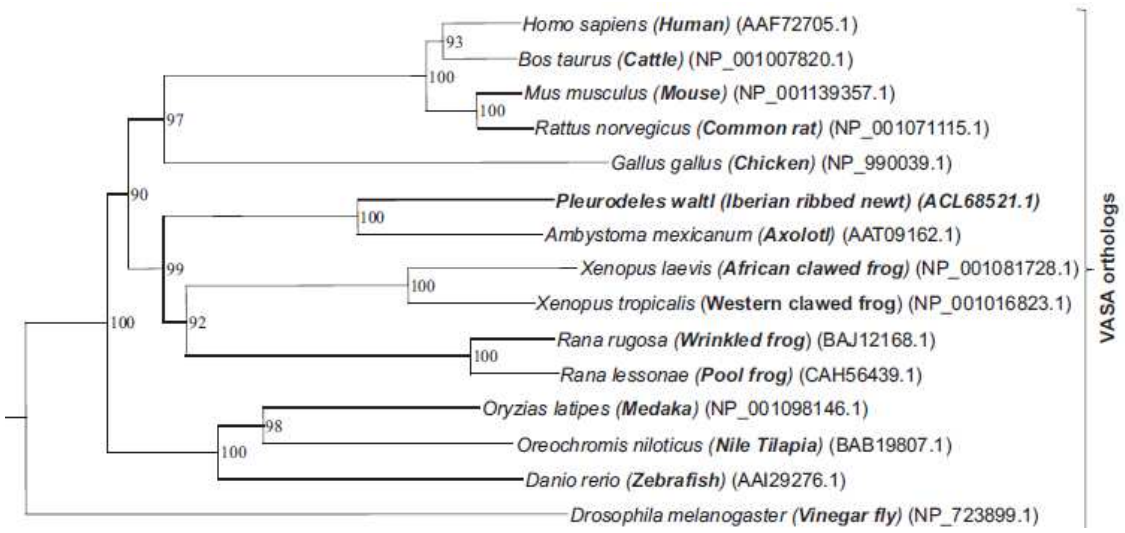
(Figure 5). En outre, l'étude de ce modèle a confirmé la contribution d'iNOS et de COX2 aux processus initiateurs de la pathologie arthrosique. Cependant, l'inhibition de l'activité de COX2 par des anti-inflammatoires non stéroïdiens (AINS) sélectifs s'est révélée neutre pour le cartilage *in vivo*, suggérant l'existence de relations entre les cibles. Les interactions avec les services de rhumatologie et de chirurgie orthopédique des hôpitaux de Nancy, nous ont permis également de confirmer une partie des données obtenues chez l'animal sur des prélèvements de tissu articulaire humain. L'intégration des données moléculaires, métaboliques et histopathologiques obtenues dans les différents modèles d'arthrose étudiés nous a permis de conclure au role primordial des adipocytokines dans cette pathologie. Ce travail est toujours poursuivi dans l'équipe actuellement.

F- Activités de recherche dans l'EA3442 (2003-2008)

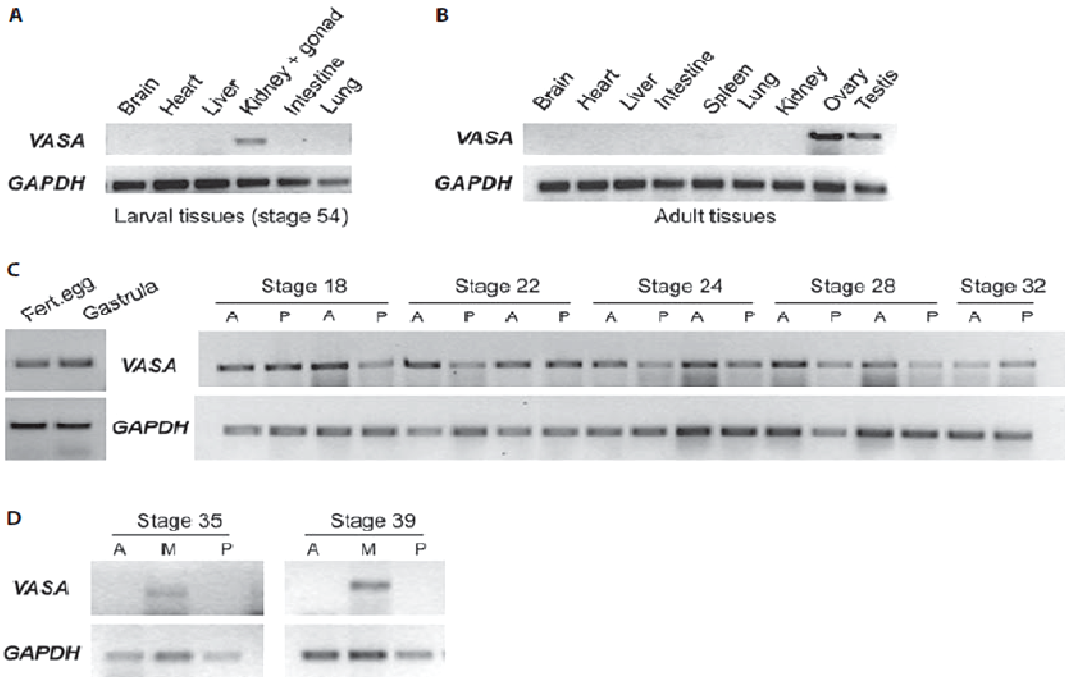
Suite à mon recrutement en tant que Maître de conférences, en septembre 2003, j'ai intégré le groupe animé par Stéphane Flament au sein de l'EA3442 « Aspects cellulaires et moléculaires de la reproduction et du développement» qui s'intéressait à l'étude de la différenciation du sexe chez le Pleurodèle. Il s'agissait pour moi d'un nouveau modèle animal, chez qui très peu d'outils génétiques et moléculaires étaient disponibles. Néanmoins, l'objectif était de transposer mes connaissances en génomique fonctionnelle et régulations cellulaires sur ce modèle en les utilisant pour comprendre les modalités moléculaires et les régulations mises en jeu lors du développement gonadique normal ou altéré par les conditions environnementales. Plus précisément, j'ai tenté de mettre en évidence les régulateurs placés en amont de l'aromatase puis je me suis focalisée sur le rôle des stéroïdes dans la différenciation sexuelle des cellules germinales. L'absence de transgénèse possible chez le pleurodèle nous a conduits à développer des modèles d'étude adaptés à cette problématique comme les parabioses embryonnaires ou la culture organotypique.

Le déterminisme du sexe est le mécanisme qui préside à la différenciation d'un individu en mâle, femelle ou hermaphrodite. Ce mécanisme est contrôlé par des déterminants génétiques et/ou environnementaux, selon les classes de vertébrés. Il induit une organogenèse sexe-spécifique de l'ébauche gonadique indifférenciée grâce à des événements cellulaires variés (Kocer, 2009). Chez tous les vertébrés, il existe des individus résultant d'inversions sexuelles, que celles-ci soient naturelles ou dues à des traitements hormonaux ou des changements environnementaux. Ces individus sont particulièrement pertinents pour mener des études ayant pour but d'identifier les facteurs et les processus morphogénétiques impliqués dans le « switch » mâle/femelle.

Figure 7: PwVASA, marqueur de cellules germinales chez *P. waltl*



Arbre phylogénétique des RNA-hélicases de la famille de VASA chez les vertébrés. La séquence de Pleurodèle est clairement apparentée aux orthologues clonés chez les amphibiens. L'arbre est raciné sur la séquence de drosophile.



Mesure par RT-PCR semi-quantitative de **l'expression du transcrit PwVASA** dans les différents tissus larvaires (A) et adultes (B), au cours du développement embryonnaire (C) et larvaire (D). PwVASA est détecté spécifiquement dans les gonades à partir de l'éclosion. A: partie antérieur de l'embryon (jusqu'au bourgeon du pronéphros); M: partie médiane (pronéphros-anus); P: partie postérieure (anus-extrémité de la queue).

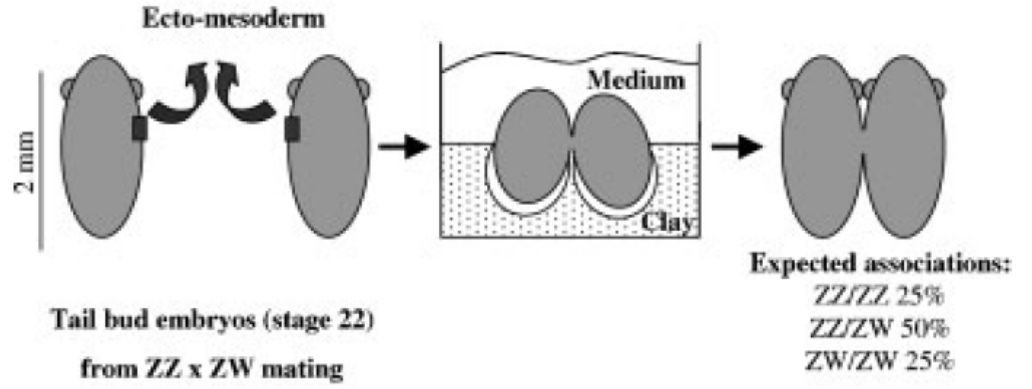
D'après Al-Asaad et al., 2012.

La thématique développée à l'EA 3442 concerne le rôle des hormones stéroïdes dans la différenciation sexuelle d'un amphibien urodèle, le Pleurodèle. Ce modèle a été choisi pour son déterminisme génétique du sexe (chromosomes sexuels ZZ chez le mâle et ZW chez la femelle) influençable par l'environnement : élevées à 32°C (au lieu de 20°C habituellement), les larves génétiquement femelles se différencient en mâles fertiles. Au laboratoire, la chronologie des événements morphologiques et moléculaires conduisant à cette différenciation est analysée. Plusieurs études ont notamment porté sur l'aromatase, enzyme qui synthétise les œstrogènes à partir des androgènes (Kuntz 2003a,b). Elles ont été poursuivies grâce au clonage et à l'étude du profil d'expression des gènes Sox9, Dmrt1 et Sf1 (Kuntz, 2006) au cours du développement (Figure 6).

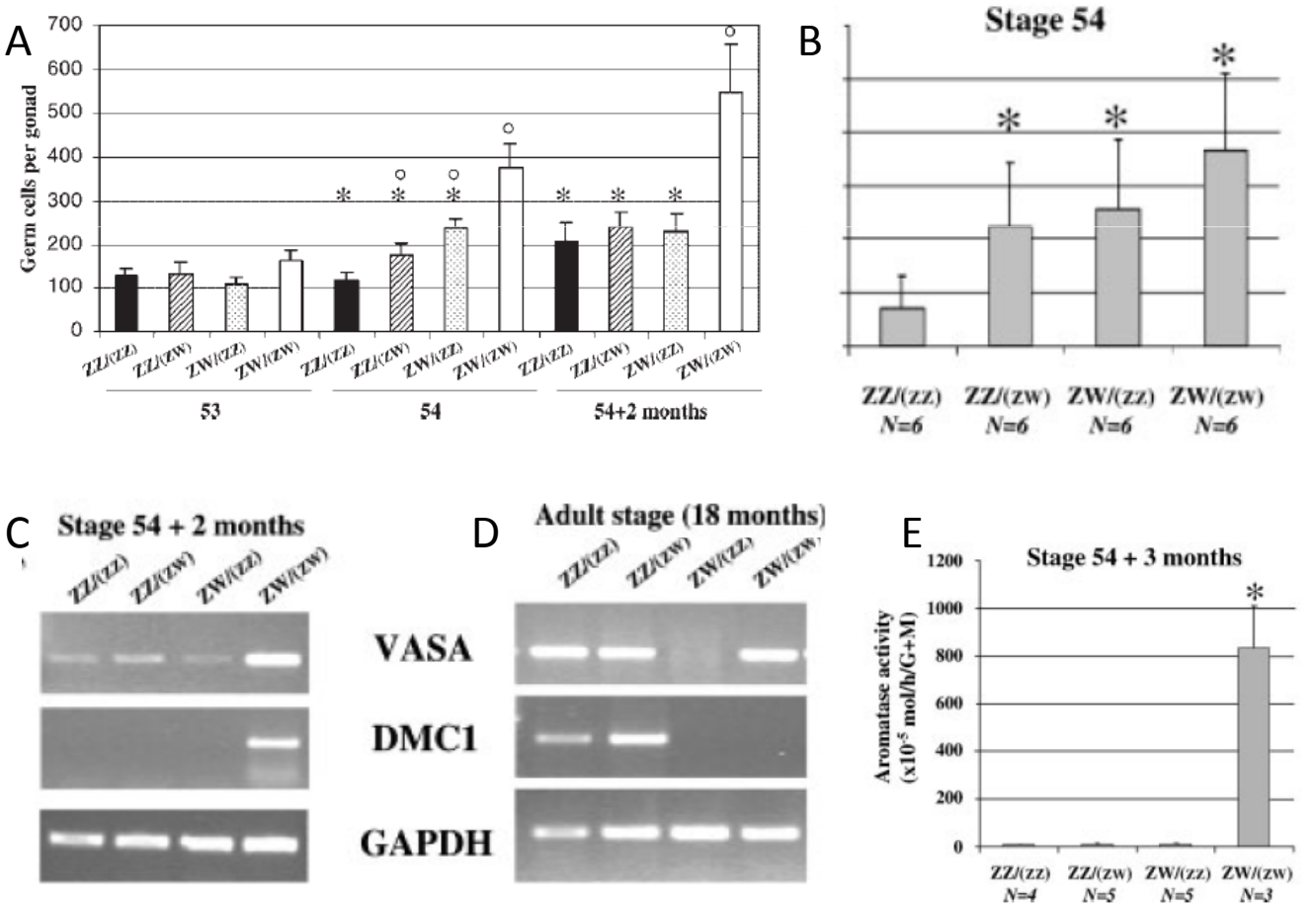
Dans le but d'identifier les principaux acteurs moléculaires responsables de la différenciation ovarienne, de nouveaux facteurs mis en évidence dans différentes espèces modèles de vertébrés sont toujours recherchés chez *Pleurodeles waltl*. C'est pourquoi, à mon arrivée au laboratoire, j'ai entrepris le clonage du gène FoxL2, un facteur de transcription dont la mutation est responsable du syndrome de blepharophimose (ou BPES pour Blepharophimosis Ptosis Epicanthus inversus Syndrome) chez l'homme (Crisponi, 2001). Plusieurs groupes ont mis en évidence l'implication de Foxl2 dans le développement et le fonctionnement de l'ovaire chez la souris, considérant ce gène comme le premier marqueur de différenciation ovarienne caractérisé à ce jour (Schmidt, 2004 ; Uda, 2004). En outre, FoxL2 régule l'expression de l'aromatase chez le poulet et les mammifères. Chez le Pleurodèle, j'ai montré que FoxL2 est également présent et exprimé dans l'ovaire adulte mais les études d'expression transcriptionnelle au cours du développement n'ont pas permis de mettre en évidence une expression sexe- ou stade-spécifique.

Parallèlement à l'étude de marqueurs de la différenciation somatique de la gonade (Dumond, 2011), je me suis attachée à développer les outils permettant d'analyser l'origine et le devenir des cellules germinales au cours de la différenciation gonadique. Plus précisément, j'ai cherché à déterminer l'importance, la nature et la cinétique des relations germen-soma au cours de la différenciation sexuelle. J'ai étudié en détail deux gènes : Vasa, marqueur des cellules germinales quel que soit leur état physiologique et Dmc1, marqueur d'entrée en méiose. Chez le Pleurodèle, au cours du développement embryonnaire, Vasa ne montre une expression spécifique de la gonade qu'au stade 35 puisqu'un transcrit maternel est présent au début du développement embryonnaire. Dmc1 code une recombinase intervenant lors des échanges de chromatides pendant la méiose. L'analyse par RT-PCR de l'expression de ce gène a permis de déterminer avec précision le moment où les cellules germinales entrent en

Figure 8: Effet free-martin chez *P.waltl*



Protocole simplifié de construction des parabioses



Effet free-martin: jusqu'au stade 54, l'expression des marqueurs somatiques et germinaux est conforme au sexe génétique des individus, quel que soit le type d'association (A, B). Au stade 54+2mois les cellules germinales ZW n'entrent pas en méiose chez les ZW/ZZ puisqu'elles n'expriment pas DMC1 (C). Elles vont disparaître (D), la gonade ZW n'exprime alors plus l'aromatase (E) mais ne présente pas de phénotype testiculaire.

D'après Dumond et al., 2008.

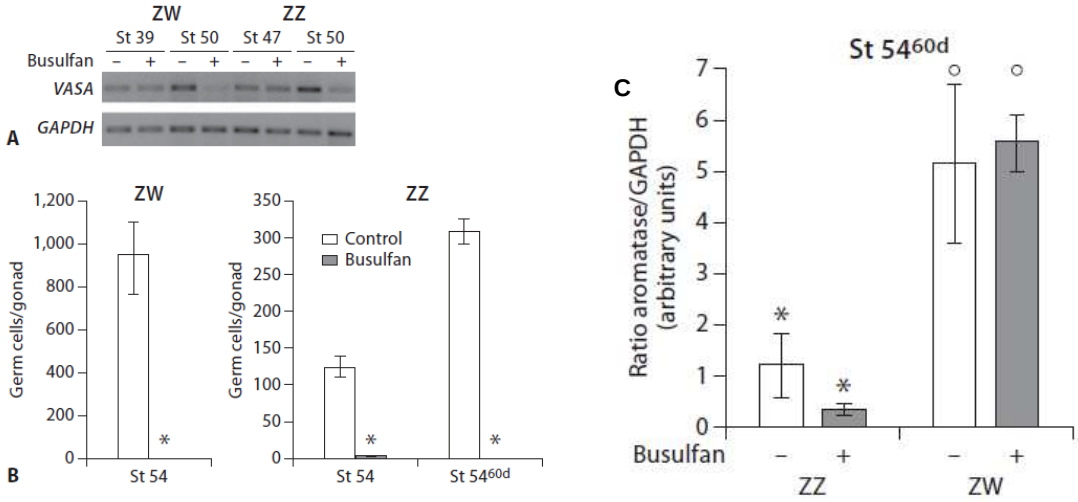
méiose : en fin de vie larvaire (stade 54+ 2 mois) pour les ovogonies et deux mois après la métamorphose pour les spermatogonies (Figure 7).

Nos outils moléculaires, marqueurs somatiques et germinaux ont permis de réaliser une étude moléculaire de la différenciation gonadique dans un modèle original de parabiose embryonnaire chez le Pleurodèle, développé en collaboration avec le docteur Jean-Pierre Maufroid (Université de Lille 1). Dans ce système, lorsqu'un embryon génétiquement mâle est associé à un embryon génétiquement femelle, ce dernier voit sa différenciation gonadique inhibée, rappelant l'effet free-martin caractéristique des bovidés (observé chez des faux-jumeaux de sexe opposé). Nous avons montré que cet effet est réellement une inhibition plutôt qu'une inversion du sexe : les cellules germinales disparaissent entre le stade 53 et le stade 55 (métamorphose), tandis que les cellules somatiques initialement nombreuses ne montrent pas d'activité aromatase, puis la gonade devient pauvre en cellules et riche en fibres de collagène (Figure 8). Chez l'adulte les marqueurs testiculaires comme dmrt1 ne sont pas exprimés dans cette gonade vestigiale. Grâce aux marqueurs des cellules germinales comme Vasa et Dmc1, nous avons montré une absence d'entrée en méiose des cellules germinales (Dumond, 2008a).

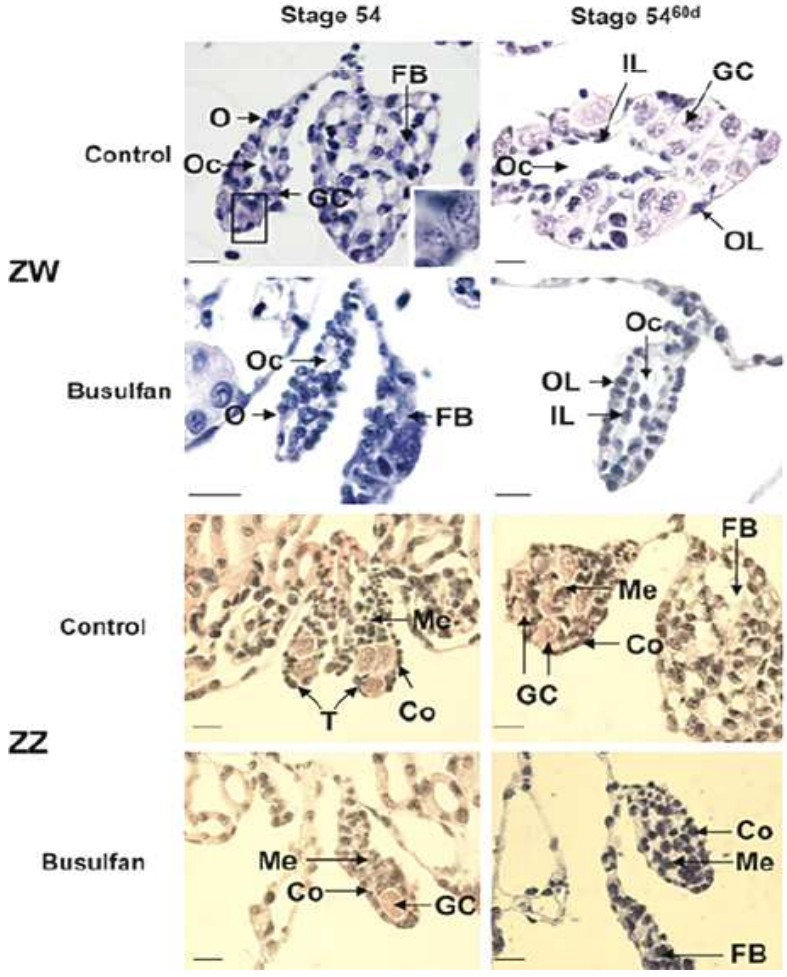
Ces données ont été complétées dans le cadre de la thèse d'Imane Al-Asaad par l'étude de la gonadogenèse dans un modèle de déplétion de cellules germinales induites par le Busulfan (diméthanesulfonate de butane-1,4-diyle) chez le Pleurodèle (Al-Asaad, 2012). Cet agent alkylant initialement utilisé dans le traitement des leucémies myéloides chroniques a été ajouté dans l'eau d'élevage des animaux pendant 3 jours au moment de l'éclosion. Nous avons montré que ce traitement induit la perte progressive des cellules germinales chez les 2 sexes. Elles sont quasiment absentes au stade 54. Néanmoins, le dimorphisme sexuel des gonades est observé chez les animaux juvéniles avec un ovaire creux chez les femelles et un testicule lobulaire avec une médulla bien développée mais dépourvue de cellules germinales chez les mâles. De plus, au stade 54+2 mois, l'activité aromatase est comparable chez les femelles traitées ou non au Busulfan (Figure 9). Ces résultats indiquent que la présence des cellules germinales n'est pas requise pour la différenciation gonadique chez le Pleurodèle, contrairement à d'autres espèces de vertébrés comme la souris (Guigon, 2006) ou le poisson-zèbre (Slanchev, 2005).

L'ensemble de ces résultats constitue une première étape dans la compréhension des relations germen-soma au cours de la différenciation gonadique chez le Pleurodèle (Dumond, 2008b). Ce travail est prolongé actuellement par l'étude de l'expression et du rôle de

Figure 9: Différenciation gonadique normale en absence de cellules germinales chez *P. waltl*



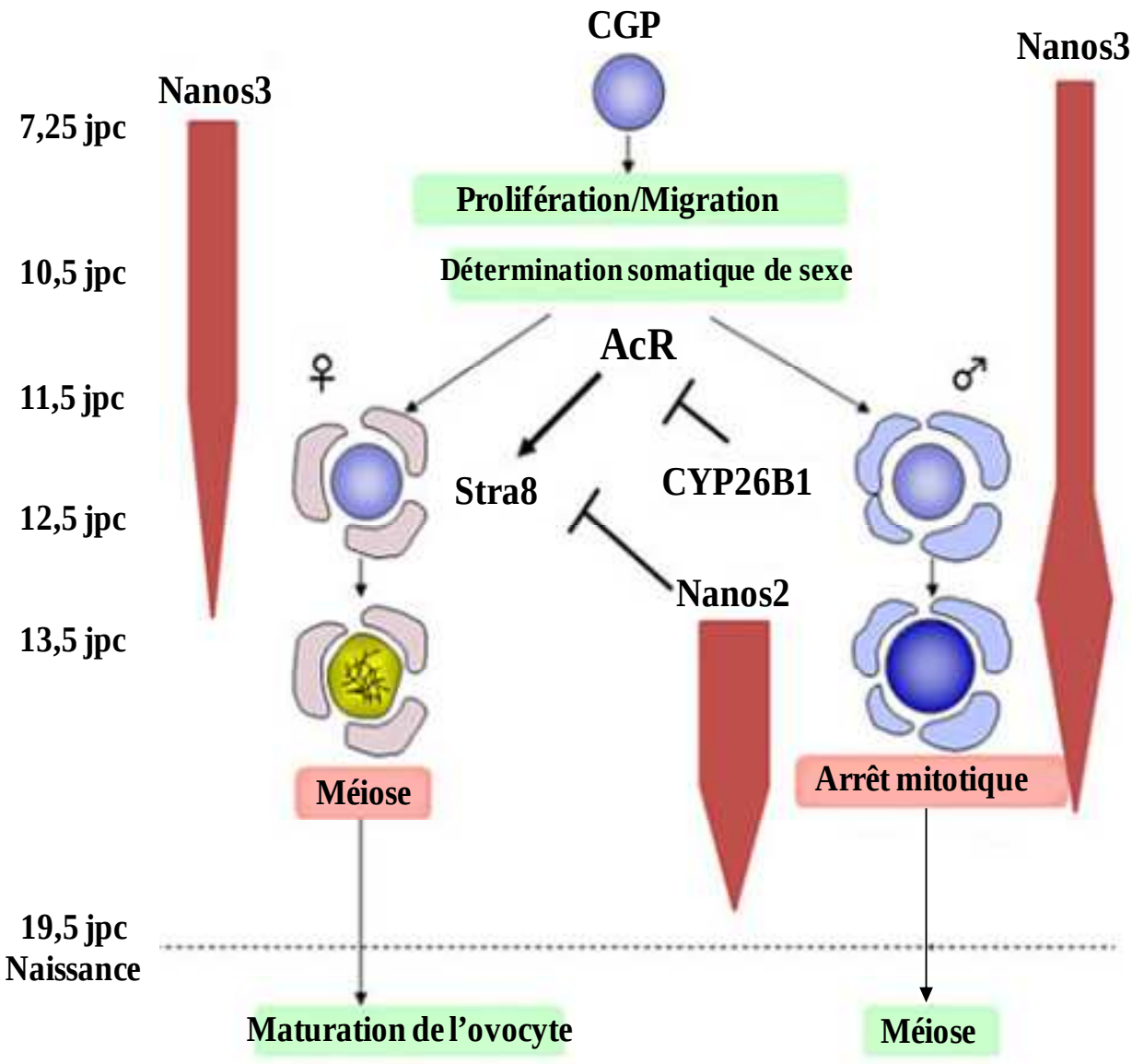
Un traitement de 72h après l'éclosion des larves de *P. waltl* conduit à une disparition progressive de l'expression de PwVASA (A) et des cellules germinales (B) mais n'affecte pas l'expression somatique de l'aromatase, spécifique des ovaires.



Histologie des gonades de larves ZZ et ZW traitées ou non au Busulphan. Chez les ZW traitées, on reconnaît un ovaire creux typique mais dépourvu de cellules germinales. Chez les ZZ, on observe une morphologie testiculaire avec une médulla épaisse d'où les cellules germinales sont absentes et un fin cortex. Co: cortex; FB: fat body; GC: germ cells; IL: inner layer; Me: médulla; O: ovary; Oc: ovarian cavity; OL: outer layer; T: testis. La barre représente 25 µm.

D'après Al-Asaad et al., 2012.

Figure 10: Rôle de l'acide rétinoïque dans la différenciation sexuelle des cellules germinales chez la souris.



Une fois les cellules germinales primordiales (CGPs) spécifiées à 7,25 jpc, elles prolifèrent et migrent vers le site des futures gonades dont les cellules somatiques commencent leur différenciation en exprimant les facteurs induisant la différenciation mâle ou femelle. L'acide rétinoïque (AcR) est produit par le mésonéphros dans les deux sexes, puis il diffusera dans la gonade. L'AcR sera dégradé par l'action de l'enzyme CYP26B1 dans la gonade fœtale mâle tandis que l'AcR induira l'expression de Stra8 dans les CGs femelles qui vont ensuite entrer en méiose. En effet, l'expression de Stra8 est réprimée d'abord par la dégradation de l'AcR par l'action de CYB26B1, ensuite par l'expression de Nanos2 qui commence à environ 13,5 jpc dans les CGs mâles. Celles-ci n'entreront en méiose qu'à la puberté. (D'après Saga, 2010).

D'après Saga, 2010.

l'hormone anti-mullérienne (Amh) au cours du développement (thèse d'I. Al-Asaad encadrée par S. Flament).

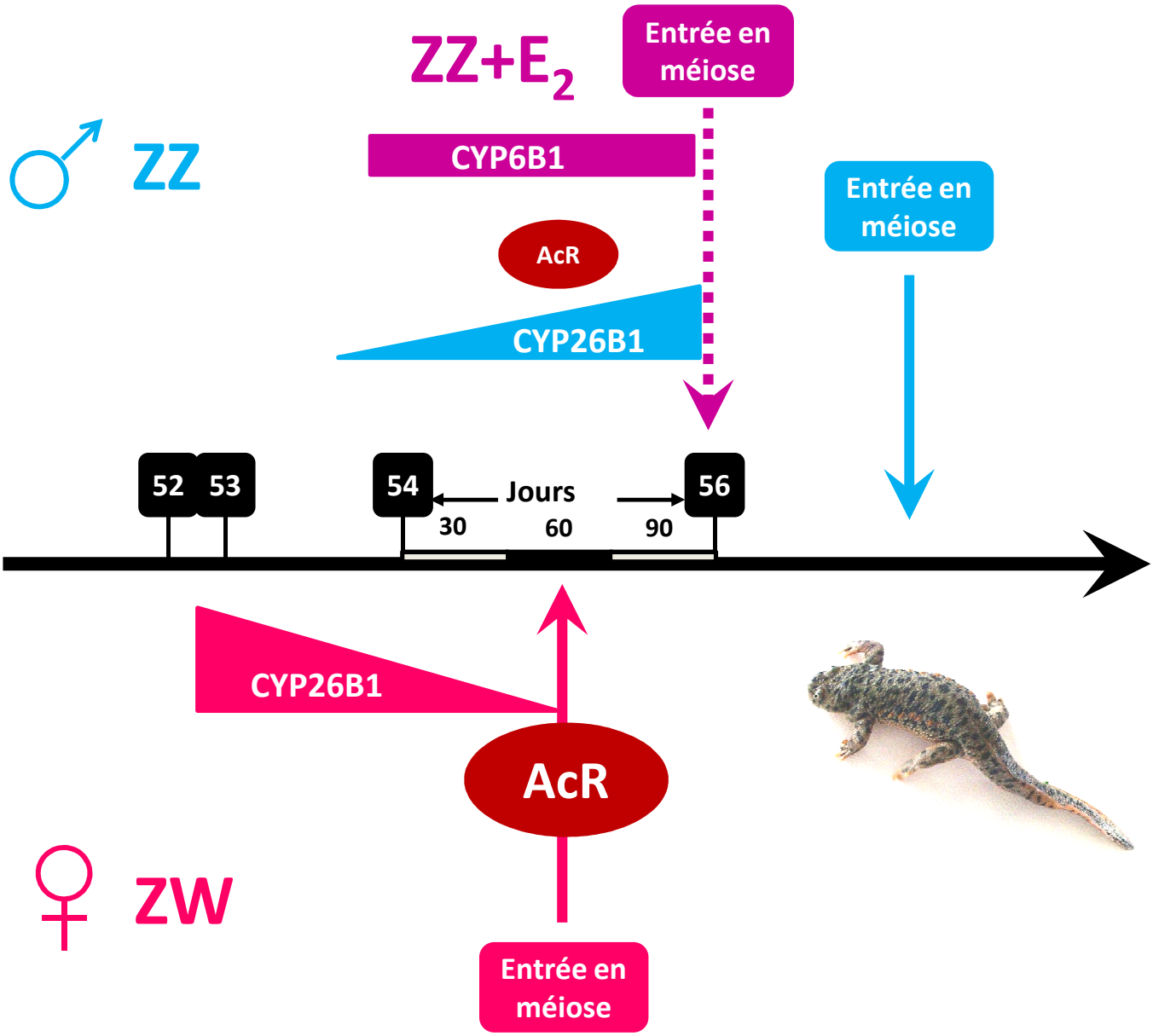
Co-encadrement du doctorat de Melle A. Wallacides

Les marqueurs moléculaires décrits ci-dessus nous ont également permis d'étudier les facteurs impliqués dans la régulation de l'entrée en méiose chez le Pleurodèle. Nous nous sommes inspirés des résultats publiés en 2006 chez les mammifères montrant que l'acide rétinoïque est impliqué et que son effet peut être modulé chez les mâles par Cyp26b1, enzyme qui dégrade l'acide rétinoïque (Koubova, 2006 ; Bowles et Koopman, 2007). L'expression fœtale de cette enzyme permettrait d'expliquer le retard d'entrée en méiose dans la gonade d'embryons de souris mâles par rapport à la gonade femelle. Nous avons également cloné le gène codant la rétinaldéhyde déshydrogénase (Raldh), enzyme permettant la synthèse de l'acide rétinoïque. Nous avons étudié l'expression de ces deux gènes au cours du développement du Pleurodèle. En outre, le groupe qui s'est constituée autour de cette thématique* a mis au point un modèle de culture organotypique et a montré que l'acide rétinoïque est capable de déclencher l'entrée en méiose chez le Pleurodèle (Figure 10). De même un inhibiteur de CYP26b1 permet l'entrée en méiose lorsqu'il est appliqué sur des cultures de complexes gonades-mésonéphros à un stade où l'entrée en méiose ne s'observe pas. Ce travail a fait en partie l'objet du stage de M2R de Angelina Wallacides et a été poursuivi au cours de sa thèse que j'ai co-encadrée avec S. Flament (Wallacides, 2009).

En utilisant des anticorps polyclonaux produits et purifiés au laboratoire, A. Wallacides a analysé par western-blot le niveau d'expression des enzymes clés du métabolisme de l'acide rétinoïque (Cyp26 et Raldh), à des stades précis de la différenciation gonadique. Elle a identifié les cellules qui expriment ces enzymes par immunofluorescence. Cette dernière étape de caractérisation a permis de confirmer le rôle crucial de l'acide rétinoïque pour l'entrée en méiose des cellules germinales chez le Pleurodèle (Figure 11). Une analyse similaire réalisée sur des animaux dont le sexe est inversé par un traitement hormonal a suggéré l'existence d'interconnections entre les voies de réponse aux stéroïdes et de signalisation par l'acide rétinoïque.

Plusieurs membres du laboratoire ont contribué à ce projet de recherches sur l'entrée en méiose des cellules germinales chez le pleurodèle en plus d'Angelina Wallacides dont c'était le sujet de thèse : **Amand Chesnel, Maître de conférences, qui a produit les anticorps polyclonaux et avec qui j'ai mis au point la culture de gonade de pleurodèle in vitro et*

Figure 11: Acide rétinoïque et entrée en méiose chez *P. waltl*



L'entrée en méiose des CGs chez *Pleurodeles waltl* a été déterminée au stade 54+2mois chez les femelles ZW et 8 semaines post-métamorphose chez les mâles ZZ. Dans un modèle de culture organotypique de gonade larvaire, l'ajout d'AcR induit une entrée en méiose précoce des CGs chez les deux sexes. Pour étudier le rôle de l'AcR endogène sur l'entrée en méiose *in vivo*, les expressions de PwRALDH2 et PwCYP26B1 ont été analysées par western blot en utilisant les anticorps polyclonaux préparés et purifiés dans notre laboratoire. PwRALDH2 est exprimée aux stades 54 et 54+60 au même niveau dans les deux sexes. En revanche, l'expression de PwCYP26B1 est moins forte chez la femelle au stade 54+60 jours, durant lequel les CGs entrent en méiose. Afin de déterminer l'effet potentiel des hormones stéroïdes sur la biosynthèse de l'AcR endogène chez le pleurodèle *in vivo*, nous avons traité les larves ZZ par l'œstradiol (100µg/L) à partir du stade 50. Les individus ZW traités développent des ovaires contenant des CGs qui entrent en méiose avant la métamorphose et montrent une expression de *PwCyp26b1* plus élevée que dans les ZZ non traités.

D'après Wallacides et al., 2009.

***Martine Chillet**, adjointe technique, qui a réalisé les études histologiques et immuohistologiques.*

G - Activités de recherche dans l'EA4421 Sigreto et conversion thématique (2009-) :

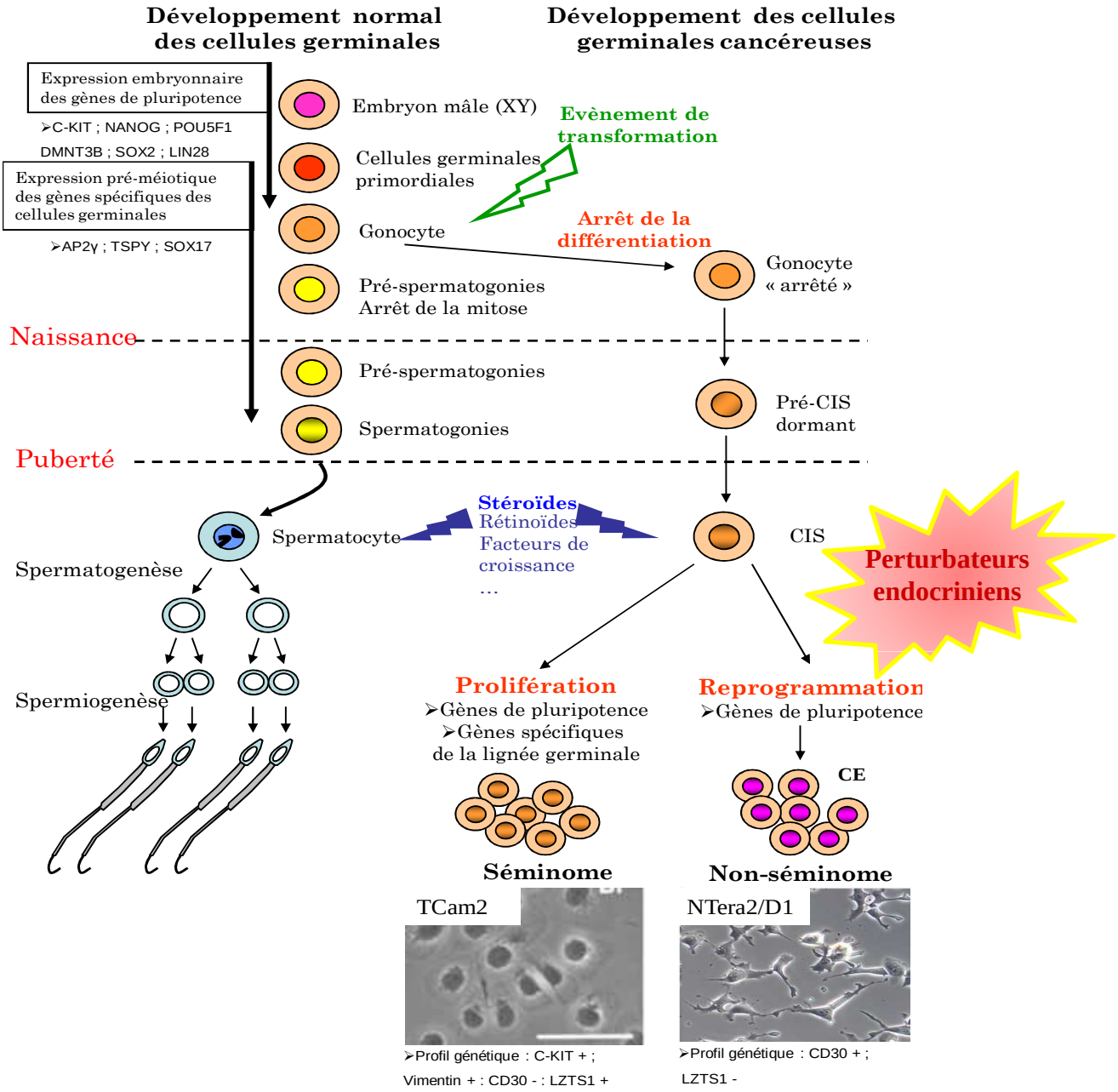
Suite à la constitution de l'EA Sigreto en 2009 et dans le cadre de la structuration d'un pôle de recherche en cancérologie à Nancy, les personnes travaillant sur les cellules germinales (A. Wallacides, A. Chesnel, H. Dumond) ont souhaité effectuer une conversion thématique. Nous avons utilisé l'expertise acquise sur le développement normal de la lignée germinale et sur les stéroïdes pour étudier les cancers testiculaires d'origine germinale dont l'initiation et la progression pourraient être liées à une exposition à des hormones stéroïdes ou des perturbateurs endocriniens (Figure 12)**.** La première partie de ce travail qui correspond à l'étude *in vitro* sur lignées issues de cancer testiculaire et à la mise au point du modèle de xénogreffe sur souris Nude a été financée par l'ANSES jusqu'en mars 2012 dans le cadre d'un appel d'offres « Santé, environnement, travail ».

Reproduction et perturbation endocrinienne

Chez les vertébrés, de nombreux signaux contrôlent l'initiation et assurent la qualité du processus reproductif. Du point de vue de l'endocrinologie comparée, il est intéressant de noter l'importante conservation des ligands, des récepteurs et des voies de signalisation mises en jeu ; l'idée étant que les systèmes de réponse ont évolué vers différents degrés de complexité mais toujours à partir des mêmes éléments de base. Ceci explique notamment pourquoi des ligands naturels issus de différentes espèces ou encore de nombreux xénobiotiques mimétiques des hormones stéroïdes ou thyroïdiennes sont susceptibles d'activer des voies de signalisation similaires chez de nombreux organismes, qui y répondent grâce à des réseaux de signalisation intégrés et complexes en aval des récepteurs mis en jeu (McLachlan, 2012). Pourtant, aucun « état de l'art » des données transcriptomiques et des voies de signalisation existantes dans les organes reproducteurs dans diverses bases de données n'est disponible. Le projet développé ci-dessous a pour objectif de contribuer à décrire ces mécanismes, ce qui permettrait de construire des modèles représentant les intensités ainsi que les dynamiques des interactions génomiques et protéiques, d'identifier les paramètres de ces modèles et ainsi de déterminer les nœuds potentiellement affectés dans le cadre de perturbations du système reproducteur.

Depuis les années soixante et l'exposition involontaire de souris de laboratoire au methoxychlore (Tullner, 1961) un grand nombre de données épidémiologiques ou

Figure 12: Développement normal et pathologique des cellules germinales dans le testicule



À gauche, le développement normal des cellules germinales mâles. Au cours du développement embryonnaire normal, l'absence d'acide rétinoïque est nécessaire pour leur entrée en quiescence. C'est à la puberté sous l'effet combiné des hormones stéroïdes et de l'acide rétinoïque que la spermatogenèse est initiée et se perpétue pendant le reste de la vie adulte.
À droite, le développement néoplasique de cellules germinales mâles. Les événements clés de l'origine et de la progression des TGCTs sont présentés en rouge. (1) arrêt de différenciation des cellules germinales primordiales (CGPs) ou des gonocytes. (2) prolifération et invasion tumorale des cellules du CIS soit en séminome avec expression des marqueurs de pluripotence et des gènes spécifiques de la lignée germinale, soit en carcinome embryonnaire avec perte de la spécificité germinale et possibilité de se différencier secondairement en d'autres types de tumeurs testiculaires.

D'après Kristensen et al., 2008.

expérimentales sur l'homme ou la faune sauvage appuient l'hypothèse selon laquelle le système reproducteur est particulièrement affecté par toutes sortes de signaux en provenance de l'environnement (Lopes-Casas, 2012 ; Duval et Simonot, 2011).

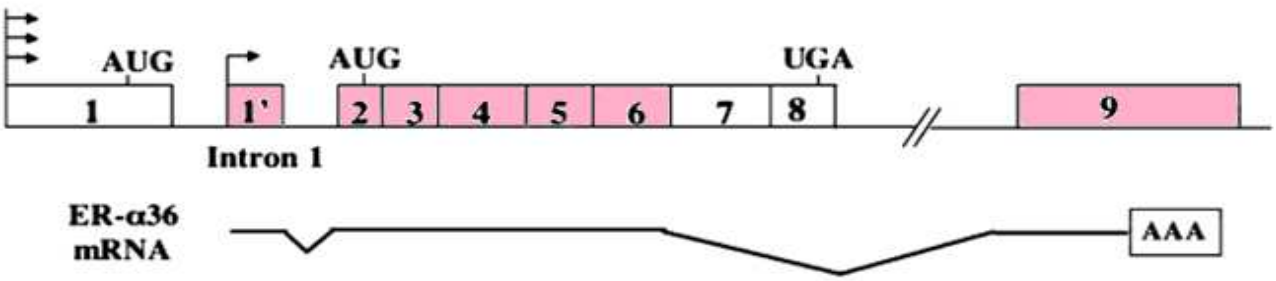
Depuis une trentaine d'années, on observe ainsi un doublement des cas de cryptorchidisme et d'hypospadias chez les nouveau-nés ainsi que des cas de cancer testiculaire chez les jeunes adultes. Parallèlement, la qualité et la quantité de sperme ont diminué d'environ 50% pendant la même période (Kristensen, 2008 ; Rouiller-Fabre, 2008 ; Delbes, 2006, Habert, 2006 ; Merlet, 2007a,b). Divers arguments laissent penser que ces anomalies sont liées entre elles. Il est clairement établi que le cryptorchidisme est un facteur de risque des trois autres altérations et que l'hypospadias et l'oligospermie sont fréquemment accompagnés de cancer testiculaire. Il en est de même pour la fertilité qui apparaît diminuée chez les hommes qui développeront ultérieurement un cancer testiculaire. Ainsi, ces quatre altérations correspondraient vraisemblablement à différents symptômes d'un même syndrome : le syndrome de dysgénésie testiculaire (TDS) (Rajpert de Meyts, 2006, Rajpert de Meyts et Hoei-Hansen, 2007).

Ces altérations de la fonction reproductive humaine sont particulièrement fréquentes dans les pays du nord de l'Europe (Finlande, Danemark) mais touchent également la France, les Etats-Unis et l'Australie. Certaines sont détectables dès la naissance (cryptorchidisme, hypospadias) même si d'autres symptômes ne se révèlent qu'à la puberté (infertilité, cancers testiculaires). L'hypothèse actuellement à l'étude et initialement formulée par Sharpe et Skakkebaek en 1993, est que l'augmentation de la fréquence des TDS résulterait d'une modification du développement du testicule due à l'exposition du fœtus puis du nouveau-né à des molécules dont la diversité et la concentration dans l'environnement augmentent régulièrement (Sharpe et Skakkebaek, 1993; Sharpe et Skakkebaek, 2008; Habert, 2006).

Les substances chimiques incriminées sont des perturbateurs endocriniens possédant une activité œstrogénique ou anti-androgénique. Par définition, un perturbateur endocrinien est un agent exogène qui interfère avec la production, la libération, le transport, le métabolisme, la liaison, l'action ou l'élimination des ligands naturels responsables du maintien de l'homéostasie et de la régulation du développement de l'organisme. Il est également défini comme n'importe quelle substance étrangère à l'organisme qui produit des effets délétères sur l'organisme ou sur sa descendance, à la suite d'une modification de la fonction hormonale (Brevini, 2005). Ces molécules vont dérégler la balance œstrogènes/androgènes en jouant sur la production et/ou l'action de ces hormones et ainsi perturber le développement gonadique. Les perturbateurs endocriniens présents dans l'environnement peuvent être d'origine naturelle comme les phyto-œstrogènes ou d'origine synthétique comme les pesticides, les fongicides,

Figure 13 : Structure génique et protéique de « ERα36 »

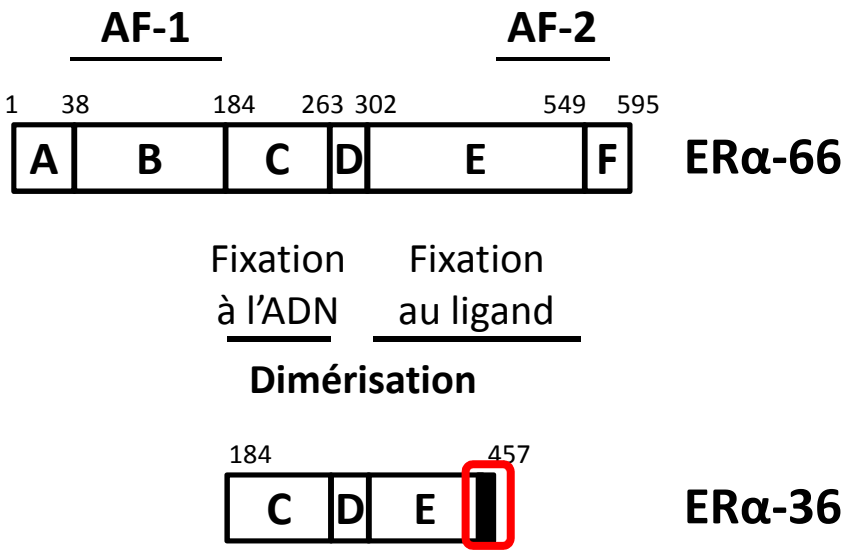
(A)



(B)

1 MAMESAKETRYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNR
61 RKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLW
121 PSPLMIKRSKKNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRE
181 LVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRN
241 QGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSILLLNSGISHVEAKKRILNLHPK
301 IFGNKWFPRV

(C)



(A) Organisation génomique du gène ERα36 humain. Les rectangles colorés en rose représentent les exons du gène ERα36. Les exons numérotés de 1 à 8 correspondent au gène Erα66. L'intron 1 contenant un exon non-codant dit « exon 1' » à partir duquel ERα36 a été généré est indiqué. AUG représente le codon start et UGA le codon stop. La structure de l'ARNm de Erα36 est indiquée (B) Séquence en acides aminés de ERα36, les derniers 27 acides aminés spécifiques de ERα36 sont soulignés. (C) Domaines fonctionnels des isoformes de ERα humain. Les domaines sont notés de A à F. Le nombre d'acides aminés ainsi que la fonction de chaque domaine sont représentés. Le domaine qui contient les derniers 27 acides aminés spécifiques de ERα36 est marqué en rouge.

D'après Wang et al., 2005.

les insecticides, les produits chimiques industriels et certains produits pharmaceutiques (Brevini, 2005).

Comme ces substances sont présentes dans l'eau, le sol, l'air et les aliments, l'homme est en contact continu avec elles dans la vie quotidienne. L'exposition à ces substances diffère d'un individu à l'autre selon son mode de vie, son régime alimentaire et son lieu de travail. Il s'agit généralement d'une exposition chronique à de faibles doses et à un grand nombre de molécules différentes. Ces polluants pénètrent dans l'organisme par ingestion, par inhalation et par absorption et peuvent traverser la barrière placentaire (Sharpe et Irvine, 2004; Main, 2010). Certaines substances chimiques telles que les retardateurs de flamme comme le hexabromocyclododecane (HBCD) persistent dans l'organisme durant plusieurs années. Elles sont en effet souvent lipophiles et sont stockées dans le tissu adipeux (Main, 2007). C'est notamment le développement précoce des individus dont le système endocrinien est inachevé et plus particulièrement les phases initiales de la sexualisation qui sont directement menacées par l'action de ces molécules présentes dans l'environnement même si les conséquences d'une exposition à ces produits ne seront observables que plusieurs décennies plus tard, voire même chez les générations suivantes.

Susceptibilité aux oestrogéno-mimétiques et signalisation oestrogénique non conventionnelle

Si les anomalies (notamment le développement de cancers de l'appareil reproducteur) induites par une exposition aux perturbateurs endocriniens sont diverses, nombreuses et bien documentées, les modalités et les conséquences à long terme des perturbations constatées demeurent mal connues. (MacCarrey, 2012),

Le récepteur nucléaire ERα66 était considéré comme seul médiateur de l'action des œstrogènes dans les cellules cancéreuses mammaires jusqu'à la découverte de nouveaux récepteurs localisés à la membrane plasmique, notamment un récepteur couplé aux protéines G (GPER) et un variant du gène codant le récepteur ERα identifié en 2005, ERα36 (Revankar, 2005 ; Wang, 2005) : ERα36 est transcrit à partir d'un promoteur alternatif localisé dans le premier intron du gène codant ERα66. Il ne possède pas les domaines de transactivation AF1 et AF2 et une partie du domaine de liaison au ligand est également manquante, ce qui lui confère des propriétés de liaison aux ligands différentes de ERα66. En outre, il présente à son extrémité C–terminale une séquence spécifique de 27 AA absente chez ERα66 qui permet de le détecter spécifiquement (Figure 13). ERα36 est localisé préférentiellement au niveau de la membrane cellulaire et médie les effets non génomiques

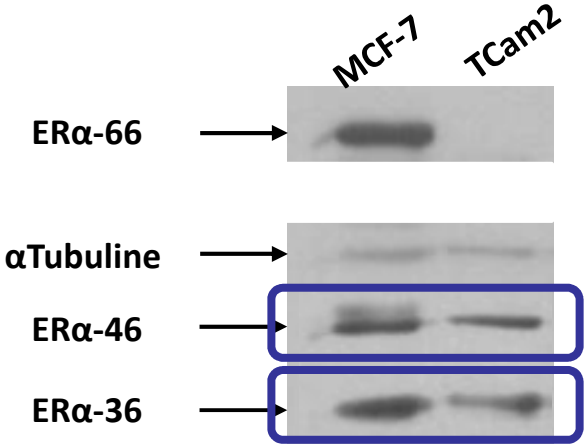
des œstrogènes en activant plusieurs cascades de kinases et/ou en déclenchant le relargage du calcium intracellulaire (Wang, 2006 ; Kang, 2010, Zhang, 2011). ERα36 est nécessaire à la prolifération induite par l'œstradiol dans les cellules séminomateuses (Wallacides, 2012). Il est exprimé non seulement dans les lignées de cellules cancéreuses mammaires ER-positives mais aussi dans les cellules ER-négatives (dépourvues d'ERα66) (Lee, 2008) et dans des échantillons de tumeurs (Zheng, 2010). L'expression d'ERα36 pourrait constituer un nouvel élément susceptible d'expliquer que (i) l'ovariectomie empêche non seulement le développement de tumeurs hormono-dépendantes mais aussi de tumeurs hormono-indépendantes (Early Breast Cancer Trialist group, 1992) (ii) un traitement aux œstrogènes stimule la croissance de cellules hormono-indépendantes MBA-MB-231 injectées à des souris immunodéficientes (Friedl et Jordan, 1994 ; Zhang, 2010).

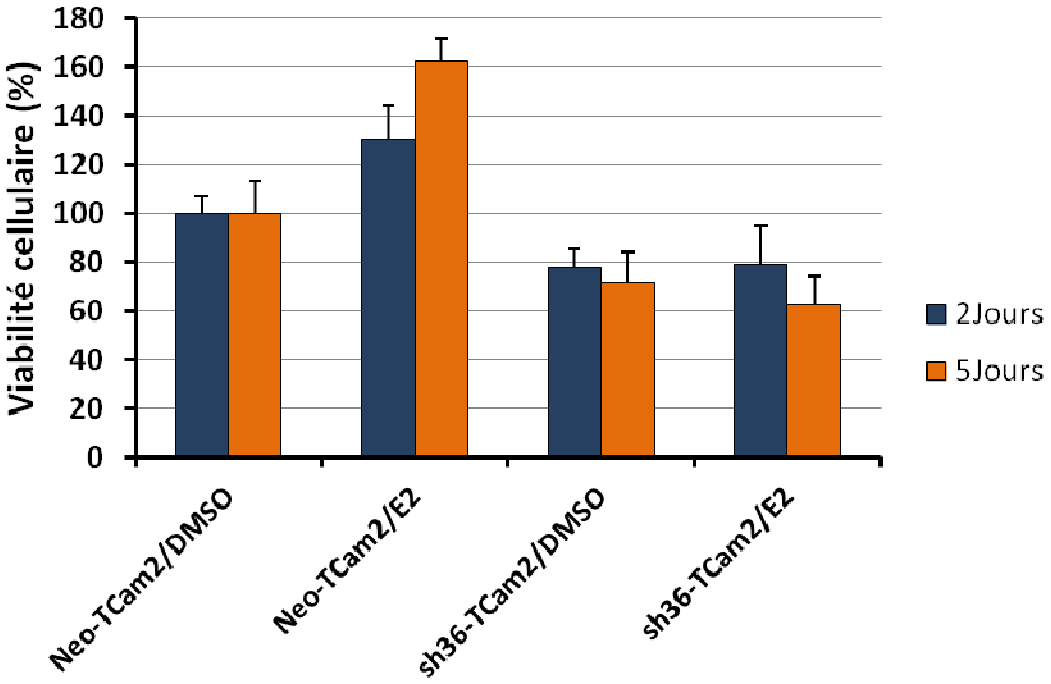
Les effets non génomiques des œstrogènes peuvent aussi être médiés par un récepteur membranaire couplé aux protéines G : GPER (G protein coupled estrogen receptor) (Maggiolini et Picard, 2010). Ce récepteur comporte 7 domaines transmembranaires et peut activer différentes voies de signalisation (PLC/Ca^{2+}, cAMP/PKA, ERK, PI3K/AKT) menant à l'expression de gènes impliqués dans la survie, la prolifération et la migration cellulaire. L'expression de GPER est requise pour la prolifération et le contrôle de l'expression d'ER36 dans les cellules séminomateuses et l'inactivation de GPER bloque la prolifération induite par l'œstradiol dans les lignées triple-négatives MDA-MB-435 et HCC1806 (Girgert, 2012). Ainsi, ERα36 pourrait médier certains des effets non génomiques des œstrogènes attribués jusqu'alors à GPER (Kang et Wang, 2010). Toutefois, les relations fonctionnelles entre ces 2 récepteurs n'ont jamais été étudiées dans des échantillons de tumeurs et il est à noter que le statut GPER est rarement étudié dans les tumeurs mammaires.

Problématique

Dans ce contexte, j'ai souhaité aborder la problématique de **l'influence des stéroïdes sur la différenciation pathologique des cellules germinales** en me fixant 3 objectifs : (i) mettre en évidence un effet éventuel des stéroïdes sexuels naturels (œstrogènes et androgènes) sur la prolifération de cellules cancéreuses d'origine germinale (ii) caractériser l'effet « perturbateur endocrinien » et comprendre les mécanismes d'action de certaines classes de molécules en utilisant ces mêmes modèles (iii) tester l'hypothèse d'une origine fœtale des tumeurs testiculaires germinales induite par ces perturbateurs endocriniens dans un modèle animal.

Figure 14: Récepteurs α des oestrogènes exprimés dans TCam-2 et leur implication dans l'action prolifératives de l'E_2





Les cellules Neo-TCam-2 sont des cellules TCam-2 transfectées par le plasmide vide, qui permet l'expression du shRNA anti-ERa36 dans la lignée sh36-TCam-2. L'estradiol (E2) n'induit pas de prolifération dans les cellules sh36-TCam-2.

D'après Thèse A. Wallacides, 2011.

Co-encadrement de Melle Wallacides (suite)

En septembre 2008, Melle Wallacides a achevé la première partie de son travail de thèse sur l'entrée en méiose des cellules germinales du pleurodèle et a souhaité s'associer, à ma conversion thématique. Dans un premier temps, nous nous sommes focalisées sur les effets des stéroïdes naturels sur les cancers testiculaires d'origine germinale. Afin de disposer d'outils cellulaires et moléculaires permettant de caractériser les voies de signalisation stimulées par l'œstradiol et la testostérone, nous avons, dans un premier temps, choisi de travailler sur des lignées cellulaires établies.

Les tumeurs testiculaires germinales (TGCTs) représentent 95 % de l'ensemble des tumeurs testiculaires. La classification des TGCTs la plus couramment utilisée est celle proposée par l'Organisation Mondiale de la Santé (OMS), revue en 2004, qui distingue notamment :

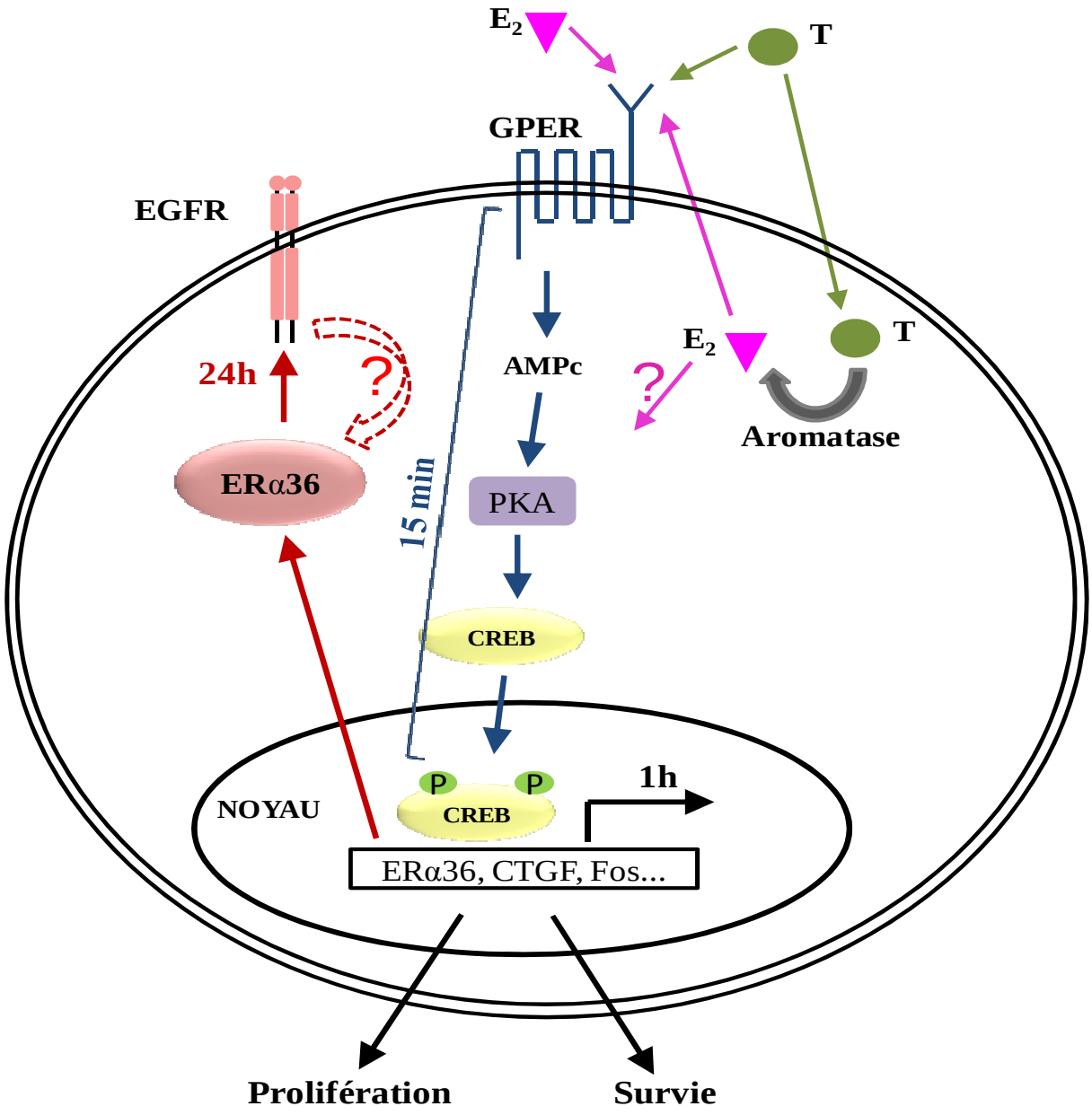
- les tumeurs séminomateuses qui représentent environ 50 % des tumeurs germinales et sont composées de cellules ayant conservé l'expression de marqueurs germinaux et exprimant des marqueurs de pluripotence,

- les tumeurs non-séminomateuses qui sont plus hétérogènes: carcinome embryonnaire, tumeur du sac vitellin, choriocarcinome, tératome, polyembryome.

Nous travaillons principalement sur la lignée TCam-2, établie en 1993 par Mizuno à partir d'une tumeur séminomateuse pure prélevée chez un patient de 35 ans (Mizuno, 1993 ; Dejong, 2008). Cette lignée a également été propagée par les mêmes auteurs après xénogreffe sur des souris SCID et représente actuellement la seule lignée séminomateuse disponible. Peu d'études ont été réalisées à ce jour sur ces cellules.

Nous avons montré que la vitesse de prolifération de cellules issues de cancers testiculaires dépend de la présence de stéroïdes et de rétinoïdes, ce qui permettrait de classer les séminomes parmi les tumeurs hormono-sensibles. Cette conclusion est d'autant plus intéressante que la lignée TCam-2 n'exprime pas le récepteur ERα66, classiquement impliqué dans les phénomènes d'hormono-dépendance (figure 14). Les travaux de Melle Wallacides et ceux de Mr Hussein Ajj (M2R) ont ainsi permis de montrer que la sensibilité aux œstrogènes et aux androgènes aromatisables comme la testostérone dépend d'un récepteur membranaire des stéroïdes (GPER) qui active une voie de transduction du signal dépendante de la protéine kinase A puis l'expression de ERα36 ((figure 15) Wallacides, 2012). Le décryptage de ces voies de signalisation non canoniques des œstrogènes pourrait conduire, à terme, à définir de

Figure 15: Représentation schématique des voies de signalisation impliquées dans la réponse à l'œstradiol dans les cellules TCam-2



Les hormones stéroïdes stimulent la prolifération des cellules séminomateuses TCam2. La testostérone agit directement via un récepteur membranaire et en partie après son aromatisation par activation des voies œstrogéniques. L'effet de l'œstradiol dans ces cellules tumorales est dépendant de la présence du récepteur membranaire GPER qui active par la suite le récepteur ERα36 localisé dans le cytoplasme via la voie AMPc/PKA.

D'après Thèse A. Wallacides, 2011.

nouveaux marqueurs d'exposition aux molécules œstrogèno-mimétiques et à mieux comprendre les mécanismes pathologiques de la perturbation endocrinienne.

Co-encadrement du doctorat de M Hussein Ajj

Ce travail fondamental est complété par une étude plus appliquée qui a pour but de mesurer l'influence de micropolluants en mélange sur la prolifération et la différenciation de tumeurs testiculaires d'origine germinale. Cet aspect de notre thématique qui est plus particulièrement développé pour la thèse de Hussein Ajj sera élargi à d'autres modèles (voir projet de recherches).

Le choix des perturbateurs endocriniens inclus dans l'étude a été guidé par (i) leur proximité chimique avec les stéroïdes déjà étudiés au laboratoire ou leur affinité pour le récepteur membranaire des stéroïdes GPER (Thomas et Dong, 2006), (ii) l'exposition potentielle ou avérée des populations sensibles (femmes en âge de procréer et/ou des jeunes enfants) à ces composés, (iii) leur caractère reprotoxique suspecté, (iv) leur capacité potentielle ou avérée à stimuler la prolifération des lignées cellulaires hormono-sensibles (Myers, 2009), (v) leur classement dans la liste des polluants émergents établie par le réseau européen de laboratoires NORMAN (Network of reference laboratories for monitoring of emerging environmental pollutants). Nous avons utilisé ces différentes molécules seules ou en mélange et à des doses faibles correspondant aux quantités retrouvées dans l'alimentation ou l'environnement.

Au cours de sa première année de thèse, Hussein Ajj a testé l'effet de faibles doses (1pM à 10nM) de mélanges de micropolluants à effet perturbateur endocrinien supposé sur la prolifération des cellules TCam-2. Un premier mélange contenant des métabolites de l'œstradiol (œstrone, œstriol) et des œstrogènes de synthèse prescrits comme contraceptifs ou anti-asthmatiques (mestranol, prednisolone) s'est révélé avoir une action stimulatrice de la prolifération et une action anti-apoptotique. Compte-tenu des données obtenues précédemment, ce mélange a été considéré comme un témoin positif puisqu'il regroupe des molécules aux propriétés proches de celles de l'œstradiol. L'analyse de l'expression de gènes cibles de l'œstradiol a confirmé l'effet œstrogéno-mimétique. Le second mélange testé contenait une quantité équimolaire de 4-nonyl- et 4-octyl-phénol. Là encore, Mr Ajj a observé une stimulation de la prolifération à très faible dose (1 pM), alors qu'à forte dose (10 nM) il décelait plutôt un effet pro-apoptotique. Par ailleurs, l'analyse d'expression de gènes cibles des œstrogènes ne permet pas de déterminer clairement un mode d'action œstrogéno-mimétique.

Au même moment, le groupe de K. Guenther en Allemagne a publié la caractérisation et le dosage de mélanges d'alkylphénols effectivement présents dans l'alimentation humaine (Raecker, 2011). Ces articles mettaient notamment en évidence la contamination ubiquitaire de populations sensibles comme les jeunes enfants par un mélange de 4-nonylphénol et de 4-tert-octyphénol [4NP + 4tOP] en rapport 30:1. Hussein Ajj réitère donc les expériences de mesure de prolifération, d'apoptose et d'expression de gènes cibles avec ce dernier mélange. La suite du projet de thèse est décrite dans la partie « Projet de recherche » (Axe 1).

A ce stade, nous avons donc montré, *in vitro*, l'influence des stéroïdes sexuels et de certaines molécules considérées comme oestrogéno-mimétiques sur la prolifération des cellules issues de cancers testiculaires. En travaillant sur des mélanges et des faibles doses, nous nous sommes approchés, autant que possible, des conditions d'exposition humaine décrites dans la littérature. Nous avons également commencé à disséquer les voies de signalisation mises en jeu et mis en exergue le rôle clef de ERα36. Parallèlement, nous avons adapté les techniques de transfection aux lignées étudiées et construits des lignées cellulaires stables sous- ou sur-exprimant ERα36. Un modèle de souris xénogreffées par les cellules étudiées en culture a été mis au point et commence à être exploité (voir la partie « projet »).
L'ensemble des ces outils et modèles, l'expertise acquise sur la problématique « cancer hormono-sensible et environnement », ainsi que les collaborations initiées nous permettent maintenant d'envisager d'élargir cette question du rôle clef d'ERα36 dans la réponse aux perturbateurs endocriniens en s'intéressant à (i) d'autres modèles de tumeurs hormono-sensibles et (ii) l'ensemble des étapes de la cancérogénèse que sont l'initiation, la progression et la dissémination des cellules malignes. ERα36 sera alors étudié en tant que médiateur de la réponse aux perturbateurs endocriniens, son expression pouvant être interprétée comme révélatrice d'une exposition passée ou présente aux molécules étudiées. Dans le contexte de cancers hormono-sensibles plus fréquents et plus morbides que le cancer testiculaire (sein, prostate…), l'expression d'ERα36 pourra également être abordée dans une optique de détection et de validation de nouveaux marqueurs pronostics de réponse aux traitements.

PROJET DE RECHERCHE
ET
D’ENCADREMENT DOCTORAL

Contexte du projet

Les organes reproducteurs comme le sein, le testicule, l'ovaire, l'endomètre ou la prostate, dont le développement ou le fonctionnement sont contrôlés par le système endocrinien, sont des cibles privilégiées pour toute molécule susceptible de lier les récepteurs des stéroïdes et/ou d'activer les voies de signalisation sollicitées en conditions normales. De plus, si l'intérêt de notre étude sur le cancer testiculaire pour la recherche translationnelle peut être considéré comme modeste puisqu'on observe plus de 95% de survie chez les patients atteints, il apparait plus nettement lorsqu'on s'intéresse à d'autres modèles comme le cancer du sein, de l'ovaire ou de la prostate. Avec 53 000 nouveaux cas en 2011, le cancer du sein est en effet le plus fréquent chez la femme avant le cancer colorectal (19 000 cas) et le cancer du poumon (12 000 cas) et se situe en tête de la mortalité, avec 11 500 décès même si le taux de mortalité diminue en France depuis près de 15 ans (chiffres INVS). Les cancers de l'endomètre et de l'ovaire sont beaucoup plus rares mais souvent de mauvais pronostic. Quant au cancer de la prostate, cancer masculin le plus fréquent, il touche essentiellement des hommes âgés (en moyenne 71 ans au diagnostic en 2005) mais sa fréquence (71000 nouveaux cas en 2011) et la mortalité induite (environ 12% des patients) demeurent élevés.

Je souhaite donc me focaliser sur 2 organes modèles déjà étudiés au laboratoire: le sein et le testicule. En effet, plusieurs caractéristiques font de ces 2 organes des modèles de choix pour l'étude des mécanismes et des conséquences de la perturbation endocrinienne :

- l'incidence des cancers mammaires et testiculaires a doublé depuis une trentaine d'années, en particulier dans les pays industrialisés où la quantité et la variété des xénobiotiques présents dans l'environnement ne cessent d'augmenter. L'hypothèse d'une cause environnementale de ces cancers est renforcée par la rareté des cancers familiaux liés à des mutations transmissibles et par la constatation que, dès la seconde génération, les migrants présentent la même incidence de cancers mammaires et testiculaires que la population de leur pays d'accueil (Beiki, 2010, Cyriac, 2012, Kratz, 2012).

- les cancers mammaires et testiculaires sont oestrogéno-sensibles : (i) plusieurs facteurs de risque du cancer du sein sont liés à l'exposition aux estrogènes endogènes (puberté précoce, ménopause tardive...) ou exogènes (contraception orale, traitement hormonal de la ménopause, alcool) (Parsa et Parsa, 2009, Pelucchi, 2011, Nelson, 2012, Seitz, 2012), (ii) les cellules issues de cancers testiculaires d'origine germinale prolifèrent plus vite après traitement par l'estradiol ou la testostérone *in vitro* et *in vivo* (Chevalier, 2012a, 2012b, Wallacides, 2012).

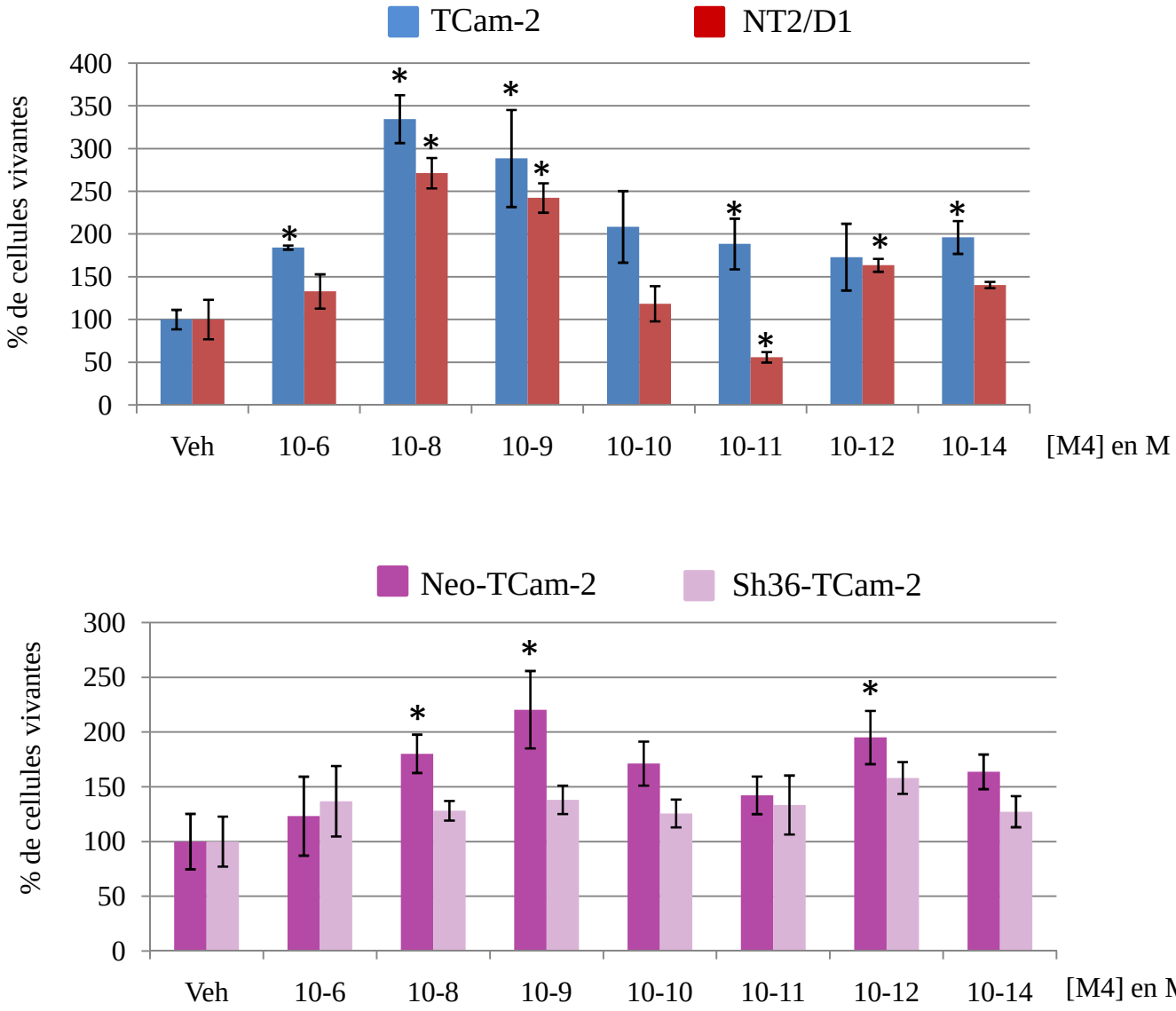
- les différents types cellulaires qui composent la glande mammaire et le testicule se différencient pendant la vie fœtale et périnatale mais n'assureront une fonction reproductive réelle qu'après la puberté, sous le contrôle étroit des hormones stéroïdes (Delbès, 2006). Cette mise en place des structures essentielles à un fonctionnement décalé dans le temps se fait donc pendant une période de sensibilité accrue aux facteurs environnementaux et une exposition chronique « tout au long de la vie » aux perturbateurs endocriniens, telle qu'elle est actuellement décrite, augmente le risque de présenter des anomalies à l'âge adulte. (Fucic, 2012),

- la présence de certains allèles ou un taux d'expression variable de gènes du métabolisme des xénobiotiques ou stéroïdes et de leurs récepteurs confèrent une susceptibilité individuelle plus ou moins grande aux cancers du sein et du testicule. (Rapley, 2007, Ferlin 2012, Kristiansen, 2011, 2012a,b ; Västermark, 2011).

Problématique

Le projet de recherche que je souhaite développer sera réalisé au sein de la future unité constituée de la fusion de l'EA4421 Sigreto et du CRAN à partir de janvier 2013. Ce projet s'inscrit dans la continuité de la conversion thématique initiée en 2009. Décliné en trois axes, il s'articule autour de 3 mots-clefs : tumeurs hormono-sensibles, perturbateurs endocriniens, voie non conventionnelle de signalisation œstrogénique. Le premier axe renferme un projet concernant les effets d'un mélange d'alkylphénols sur l'initiation (1b) et la progression tumorale (1a) dans un modèle de cancer testiculaire d'origine germinale puis de cancer mammaire (1c) *in vitro* et *in vivo*. Le but est de caractériser le rôle éventuel de ERα36 dans ces processus afin de valider cette forme tronquée du récepteur ERα66 comme marqueur d'exposition aux perturbateurs endocriniens dans les cellules hormono-sensibles, en lien avec les cancers testiculaires (1a, 1b, 1c) et mammaires (1b, 1c). Le second axe s'inscrit dans un objectif à plus long terme qui sera de valider ERα36 comme marqueur prédictif de réponse aux traitements anti-tumoraux dans les cancers hormono-sensibles et en particulier dans le cancer du sein.

Figure 16: Stimulation de la prolifération de différentes lignées cellulaires induite par un traitement de 48h avec le mélange M4



Les doses de M4 sont indiquées en abscisse (par exemple -6 = 10-6M soit 1µM). Les cellules Neo-TCam-2 sont des cellules TCam-2 transfectées par le plasmide vide, qui permet l'expression du shRNA anti-ERα36 dans la lignée sh36-TCam-2. L'œstradiol (E2) n'induit pas de prolifération dans les cellules sh36-TCam-2. *, significativement différent des cellules de la lignée correspondante, traitées par le solvant seul ($p<0,05$).

Axe 1- Effets de perturbateurs endocriniens sur la croissance des tumeurs hormono-sensibles de l'appareil reproducteur (testiculaires et mammaires)

a) Progression tumorale dans les tumeurs testiculaires d'origine germinale

De nombreuses observations réalisées au cours des 30 dernières années confortent l'hypothèse initialement formulée par Sharpe et Skakkebaek (Skakkebaek, 1975) selon laquelle les problèmes répertoriés dans le domaine de la reproduction masculine pourraient résulter de l'exposition à des perturbateurs endocriniens comme les phtalates ou les alkylphénols (Hutchison 2008a, 2008b ; Scott 2007 ; Lambrot 2009 ; Habert, 2009 ; Balabanic, 2011 ; Schug, 2011 ; Muczynski, 2012).

Suite aux résultats préliminaires obtenus pendant la première année de thèse de H. Ajj, nous avons évalué l'effet de faibles doses (pM) d'un mélange de [4NP + 4tOP] sur la croissance de tumeurs testiculaires *in vitro* et *in vivo*. Les 4-tert-octylphénol (4tOP) et 4-nonylphénol (4NP) sont des perturbateurs endocriniens persistants classés comme substances prioritaires par la directive européenne sur l'eau (WFD 2000/60/CE). Ils sont produits par les processus de biodégradation des effluents urbains à partir des éthoxylates correspondants. En outre, le 4NP est utilisé comme antioxidant dans les emballages alimentaires mais aussi dans les produits d'entretien, les agents dispersants et émulsifiants, et le 4tOP comme intermédiaire dans la production des gommes de pneu.

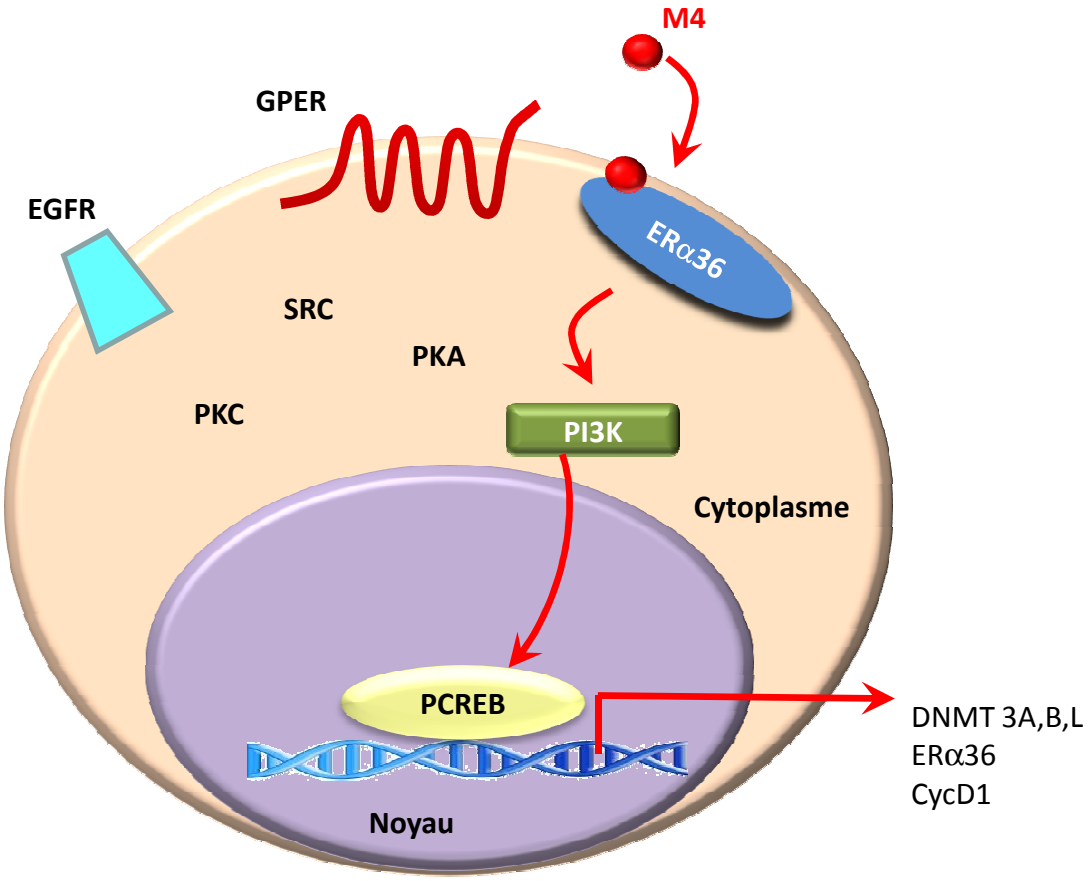
Ces molécules sont particulièrement persistantes et toxiques car elles peuvent s'accumuler dans les organismes aquatiques et les sédiments. Plusieurs études *in vitro* et *in vivo* suggèrent le potentiel œstrogénique et anti-androgénique des 4tOP et 4NP, posant ainsi la question de leur impact potentiel sur la santé humaine. Sachant que la contamination par les alkylphénols concerne au moins 50% de la population, nous avons posé la question de leur impact sur la croissance et la progression des tumeurs testiculaires en associant plusieurs modèles tumoraux :

- (i) une étude *in vitro* sur la lignée séminomateuse TCam-2 comportant

- une première étape de mise en évidence des effets des mélanges de micropolluants sur la prolifération cellulaire et l'apoptose qui a révélé une augmentation des marqueurs de prolifération cellulaire associée à une accélération de la multiplication des cellules (Figure 16).

- la caractérisation des voies de signalisation mises en jeu en se basant sur les résultats acquis au cours du doctorat de A. Wallacides. Cette analyse révèle 3 points majeurs, que sont l'implication de la cascade de kinases dépendante de la PI3K/Akt, de ERα36 mais pas de

Figure 17: Voies de signalisation activées par le mélange M4 dans les cellules TCam-2



Un traitement par 1nM de M4 active en quelques minutes la phosphorylation de CREB par la PI3kinase de façon dépendante de ERα36 mais indépendante de GPER, EGFR, src, PKC et PKA. Dans un délai de 1 à 24h, ces évènements conduisant à l'augmentation d'expression de ERα36 et Cycline D1 et à la répression de Dnmt3B et 3L.

GPER, contrairement à ce qui avait été observé pour la réponse à l'oestradiol (Wallacides, 2012 ; Figure 17).

- une seconde étape d'analyse transcriptomique encore en cours, mais dont les premiers résultats indiquent que le mélange [4NP + 4tOP] module l'expression de facteurs impliqués dans les modifications épigénétiques. Ce dernier résultat est particulièrement intéressant puisque plusieurs études soulignent l'impact des dérèglements épigénétiques induits par les alkylphénols (notamment le bisphenol A) à court terme chez les individus exposés mais également à plus long terme selon des processus transgénérationnels (Wolstenholme, 2012; Mannikam, 2012).

- (ii) le développement d'un modèle de souris xénogreffées par des cellules de carcinome embryonnaire (NT2-D1) pour une analyse *in vivo* (l'analyse des animaux (tumeurs xénogreffées + testicules) ayant subi un traitement pendant 28 jours est en cours). Les premiers résultats suggèrent, là aussi, une accélération de la progression tumorale induite par le mélange [4NP + 4tOP]. (Figure 18).

L'ensemble de ces résultats sont présentés dans une publication (Ajj et al.), actuellement soumise à Environmental Health Perspectives.

Je souhaite poursuivre et développer cette caractérisation des médiateurs de croissance et progression tumorale induites par les alkyphénols, en articulant plusieurs approches.

A partir de lignées cellulaires représentatives des 2 principaux types de cancers testiculaires que sont les séminomes (lignée TCam-2) et les carcinomes embryonnaires (lignée NT2/D1), je souhaite :

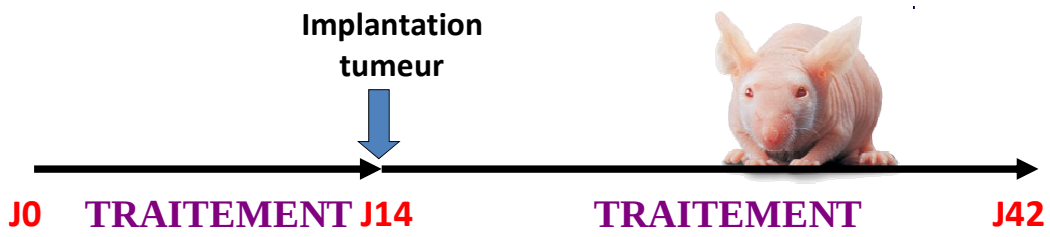
- poursuivre la validation des gènes cibles du mélange [4NP + 4tOP] identifiés lors de l'analyse transcriptomique, notamment ceux pouvant être impliqués dans le contrôle des effets prolifératifs à long terme (cycle cellulaire, pluripotence, modifications épigénétiques…)

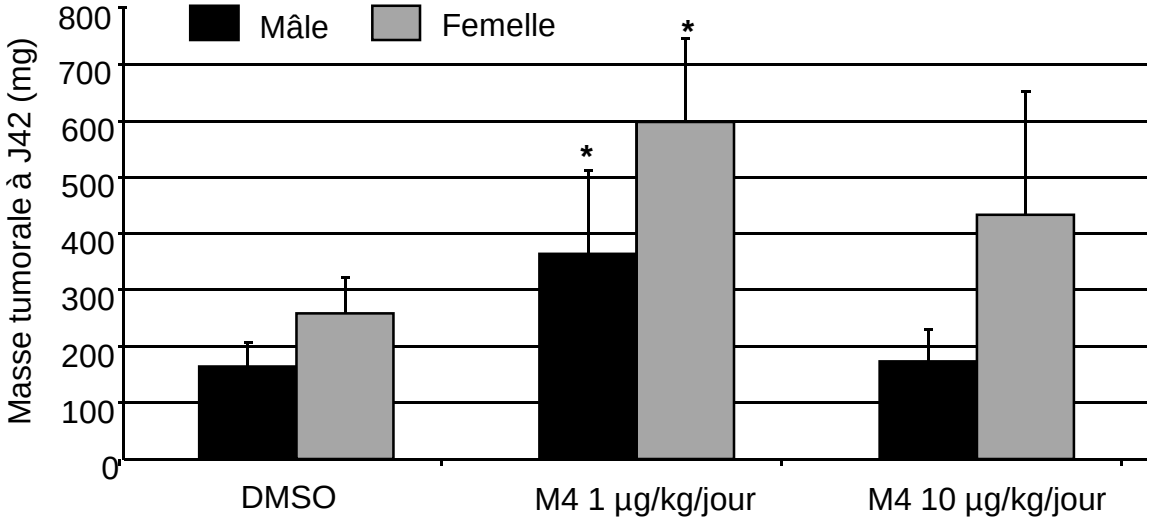
- préciser le rôle de ERα36 dans ces mécanismes en tant que récepteur direct des perturbateurs endocriniens et déclencheur des cascades mises en jeu, co-régulateur transcriptionnel ou simple médiateur des effets observés. Une collaboration s'initie avec l'équipe de Patrick Ballaguer (IRCM, Montpellier) pour cribler les molécules capables de lier ERα36 et ainsi modéliser les interactions physiques entre le récepteur et ses ligands.

A partir de biopsies de tumeurs humaines, je souhaite :

- évaluer le niveau d'expression et la localisation d'un certain nombre de ces marqueurs dans des prélèvements de carcinomes embryonnaires et de séminomes humains

Figure 18: Effet d'un traitement par le mélange M4 sur la croissance de tumeurs dérivées de cellules NT2/D1, xénogreffées chez la souris Nude





Trois groupes de souris Nudes, mâles ou femelles, ont été traités par injection quotidienne sous-cutanée pendant 14 jours avec le diluant (DMSO), ou bien 1µg/kg/J ou 10µg/kg/J du mélange 4NP/4tOP. A J14, elles ont reçu un greffon de tumeur issue de cellules NTera2/D1 (carcinome embryonnaire). Le traitement a été poursuivi pendant 28 jours. La figure montre une **augmentation significative de la masse tumorale à l'exérèse après traitement des souris avec la dose de 4NP/4tOP correspondant à l'exposition humaine** (1µg/kg/jour).

conservés dans la tumorothèque de la Faculté de Médecine de Nancy ou fournis par le laboratoire de E. Rajerts-de-Meyts (Danemark),

- créer une banque de xénogreffes de tumeurs testiculaires d'origine humaine chez la souris Nude afin de disposer, à terme, de modèles *in vivo* permettant de tester l'effet de polluants émergents (Liste établie par le réseau européen de laboratoires NORMAN). Ces 2 derniers points pourront être réalisés en collaboration avec la « Plateforme xénogreffes » localisée à la Faculté de Médecine.

L'axe 1a du projet décrit ci-dessus s'inscrit dans la continuité de la thèse de Hussein Ajj que je co-encadre à 50% avec S. Flament. Amand Chesnel, Maître de Conférences, contribuera à ce projet en travaillant plus particulièrement in vitro avec la lignée NT2-D1. Martine Chillet, adjointe technique, participe à cette thématique pour les analyses d'expression protéiques (western blot, immunohistochimie, immunocytologie…).

b) Transformation néoplasique et initiation tumorale

Les jeunes enfants, chez qui le développement de l'axe gonadotrope est inachevé, représentent une population particulièrement sensible et exposée aux effets des perturbateurs endocriniens. Récemment, une étude réalisée en Allemagne sur les aliments destinés aux jeunes enfants (0-3 ans) indique la présence ubiquitaire de 4NP et de 4tOP (dans un rapport 30:1) dans les denrées analysées (Raecker, 2011). En se basant sur des estimations de consommation quotidienne de ces aliments, Raecker et ses collaborateurs ont ainsi estimé la contamination des jeunes enfants à environ 0,5µg/kg/jour.

La présence de ces molécules dans les eaux de boisson et les denrées alimentaires d'origine industrielle entraine une exposition prolongée, même à faible dose, qui pourrait en partie être responsable d'une augmentation de troubles de la reproduction ou de l'incidence de cancers hormono-dépendants (Fucic, 2012).

De plus, l'étude de l'ontogenèse des effets des œstrogènes *in vitro* pendant la vie fœtale et néonatale a mis en évidence l'existence de périodes critiques de sensibilité du testicule aux œstrogènes. Dans un modèle de culture organotypique de testicule, l'équipe de Habert a ainsi démontré qu'à la différence de l'adulte, les œstrogènes endogènes ont un effet négatif sur les fonctions et le développement du testicule pendant la vie fœtale et néonatale (Rouiller-Fabre, 2009). Ces variations de sensibilité au cours du développement pourraient être liées à des variations de la capacité du testicule à produire des œstrogènes endogènes qui masqueraient l'effet des œstrogènes exogènes (Delbès, 2007).

Dans ce contexte, l'objectif est de générer et de valider un modèle animal d'exposition fœtale et néonatale à un mélange de polluants permettant de déterminer son impact sur le développement et de mesurer les effets reprotoxiques et cancérogènes, à des doses comparables à celles observées chez les populations humaines sensibles (femmes enceintes, nourrissons, jeunes enfants). Dans le contexte de cellules séminomateuses hormono-sensible nous avons mis en évidence ERα36 en tant que biomarqueur potentiel d'exposition aux alkylphénols, suspects d'être promoteurs de cancers. Nous souhaitons maintenant valider *in vitro* et *in vivo* la fonction d'ERα36 comme marqueur de susceptibilité aux alkylphénols et modéliser les mécanismes de perturbation endocrinienne engendrée par les alkylphénols, qui dépendent de ERα36.

Pour cela, nous mesurerons les effets reprotoxiques et cancérogènes (testicule, glande mammaire) du mélange [4tOP+4NP] à des doses comparables à celles observées chez l'homme *in vivo*

(i) au cours du développement puis à l'âge adulte chez des souris sauvages ou transgéniques pour ERα36. Le promoteur de ERα36 étant absent de l'intron 1 du gène ERα66 chez la souris, nous souhaitons produire deux lignées de souris knocked-in pour ERα36 : une dans laquelle l'expression du transgène humain sera sous le contrôle d'un promoteur spécifique de la lignée germinale (ex : Mouse Vasa Homolog) et l'autre sous le contrôle d'un promoteur spécifique de la glande mammaire (ex : β-lactoglobulin) (Brandt, 2000).

(ii) Chez les descendants de souris sauvages gestantes exposées au [4tOP+4NP] (ainsi que des souris KI si leur phénotype le permet) afin de déterminer un éventuel effet direct et/ou transgénérationnel de l'exposition jusqu'en F3. Cette génération F3 est en effet la première dont aucune cellule n'aura été directement exposée au mélange (Mannikam, 2012)

Les analyses porteront

(i) sur des marqueurs d'ontogenèse de l'appareil reproducteur mâle et de la glande mammaire,

(ii) sur des marqueurs précoces de transformation néoplasique et de progression tumorale des organes concernés chez des souris exposées ou non au mélange [4tOP+4NP] tout au long de la vie.

Dans cette approche, une attention particulière est portée à (i) l'exposition à ces molécules « en mélange », à « faibles doses », « tout au long de la vie », mimant ainsi l'exposition humaine; (ii) l'évaluation de paramètres révélateurs du risque reprotoxique ET du risque cancérigène, souvent associés comme promoteurs des cancers du sein ou du testicule. Ce projet pourrait ainsi conduire à définir de nouveaux marqueurs précoces de transformation néoplasique en lien avec l'exposition aux alkylphénols tout au long de la vie. Ce point est particulièrement intéressant dans le contexte de la sensibilité à des perturbateurs endocriniens oestrogéno-mimétiques comme les alkylphénols car il permettra de mieux prédire les effets d'une exposition prolongée à ces molécules en fonction des voies de signalisation déjà identifiées dans les différents types cellulaires. L'analyse de l'incidence des pathologies cancéreuses dans les organes hormonosensibles adultes et des éventuelles anomalies

transmises de façon transgénérationnelle contribuera à tester l'hypothèse de perturbations épigénétiques causés par les alkylphénols.

Modèle d'exposition des animaux au mélange [4tOP+4NP]

Le modèle d'exposition consistera en l'ingestion quotidienne d'un aliment contaminé avec le mélange [4NP + 4tOP] par des femelles gestantes et allaitantes, ce qui correspond à la voie d'entrée principale observée chez l'homme puisque ces molécules sont retrouvées dans les aliments et les eaux de boisson. Les animaux seront exposés au mélange de contaminants via un protocole d'alimentation libre qui, du fait des conditions de réalisation, permet de s'assurer de l'ingestion totale de la dose quotidienne de contaminants. Brièvement, ce protocole vise à conditionner les animaux pour qu'ils ingèrent durant un laps de temps de courte durée une boulette de nourriture additionnée avec une solution contenant le mélange [4NP + 4tOP] selon un volume qui est ajusté selon le poids de l'animal. Une fois cette fenêtre de temps passée, les animaux sont remis dans des conditions standard d'élevage avec un accès libre à la nourriture (Crepeaux, 2012). Les souriceaux constituant la génération F0 seront exposés (ou non) pendant la vie fœtale par administration à leur mère d'aliments contenant la quantité de mélange ajustée au poids de l'animal. Après la naissance, pendant la période néonatale puis tout au long de la vie, ils continueront à être alimentés avec l'aliment contaminé ou non.

L'étude inclura 6 lots expérimentaux comprenant 6 portées par lot: le lot "témoin" qui recevra l'aliment non contaminé et 5 lots "traités" qui recevront l'aliment contaminé, l'un contenant une dose de [4NP + 4tOP] de 0,5 µg/kg/jour correspondant à la quantité journalière ingérée par l'homme, les autres une dose 10 ou 100 fois moins ou plus élevée (0,005 ; 0,05 ; 5 ; 50 µg/kg/jour). Cette gamme d'exposition pourra éventuellement rendre compte d'une dose-réponse non monotone, comme cela est souvent observé avec les hormones ou les oestrogéno-mimétiques (exemple du bisphénol A).

Au sein de chaque portée, trois souriceaux de chaque sexe seront sacrifiés à des temps prédéterminés et différents prélèvements réalisés incluant le sang, les gonades et la glande mammaire chez les femelles. Les temps de prélèvement ont été choisis de manière à étudier les effets de la contamination pendant 2 périodes différentes de haute sensibilité du développement général et sexuel de l'individu : une exposition fœtale (prélèvements à J3), une exposition fœtale et périnatale (prélèvements à J21). Les effets à l'âge adulte seront aussi étudiés (effets tardifs sur le développement de cancers testiculaires, la spermatogenèse et les capacités reproductives à J60). Au plan de l'analyse statistique des résultats, la moyenne des

résultats obtenus avec chaque souriceau d'une même portée sera utilisée de manière à utiliser la portée et non l'individu comme unité statistique et ainsi éviter l'influence des effets intra-portées.

Paramètres analysés in vivo

Les analyses qui seront réalisées sur les prélèvements des animaux de la génération F0 porteront sur des marqueurs de l'ontogenèse testiculaire et de la glande mammaire, ainsi que sur des marqueurs précoces de transformation néoplasique et de progression des tumeurs mammaires et testiculaires :

- Mesure de la distance ano-génitale à la naissance
- Analyse par PCR quantitative de marqueurs de tumeurs testiculaires : transformation néoplasique (KITL, AP2gamma...) et progression tumorale (CycD1, c-myc, HER2, GPER, PR, ER66...).
- Histologie testiculaire et mammaire aux différents temps de prélèvement
- Analyse immunohistochimique de marqueurs des différents types cellulaires du testicule (3betaHSD, SMA, AMH, POU5F1) et du sein (ERα66, HER2, PR…) aux différents temps de prélèvement. Si des anomalies sont détectées, ces mêmes échantillons pourront, à terme, être utilisés pour évaluer (i) l'apoptose des cellules germinales (test TUNEL), (ii) des modifications épigénétiques associées à une mal-différenciation de la lignée germinale, (iii) la présence de marqueurs de pluripotence et de cellules germinales primordiales normalement absents chez les animaux adultes sains.
- Dosages de stéroïdes circulants par ELISA

Cette étude permettra de tester l'hypothèse d'une programmation fœtale de certains cancers. A terme, la possibilité d'une transmission aux générations suivantes du risque accru de développer un cancer d'origine germinale pourra être appréhendée.

Production des souris KI

L'ensemble des étapes de clonage, transfection des cellules ES, implantation chez la souris gestante, croisements…conduisant à la production de souris hétérozygotes KI pour ER36 humain dans les cellules germinales ou la glande mammaire sera pris en charge, sous la forme d'une prestation de service par l'Institut Clinique de la Souris à Illkirch. Il en est de même pour l'analyse phénotypique de la génération F0 non traitée au mélange [4NP + 4tOP].

Si les souris ainsi obtenues s'avèrent viables et fertiles, elles seront soumises au protocole d'exposition décrit ci-dessus pour les souris sauvages. Une attention particulière sera alors

portée au suivi de l'expression (transcriptionnelle et protéique) de ERα36 dans les différents types cellulaires constituant le testicule et la glande mammaire aux différents temps de prélèvement afin de caractériser son éventuelle implication dans les effets observés.

Analyse de l'effet transgénérationnel d'une exposition au [4NP + 4tOP]

Des animaux mâles et femelles F0 exposés aux différentes doses de [4NP + 4tOP] ou non seront conservés dans le but d'étudier un éventuel effet transgénérationnel d'une exposition aux alkylphénols. S'ils semblent présenter un phénotype (macroscopique, histologique ou moléculaire) de l'appareil reproducteur et que leur taux de fertilité le permet, les croisements seront poursuivis jusqu'en F3 pour les sauvages et les deux lignées KI ERα36. A chaque génération, les paramètres précédemment cités seront analysés.

L'axe 1b du projet décrit ci-dessus pourra être développé avec le laboratoire URAFPA de Nancy (H. Schoeder) et ou avec les animaleries centrale et transgénique de la Faculté de Médecine de Nancy pour les modèles d'exposition. Amand Chesnel, Maître de Conférences, participera à ce travail en apportant notamment ses compétences en épigénétique et en biologie de la reproduction. Martine Chillet, adjointe technique assurera le traitement histologique et immunohistochimique des prélèvements de rat.

c) Modélisation de réseaux de gènes dépendants de ERα36, initiation, progression tumorale et potentiel métastatique

Au sein de l'EA SIGRETO, notre équipe s'intéressait depuis plusieurs années à la signalisation des œstrogènes dans les cellules cancéreuses mammaires et depuis peu dans les cellules séminomateuses. Les travaux ont porté notamment sur les effets non génomiques de ces hormones qui stimulent ces deux types de cellules cancéreuses. Par exemple, le laboratoire a mis en évidence une activation ligand-indépendante du récepteur ERα66 dans les cellules d'adénocarcinomes mammaires MCF7 exposées à l'éthanol (Etique et al., 2007 ; 2009). Depuis 2009, nous avons mis en évidence le rôle clef de ERα36, une forme tronquée du récepteur alpha aux œstrogènes dans la sensibilité aux stéroïdes de la lignée séminomateuse TCam-2 (Wang, 2005, 2006 ; Lee, 2008 ; Shi, 2009 ; Zhang, 2011 ; Wallacides, 2012).

Dans ce contexte, et parallèlement aux analyses *in vivo* décrites ci-dessus, je souhaite poursuivre ma conversion thématique en cancérologie dans le cadre d'un projet étendu à la modélisation des réseaux de gènes impliqués dans l'initiation, la progression tumorales et la promotion du potentiel métastatique des tumeurs mammaires et testiculaires. Cette partie du projet sera transversal au sein de CRAN-Sigreto et associera plusieurs équipes nancéiennes.

L'étude sera réalisée dans des lignées cancéreuses (i) mammaires immortalisées non transformées (MCF10A), hormono-dépendantes (MCF7, MCF10A) ou non (MDA-MB231) ou (ii) testiculaires (TCam-2, NT2/D1) exprimant ERα36 à des niveaux variables et contrôlés. Nous réaliserons une analyse transcriptomique des gènes et des miRNA pour lesquels le [4tOP+4NP] induit des changements d'expression dans les différentes lignées et contextes génétiques. Une analyse des modifications épigénétiques cibles d'ERα36 ainsi identifiées sera initiée. Une modélisation des voies de signalisation sera menée en utilisant des algorithmes de calcul de corrélations non linéaires et d'entropie. Cette étape sera basée d'abord sur des données extraites des différentes banques disponibles (par exemple la banque de données d'expression GEO http://www.ncbi.nlm.nih.gov/gds) puis sur l'inclusion des résultats obtenus dans le cadre du projet.

En se focalisant sur le cancer du sein et le cancer des testicules dont l'incidence est clairement augmentée dans certains contextes d'exposition environnementale, nous tenterons de préciser le rôle de ERα36 dans les événements moléculaires et de modéliser les voies de signalisation

à l'origine des éventuelles anomalies observées *in vivo*, en particulier l'initiation tumorale et la promotion du potentiel métastatique .

Construction des lignées stables ERα36^{-}

L'EA4421 Sigreto travaille en routine sur les différentes lignées de cancer mammaire (MCF-7, MDA-MB231 et MCF10A) et testiculaire (TCam-2) concernées par le projet. Lees MCF-7 sont des cellules hormono-sensibles ERα66^{+}, ERα36^{+} ; les MDA-MB231 sont triples négatives mais ERα36+ et les MCF10A sont des cellules mammaires immortalisées non cancéreuses ERα36±. Les TCam-2 représentent actuellement la seule lignée séminomateuse disponible et les NT2/D1 sont issues d'une tumeur testiculaire non séminomateuse. Les lignées stables TCam-2 et NT2/D1 dans lesquelles l'expression d'ERα36 est bloquée par un shRNA ont déjà été produites au laboratoire (Wallacides, 2012 ; Ajj, soumis). Les mêmes vecteurs shRNA seront utilisés pour transfecter les lignées cancéreuses mammaires. Après 15 générations de sélection par antibiotique, la pression de sélection est éliminée pour 5 générations puis réappliquée régulièrement ainsi qu'après chaque décongélation. Nous possédons également les vecteurs permettant de surexprimer ERα36 de façon stable dans les différentes lignées (Wang, 2006).

Analyses transcriptomiques

Afin de préciser le rôle de ERα36 dans les évènements moléculaires et de modéliser les voies de signalisation à l'origine des éventuelles anomalies observées, en particulier la promotion tumorale, des approches globales (transcriptome, miRNome) sont envisagées et seront réalisées en collaboration avec la société PartnerChip, prestataire de services avec laquelle nous avons déjà travaillé (projet ANSES), dans le domaine des bio-puces à haute densité et analyses bioinformatiques associées.

Les analyses transcriptomiques, impliqueront les 4 lignées (MCF7, MDA, MCF10 et TCam-2) présentant les 2 contextes génétiques étudiés à savoir ERα36+ et ER3a6-. Chacune des lignées, sous ses 2 formes (ERα36+/ERα36-) sera ou non exposée au mélange [4NP + 4tOP]. Afin d'obtenir des données statistiquement exploitables, chaque analyse sera répétée 3 fois à partir de trois extractions d'ARNs différentes (réplicats biologiques) soit 48 conditions distinctes (une demande de financement à l'ANSES est actuellement en cours d'évaluation sur cette thématique).

Analyse des miRNAs

Les miRNAs étant démontrés comme participant à de plus en plus d'évènements régulationnels notamment en oncologie, plusieurs sociétés proposent d'ores et déjà des outils permettant l'analyse simultanée des 500 à 1800 miRNAs décrits et validés.

PartnerChip aura la charge de procéder à l'analyse la plus exhaustive possible de tous les miRNAs murins décrits. Pour cela elle utilisera une puce murine haute –densité produite par la société japonaise Toray (1719 mRNAs), dont elle a l'exclusivité d'exploitation. Cette analyse sera réalisée selon un schéma identique à celui décrit pour l'analyse transcriptomique mais à partir d'extractions spécifiques préservant les ARNs de très petite taille que sont les miRNAs (48 conditions expérimentales).

PartnerChip aura en charge les contrôles qualité et la quantification des miRNAs extraits, les marquages et hybridations, la lecture des puces haute densité, l'extraction des données brutes ainsi que l'analyse statistique associée conduisant aux différentes listes de miRNAs différentiellement exprimés.

Suite à ces approches globales une étape de validation par puce dédiée ou par séquençage haut-débit ciblant les gènes ou miRNAs mis en évidence pourra être initiée en proposant un design propriétaire pour la capture des régions d'intérêt soit sur puce, soit en solution, capture suivie d'un séquençage exhaustif (Solexa d'Illumina ou Roche 454).

*Modélisation des voies de réponse au [4tOP+4NP] dépendantes de ER*α36

Dans un premiers temps, il s'agit de faire un état de l'art des données transcriptomiques et des voies de signalisations décrites (i) dans les expériences de traitement de mammifères par les alkylphénols au cours du développement, (ii) dans les tumeurs mammaires et testiculaires dans diverses bases de données disponibles. L'extraction et l'analyse de données permettront de proposer des modèles à valider expérimentalement (Van den Akker, 2011). Ces modèles doivent représenter les intensités ainsi que les dynamiques des interactions génomiques et protéiques ce qui permettra de les classer plus finement. Cette phase, où seront impliquées trois équipes, (i) CRAN-SIGRETO pour l'aspect biologique, hypothèses, validation de modèles, (ii) LORIA pour les aspects de recherche et d'extraction de données à partir de bases existantes et (iii) CRAN pour l'aspect proposition de modèles et identification des paramètres de ces modèles, permettra d'explorer différentes méthodologies de modélisation et de faire le point sur les capacités de chaque type de modèles à représenter à la fois les régulations géniques et les voies de signalisation associées.

La méthodologie développée dans la première partie sera appliquée afin de mettre en lumière le rôle exact joué par ERα36 dans la réponse au [4tOP+4NP] en lien, ou non avec une stimulation du potentiel métastatique des tumeurs mammaires Cela permettra de compléter les modèles obtenus. Par ailleurs, l'intégration de données transcriptomiques et interactomiques, permettrait d'envisager de nouvelles cibles thérapeutiques ou de nouveaux marqueurs précoces de développement du potentiel métastatique des cellules tumorales.

Retour in vitro pour valider les hypothèses issues du modèle proposé

La validation première de ces cibles et marqueurs pourra ensuite être réalisée plus aisément *in vitro* sur les lignées déjà utilisées et *in vivo* à partir d'échantillons de tumeurs mammaires ou sur modèles de xénogreffes.

L'axe 1c du projet décrit ci-dessus sera développé en collaboration avec M.D. Devignes et M. Smail-Tabbone de l'équipe Orpailleur du LORIA (UMR 7503 ; CNRS - Université de Lorraine qui mettront à profit leur expertise en intégration et fouille de données biologiques hétérogènes et complexes. Elles réaliseront l'inventaire des données apparentées au projet et déjà accumulées dans les bases de données de transcriptomique et d'interactomique (GEO, INTACT, etc.). Les jeux de données les plus pertinents seront sélectionnés et serviront à tester les différentes approches de modélisation proposées par T. Boukhobza du CRAN qui apportera ses compétences en modélisation, identification, planification d'expériences, traitement du signal et analyses structurelle de modèles dynamiques. A partir des données expérimentales, des modèles permettant de traduire l'effet et le rôle de ERα36 dans les voies de signalisation à l'origine des éventuelles anomalies observées seront proposés. A partir de ce cas d'étude, il sera possible de développer une méthodologie d'analyse applicable à la caractérisation du rôle d'autres gènes dans les anomalies de l'appareil reproducteur.

Axe 2 - Evaluation de la pertinence d'une forme tronquée du récepteur alpha des œstrogènes (ERα36), comme marqueur de réponse aux traitements anti-tumoraux dans les cancers mammaires: études sur lignées cellulaires, souris xénogreffées et échantillons de tumeurs humaines.

Le cancer du sein est le cancer féminin le plus fréquent et représente la seconde cause de mortalité induite par cancer dans cette population après le cancer du poumon. Les tumeurs mammaires peuvent être séparées en au moins 4 sous-groupes définis par leur statut ERα66 (récepteur alpha des œstrogènes), PR (récepteur de la progestérone) et HER2 (Human Epidermal growth factor Receptor 2) (Sorlie, 2004, Gatza, 2010). Ces marqueurs ont conduit au développement d'agents thérapeutiques contre les tumeurs HER2-positives et d'agents endocrines contre les tumeurs ERα66-positives. Cependant, environ 25% des patientes atteintes de formes hormono-sensibles de cancer du sein ne répondent pas à l'anti-œstrogène tamoxifène et la moitié des patientes traitées au tamoxifène développent un phénotype tamoxifène-résistant (EBCTC, 2005). En outre, aucune thérapie ciblée n'existe pour les patientes développant des tumeurs mammaires triples-négatives, n'exprimant ni récepteurs hormonaux, ni HER2 et ces tumeurs récidivent souvent.

Nous nous focaliserons plus particulièrement sur le rôle présumé de ERα36 dans la résistance à l'hormonothérapie et le développement du potentiel métastatique.

ERα36 est exprimé non seulement dans les cellules cancéreuses mammaires ERα66-positives, dans les cellules ERα66-négatives (Lee, 2008), mais également dans des échantillons de tumeurs triples négatives (Zheng, 2010). *In vitro*, les cellules triples négatives MDA-MB-231 et MDA-MB-436 voient leur prolifération stimulée par le 17β-œstradiol ($1x10^{-9}$ ou 10^{-10}M) dans un milieu pauvre en sérum de veau (2,5%) (Zhang, 2010). Ces effets ne sont plus observés lorsque l'expression d'ERα36 est inhibée par shRNA. Dans ces cellules il a aussi été montré qu'ERα36 interagit physiquement avec le complexe EGFR/src/shc et médie la phosphorylation d'EGFR, de src et des ERK en réponse à l'œstradiol. En outre, l'EGF stimule la transcription d'ERα36 et il existe donc une boucle de rétrocontrôle entre ces deux récepteurs.

Enfin, dans les cellules qui surexpriment ERα36, le tamoxifène ne peut empêcher l'activation des ERK médiée par ERα36 et de façon surprenante, il stimule la croissance cellulaire (Wang, 2006). De ce fait, ERα36 pourrait être impliqué dans la résistance *de novo* au tamoxifène. Cette hypothèse est étayée par le fait que la surexpression d'ERα36 est

associée à une plus faible survie sans récidive chez les patientes présentant des tumeurs mammaires ERα66-positives traitées au tamoxifène (Shi, 2009).

Récemment, Chaudhri et ses collaborateurs ont montré dans la lignée triple-négative HCC38 que l'œstradiol couplé à la BSA (forme qui ne pénètre pas dans les cellules) réduit l'apoptose induite par le tamoxifène (agent endocrine utilisé dans le traitement des tumeurs ERα66+) alors qu'il stimule la prolifération et la migration de façon ERα36-dépendante (Chaudhri, 2012). Cet effet « promoteur du potentiel métastatique » reste à confirmer *in vivo* mais pourrait en partie expliquer le mauvais pronostic des patientes ERα36^{++}.

Enfin, le statut GPER étant rarement étudié dans les tumeurs mammaires, il n'est pas exclu que certains des effets non génomiques des œstrogènes attribués jusqu'alors à GPER soient médiés par ERα36 (Kang et Wang, 2010).

Au vu des résultats récents décrits ci-dessus sur l'isoforme ERα36 et de ses implications suspectées dans l'évolution des tumeurs mammaires et dans les phénomènes de résistance à l'hormonothérapie, nous souhaitons étudier le statut ERα36, Her2 et GPER dans des échantillons de tumeurs mammaires hormono-dépendantes (ERα66-positives) et hormono-indépendantes (ERα66-négatives).

En juillet 2011, nous avons initié une collaboration avec le Professeur Abecassis du Centre Paul Strauss de Strasbourg pour l'analyse de cDNA extraits à partir de 92 tumeurs mammaires stratifiées selon leur grade et leur statut ER/PR.

Le niveau d'expression transcriptionnelle de GPER, ERα36, HER2 et ERα66 a été mesuré par PCR quantitative dans chacun des échantillons puis les données ont été analysées par Taha Boukhobza (CRAN, Nancy).

Les premiers résultats indiquent notamment que l'expression de GPER, ERα36 et ERα66 est corrélée dans les tumeurs RE^{+}/RP^{+} de grade 2. Les patientes porteuses de ces tumeurs de bas grade sensibles aux œstrogènes sont celles auxquelles on prescrit généralement un traitement anti-hormonal à base de tamoxifène, lui-même agoniste de ERα36. Nous proposons donc de vérifier ces données dans le cadre d'une étude rétrospective de plusieurs centaines d'échantillons de tumeurs mammaires afin de préciser le champ d'application de la classification « ER^{+}/ER^{-}» actuelle et de fournir au clinicien une aide au choix thérapeutique.

Fort des résultats préliminaires déjà obtenus et des données récentes de la littérature scientifique, le projet repose sur le renforcement et le développement de la collaboration entre les équipes de Nancy et de Strasbourg. Il vise à comprendre les bases moléculaires des échecs thérapeutiques observés chez les patientes ER^{+} en déterminant l'implication de réseaux de

signalisation dépendants des récepteurs membranaires des œstrogènes dans le potentiel métastatique des tumeurs mammaires.

La stratégie d'étude s'articulera autour de 2 questions générales :

1 - Les récepteurs membranaires des œstrogènes (ERα36, GPER) sont-ils des marqueurs prédictifs de réponse thérapeutique dans les échantillons de tumeurs ER^+ traitées au Tamoxifène (ER^+, TAM^+) disponibles pour cette étude rétrospective?

Nous analyserons 120 échantillons de tumeurs ER^+ issues de patientes traitées au tamoxifène (TAM^+) ainsi que 60 échantillons de tumeurs triple-négatives (ER^-, TAM^-). L'expression d'ERα36 et GPER sera analysée par PCR quantitative sur des ARNs extraits et rétrotranscrits à partir de 6 groupes d'échantillons : les tumeurs de statut ER^+ (120 échantillons) ou ER^- (60 échantillons), de grade 1, 2 ou 3. Des analyses de corrélations seront ensuite effectuées entre le niveau d'expression des gènes et les paramètres histocliniques tels que le type de traitement, le grade, l'extension ganglionnaire, la récidive, l'existence de métastases et la survie globale des patientes. La localisation membranaire ou intracellulaire d'ERα36 et GPER sera vérifiée par immunohistochimie dans les échantillons de tumeur de chaque groupe (ER^+, TAM^+ ou ER^-, TAM^-).

2 - L'expression forte de ces récepteurs est-elle un facteur de mauvais pronostic parce qu'ils stimulent le potentiel métastatique des tumeurs primaires ?

Sur les mêmes groupes d'échantillons que précédemment, nous déterminerons s'il existe des corrélations entre l'expression des récepteurs ERα36 et GPER et des marqueurs métastatiques après mesure de leur expression en QPCR (snail, RANKL, DDB2, VIM, MMP9 et Cadh principalement) puis par immuno-histochimie.

Une identification des relations et des corrélations multivariables entre les gènes étudiés afin d'établir des modèles des voies de signalisation seront menées en utilisant des algorithmes de calcul de corrélations non linéaires et d'entropie (Chen, 2010, Huang, 2009).

La dernière partie du travail consistera en une étude *in vitro.* L'objectif sera d'une part de valider les cibles suggérées lors de l'analyse des échantillons de tumeurs et d'autre part d'identifier les voies de signalisation activées en aval des récepteurs membranaires des œstrogènes et contrôlant le potentiel métastatique.

Cette validation dynamique sera réalisée sur des lignées cancéreuses mammaires hormono-dépendante (MCF7; $ERα66^+$, $ERα36^+$) ou triple négative (MDA-MB-231 ($ERα36^+$). Des cinétiques d'exposition à l'oestradiol ou au tamoxifène permettront de confirmer la variation d'expression des gènes cibles marqueurs métastatiques suspectés grâce à l'analyse de

corrélation faite sur les tumeurs. Le blocage des récepteurs membranaires ERα36 et/ou GPER par siRNA ou par des antagonistes (G15 pour GPER) permettra de confirmer l'implication du (des) récepteur(s) membranaire(s) des oestrogènes dans cet effet.

Des lignées stables dans lesquelles l'expression d'ERα36 est bloquée par un shRNA sont en cours de sélection au laboratoire. Nous possédons également les vecteurs permettant de surexprimer ERα36 de façon stable dans les différentes lignées (Wang, 2006). Ces lignées seront utilisées en plus de celles citées ci-dessus.

Une fois l'implication du(des) récepteur(s) membranaire(s) confirmée, l'identification des voies de signalisation en aval se fera par traitement à l'œstradiol et/ou au tamoxifène, en présence de différents inhibiteurs des voies de signalisation connues en aval de ces récepteurs membranaires (PKA, MAPK, PI3K/Akt, Src/EGFR). Cela permettra de savoir lesquelles aboutissent aux gènes cibles impliqués dans l'augmentation du potentiel métastatique.

Nous souhaitons donc confirmer les résultats préliminaires déjà obtenus sur une cohorte plus conséquente et *a posteriori* déterminer si l'expression des marqueurs analysés est corrélée avec le taux de récidives de ces patientes.

A plus long terme, nous nous intéresserons aux mécanismes de la signalisation médiée par GPER et ERα36 dans les lignées cellulaires et les échantillons de tumeurs mammaires.

Afin de valider ERα36 comme marqueur de réponse aux traitements dans les cancers mammaires, je souhaite également développer un modèle *in vivo* en collaboration avec la « Plateforme xénogreffes » localisée à la Faculté de Médecine de Nancy. Il s'agirait de cellules MDA-MB231 KO pour ERα36 ou non, xénogreffées sur des souris Nude et permettant l'analyse des effets de traitements *in vivo*. Cet aspect du travail pourra être étendu à l'étude de xénogreffes issues de lignées T47D et MCF-7 KO ERα36.

Ce type d'étude contribuera à une meilleure connaissance du réel statut hormono-sensible des tumeurs incluant le rôle des formes tronquées des récepteurs nucléaires dans la progression tumorale et la résistance aux traitements anti-cancéreux. Cette problématique est notamment développée pour les isoformes du récepteur des androgènes dans les cancers du testicule et de la prostate, respectivement dans l'équipe de Rajpert-de Meyts au Danemark et dans celle de Céraline à Strasbourg. Enfin, dans le cadre de l'activité de recherche translationnelle du CRAN, la validité de ERα36 comme marqueur prédictif de réponse aux traitements pourrait ensuite être évaluée en situation clinique afin de mieux orienter les choix thérapeutiques dans une démarche de traitement personnalisé et éventuellement de mettre en évidence de nouvelles cibles thérapeutiques dans les tumeurs triple-négatives. A terme, ce

projet pourra servir de base pour la mise en place d'une étude prospective à grande échelle afin de valider le rôle clef des récepteurs membranaires des œstrogènes dans la réponse aux traitements du cancer du sein.

J'ai développé l'axe2 du projet décrit ci-dessus dans le cadre de mon CRCT (1er semestre 2012) en collaboration d'une part avec J. Abecassis (Centre Paul Strauss, Strasbourg) et d'autre part avec T. Boukhobza (CRAN, Nancy) pour le traitement des données dans le cadre des actions transversales menées parallèlement au rapprochement Sigreto-CRAN (prochain contrat quinquennal). La mise en place du projet contribuera à renforcer les collaborations scientifiques entre des équipes de chercheurs et de cliniciens, localisées à Nancy et à Strasbourg, qui émergent au sein du Cancéropôle Grand Est. La pérennité de ce type de consortium interrégional est susceptible, à terme, de permettre le développement de projets de recherche d'envergure nationale en réponse à des appels à projets de l'INCa ou de l'ANR.

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PROJET D’ENSEIGNEMENT

Depuis mon recrutement en tant que Maître de conférences à l'UHP (septembre 2003), j'ai enseigné principalement la biologie animale et la biologie cellulaire en Licence et la biologie de la reproduction en Master. Ces enseignements correspondaient à la fois aux thématiques abordées dans mes expériences de recherches antérieures et à celles développées depuis mon recrutement.

Dans le cadre de l'habilitation 2013-2018, j'ai souhaité prendre la responsabilité de plusieurs UE de biologie communes à Metz et Nancy en Licence de Sciences du Vivant et de l'Environnement

- Biochimie et Biologie cellulaire L1
- Techniques expérimentales en biologie cellulaire L2

Je participerai activement à la refonte et assurerai des enseignements de biologie cellulaire du L1 au L3 afin de permettre une certaine cohérence des contenus sur l'ensemble de la Licence.

Suite à ma reconversion thématique, je proposerai également une UE optionnelle dans le Master **«** Sciences Biologiques et Ingénierie pour la Santé », Parcours M1 Biologie cellulaire et physiologie. Cette UE « Perturbateurs endocriniens et risque pour la santé humaine » reprendra certaine bases d'endocrinologie, et prendra l'exemple des œstrogéno-mimétiques pour illustrer les différents aspects de la perturbation endocrinienne et du risque pour la santé humaine. Une conférence d'un spécialiste de l'ANSES est d'ores et déjà prévue dans la maquette.

A terme, je souhaite participer au parcours européen de Cancérologie en Master 2 de Sciences Biologiques et Ingénierie pour la Santé qui sera mis en place en 2013 et qui regroupera *a minima* les Universités de Lorraine, du Luxembourg, de Liège et de la Sarre.

ANNEXE

5 PUBLICATIONS MAJEURES

Mol Gen Genet (1997) 257: 62–70

ORIGINAL PAPER

P. Billard · H. Dumond · M. Bolotin-Fukuhara

Characterization of an AP-1-like transcription factor that mediates an oxidative stress response in *Kluyveromyces lactis*

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Abstract The *KlYAP1* gene, encoding the transcription factor Yap1p from *Kluyveromyces lactis*, was cloned by functional complementation of the cadmium hypersensitivity phenotype of a *Saccharomyces cerevisiae* strain lacking functional *YAP1* and *YAP2* genes. The *KlYAP1* gene product is 41% identical to Yap1p, the sequence similarity being centered on the bZip domain and extending into the C-terminal portion of both proteins. When expressed in *S. cerevisiae*, this gene efficiently complements some of the phenotypes associated with both *yap1* and *yap2* mutations and also mediates AP-1 response element-dependent transcriptional activation in response to H_2O_2. Gene disruption experiments in *K. lactis* indicated that the *KlYAP1* gene is involved in both the oxidative and cadmium response pathways. We also demonstrate the existence in *K. lactis* of inducible protective stress responses to both peroxides and superoxides and investigate the role of the Klyap1p protein in these responses.

Key words YAP1 · *Kluyveromyces lactis* · *Saccharomyces cerevisiae* · Transcriptional activator · Oxidative stress response

Introduction

When exposed to stressful environmental conditions, cells trigger specific responses which allow them to survive. These responses can often lead to transcriptional activation of a set of genes whose products protect against the deleterious effects of the encountered stress. In the yeast *Saccharomyces cerevisiae*, two transcription factors have been identified that mediate some of these cellular stress responses, namely Yap1p and Yap2p. They both belong to the Jun family of transcriptional activators, recognize the same AP-1 response element (ARE) sequence target in vitro, and promote ARE-dependent transcriptional activation. Initial identification of Yap1p was based on its biochemical similarity to human AP-1 (Moye-Rowley et al. 1989). Further studies delineated the physiological importance of *YAP1* and *YAP2* by demonstrating their ability to confer pleiotropic drug resistance when overexpressed in vivo (*PDR4*/*SNQ3*/*PAR1*, Hertle et al. 1991; Hussain and Lenard 1991; Schnell and Entian 1991; *CAD1*, Bossier et al. 1993; Wu et al. 1993; Hirata et al. 1994). Despite the structural similarity between Yap1p and Yap2p, the phenotypes associated with the deletion of the corresponding genes differ. For example, Δ*yap1* mutants are hypersensitive to cadmium, while a Δ*yap2* strain is not significantly affected (Wu et al. 1993; Hirata et al. 1994). The large number of phenotypes produced by changes in dosage of both *YAP1* and *YAP2* suggested that several genes are under their control. Further investigations demonstrated that Yap1p directs transcriptional activation of antioxidant genes, including those coding for thioredoxin (*TRX2*, Kuge and Jones 1994), γ-glutamylcysteine synthetase (*GSH1*, Wu and Moye-Rowley 1994), glutathione reductase (*GLR1*, Grant et al. 1996a), and the *YCF1* gene encoding an ATP-binding cassette protein involved in cadmium tolerance (Wemmie et al. 1994b). However, no target gene for Yap2p has yet been found.

Aerobically growing organisms have to cope with toxic, reactive oxygen species that are produced during normal cell metabolism. Respiration processes partially reduce oxygen to superoxide and H_2O_2, which are only moderately reactive but can form hydroxyl radicals that react with almost all cellular components and may be

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responsible for most of the oxidative damage from reactive oxygen species. To counteract these deleterious effects, aerobic cells are endowed with enzymatic defenses including superoxide dismutase, catalase, peroxidases, and antioxidant molecules such as vitamins C and E, thioredoxin, and glutathione. Among all organisms investigated so far, *Escherichia coli* is the one whose response to oxidative stress is best understood (for review see Storz et al. 1990; Farr and Kogoma 1991). This bacterium has been shown to possess adaptive mechanisms that protect against oxidative injury: exposure to low concentrations of H_2O_2- or superoxide-generating agents makes cells resistant to a subsequent encounter with elevated doses of the same oxidants. Such an adaptive response also exists in three well studied yeasts, *S. cerevisiae* (Collinson and Dawes 1992; Jamieson 1992), *Schizosaccharomyces pombe* (Lee et al. 1995), and *Candida albicans* (Jamieson et al. 1996). Recently, the *YAP1* and *YAP2* genes have been demonstrated to play a major role in regulating the adaptive oxidative stress response of *S. cerevisiae* (Stephen et al. 1995). Deletion of either *YAP1* or *YAP2* drastically reduces the level of protection afforded by induction of the H_2O_2 adaptive stress response.

To obtain more information about these two regulatory genes and their precise contribution to the global stress response, we decided to study their homologs in the "petite-negative" yeast *Kluyveromyces lactis*. This yeast is related to *S. cerevisiae*, but relies predominantly on respiratory metabolism of glucose under aerobic growth conditions and may therefore have to deal with constitutive production of reactive oxygen species. However, little is known about either oxidative or global stress responses in *K. lactis*. Here we report the isolation and characterization of a gene designated *KlYAP1* that is functionally and structurally homologous to the *YAP1* gene. Phenotypes produced by variation in the *KlYAP1* gene dosage in *K. lactis* are essentially the same as those observed in *S. cerevisiae* upon variation of *YAP1* copy number, thus suggesting that the corresponding proteins play similar roles in both yeasts. We also report on the characteristics of adaptation of *K. lactis* cells against oxidative stress, using either H_2O_2 or the superoxide-generating agent menadione, and the role played by *KlYAP1* in the regulation of this adaptive response.

Materials and methods

Strains and media

The *K. lactis* strain used in this study, MW179-1D [MATα *lac4-8 uraA1-1 leu2 metA1-1* Ade$^-$ *trp1* Rag$^+$ (pKD1$^+$)], has been previously described (Chen et al. 1992). The isogenic *S. cerevisiae* strains W303α, Δ*yap1*, Δ*yap2*, and Δ*yap1*Δ*yap2* were obtained from C. Rodriguez-Pousada and are described elsewhere (Bossier et al. 1993). Yeast strains were grown either in nutrient-rich liquid medium (YPG; 1% yeast extract, 2% bactopeptone, 2% glucose) or in SD minimal medium [0.17% yeast nitrogen base without ammonium sulfate and amino acids (YNB; Difco), 0.5% ammonium sulfate, 2% glucose] supplemented with amino acids or bases when required. Media were solidified with 2% agar.

The *E. coli* strains used were: DH5α [**F**$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* ***deoR*** (*ϕ80dlac*Δ(*lacZ*)***M15***)]; M8820 [**F**$^-$ *araD139* Δ(*ara-leu*)*7697 proAB* Δ(*argF-lacIPOZYA*)*XIII rpsL* (Sm^r)]; JM109 [***F′ traD36 proAB lacI^q* Δ(*lacZ*)*M15*** / *recA1* Δ(*lac-proAB*) *endA1 gyrA96* (***Nal^r***) ***thi*** *hsdR*17 (r_K^- m_K^+) *supE44 relA1*].

Isolation and sequencing of the *KlYAP1* gene

All standard molecular cloning techniques were carried out as described by Sambrook et al. (1989). A high-copy-number *K. lactis* library (kindly provided by M. Wésolowski-Louvel) of *Sau*3A partial fragments cloned in the *Bam*HI site of plasmid pSK1 (Prior et al. 1993) was transformed into the *S. cerevisiae* Δ*yap1*Δ*yap2* strain by the modified lithium acetate procedure (Schiestl and Gietz 1989). Ura$^+$ transformants were replica plated on YNB plates containing 0.3 mM $CdSO_4$, and two cadmium-resistant clones were selected for further analysis. Plasmid-borne cadmium resistance was verified by plasmid rescue (Hoffman and Winston 1987) and retransformation into the same Δ*yap1*Δ*yap2* strain. Transposon insertion mutagenesis of one of the corresponding plasmids, named pSY1 (see below), was performed with the mini-Mu derivative MudIIPR13, as previously described (Daignan-Fornier and Bolotin-Fukuhara 1988). Plasmid DNA of transductants was used to transform the Δ*yap1*Δ*yap2* strain, and transformants that could not grow on SD plates containing 0.3 mM $CdSO_4$ were picked for further study. Location of the mini-Mu insertion site was determined by restriction mapping and sequence analysis. DNA sequence analysis on both strands of the *KlYAP1* gene was carried out by subcloning in pBluescript KS+ and dideoxynucleotide sequencing (Sanger et al. 1977).

Plasmids and β-galactosidase assays

Plasmids pSY1 and pSY3 are the episomal *K. lactis*/*S. cerevisiae*/*E. coli* shuttle plasmid pSK1 (Prior et al. 1993) with genomic inserts containing the *KlYAP1* gene. A 3.5-kb *Spe*I–*Sal*I fragment, spanning the entire *KlYAP1* open reading frame (ORF) and flanking sequences from pSY1, was inserted into the *Spe*I and *Xho*I sites of pBluescript KS+, yielding pKYSS. This plasmid was cleaved with *Spe*I, filled in by Klenow enzyme treatment, and then cleaved by *Sal*I. The resulting fragment was cloned in the *Sal*I and *Sac*I sites of the multicopy vector pCXJ23 and the centromeric plasmid pCXJ19 (Chen 1996) to create pCY231 and pCY193, respectively. Plasmid pGM26 is a pBR322 derivative containing the *URA3* gene and the 2-μm plasmid origin fragment of *S. cerevisiae* cloned in the *Hin*dIII and *Sal*I sites, respectively. Plasmid pGM26-YAP1 was constructed by integrating a 3.5-kb *Nhe*I–*Sph*I fragment from plasmid pPR2.2F (Bossier et al. 1993) containing the *YAP1* gene into pGM26 cleaved by *NheI* and *Sph*I. Similarly, a 2.2-kb *Xho*I–*Bam*HI fragment from plasmid pKS-YAP2 (Bossier et al. 1993) carrying the *YAP2* gene was filled in with Klenow enzyme and inserted in the blunt-ended *Bam*H1 site of pGM26, creating the plasmid pGM26-YAP2. Plasmid pEPC11 was obtained from W. S. Moye-Rowley and has been described elsewhere (Wu and Moye-Rowley 1994). β-Galactosidase assays were performed as described by Ruby et al. (1983).

Disruption of *KlYAP1* and RNA analysis

The *KlYAP1* gene disruption plasmid was constructed by cleavage of plasmid pKYSS with *Hin*cII and *Pst*I, removing a fragment spanning 85% of the *KlYAP1* coding sequence and 230 nucleotides of its promoter, and replacement of this fragment by a 1.7-kb *Sma*I–*Pst*I DNA derived from plasmid YDp-L (Berben et al. 1991) containing the *LEU2* gene of *S. cerevisiae*. The resulting plasmid (Fig. 1) was cleaved with *Not*I and used to transform a haploid MW179-1D strain to leucine prototrophy by the method of

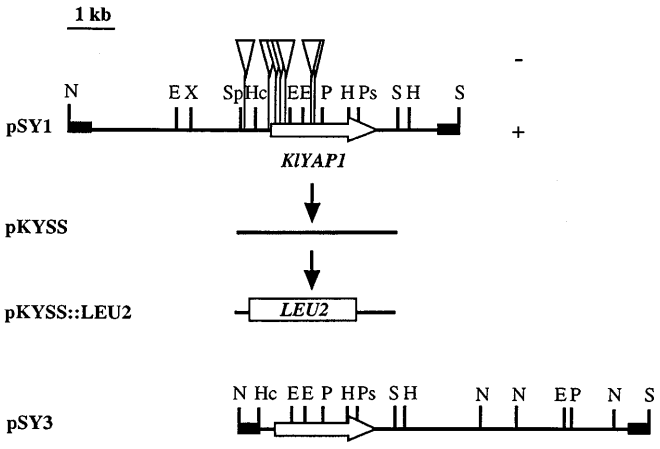

Fig. 1 Restriction map of plasmids pSY1 and pSY3 and localization of the *KlYAP1* gene in the plasmid pSY1. The *boxes* indicate pSK1 vector sequences, the *lines* represent yeast sequence. The *open arrow* indicates the open reading frame of *KlYAP1* and the direction of transcription. The MudIIPR13 insertional events that abolish the heterologous complementation of the *Saccharomyces cerevisiae* Δ*yap1*Δ*yap2* mutant are indicated by *triangles* above the restriction map. Plasmids pKYSS and pKYSS::LEU2 were required for the disruption strategy. (*E Eco*RI, *H Hin*dIII, *Hc Hin*cII, *N Nhe*I, *P Pst*I, *S Sal*I, *Sp Spe*I, *X Xba*I, + complementation for growth in the presence of 0.3 mM $CdCl_2$, − no complementation)

Dohmen et al. (1991). Proper deletion of the *KlYAP1* locus was confirmed by Southern blot (data not shown) and RNA analysis. Total RNA was extracted from cells grown to an A_{600} of 1–1.5 according to Carlson and Botstein (1982), fractionated in an agarose/formaldehyde denaturing gel, and blotted onto Hybond-C^+ membrane (Amersham). Hybridization was performed at 42° C in 50% formamide with a 348-bp PCR fragment corresponding to positions 471–819 of the *KlYAP1* gene and an *Eco*RI–*Hin*dIII fragment of plasmid pKL7 (Larson and Rossi 1991) containing the *K. lactis* actin gene. Both fragments were labelled with ^{32}P using the Ready to Go DNA labelling kit (Pharmacia).

Inhibitor resistance assays

Yeast strains to be tested for their resistance to inhibitory compounds were grown to an A_{600} of approximately 1. Appropriate dilutions were made and about 1000 cells in a 4-μl volume were spotted on YPD plates supplemented with various concentrations of the chemicals and incubated at 30° C for 2–4 days. Sensitivity to oxidants was assessed using zone-inhibition assays. Approximately 10^6 stationary phase cells grown in YPG were plated onto YPG agar. A filter disk soaked with 4 μl of either 33% H_2O_2 or 1 M menadione was placed in the center of the plates. Plates were then incubated at 30° C for 3 days before measuring the diameter of the zone of growth inhibition.

Sensitivity and adaptation to oxidative stresses

H_2O_2 and menadione adaptation experiments were carried out essentially as described by Jamieson (1992). Sensitivity to oxidants was assessed by treating 1 ml of exponentially growing cultures (A_{600} = 0.15, ca 3×10^6 cells) or stationary phase cultures (A_{600} = 15–20 diluted to A_{600} = 0.15) of MW179-1D and the isogenic Δ*klyap1* strains with a range of concentrations of H_2O_2 and menadione for 1 h in YPD at 30° C, after which appropriate dilutions were plated onto YPD plates to determine viability. The adaptive response was quantified by pretreating cells with non-lethal doses of either H_2O_2 (0.2 mM) or menadione (1 mM) for 1 h. Cells were harvested by centrifugation, washed once with distilled water, resuspended in an equal volume of fresh prewarmed YPD medium, and subsequently challenged with higher doses of the same oxidant before plating as above.

Results

Isolation of a *K. lactis* DNA fragment conferring cadmium resistance on *S. cerevisiae*

The Δ*yap1*Δ*yap2* strains of *S. cerevisiae* are highly sensitive to cadmium and H_2O_2 and are not able to form colonies on minimal media containing $CdCl_2$ or H_2O_2 at concentrations in excess of 150 μM and 2 mM, respectively. In an attempt to identify factors involved in stress tolerance, we sought to isolate *K. lactis* genes that reverse cadmium and H_2O_2 toxicity in *S. cerevisiae* lacking functional *YAP1* and *YAP2* genes. A *K. lactis* genomic library, cloned in the multicopy shuttle vector pSK1 (Prior et al. 1993), was used to transform a Δ*yap1*Δ*yap2* strain of *S. cerevisiae*. Out of 30 000 transformants screened, a total of 26 cadmium-resistant clones were isolated that displayed strong growth on plates containing 300 μM $CdCl_2$, whereas no colonies were found on plates containing 3 mM H_2O_2. These cadmium-resistant transformants were further analyzed by examining whether or not they were able to grow on SD plates containing cycloheximide, *o*-phenanthroline or H_2O_2. All clones were found to allow growth at drug concentrations that are normally lethal for the wild-type strain, W303α (Table 1). Similarly, H_2O_2 toxicity was partially suppressed in all transformants. Based on these observations, plasmids extracted from one clone, designated pSY1, were transformed into *E. coli* and reintroduced into the *S. cerevisiae* strain W303α and the isogenic strains lacking *YAP1, YAP2* or both *YAP1* and *YAP2*. The degree of resistance to toxic compounds conferred by pSY1 in these strains was compared to that mediated by overexpression of *YAP1* and *YAP2*. As shown in Table 1, the presence of multiple copies of pSY1 confers, albeit to a lesser extent, the same multiple chemical resistance to *S. cerevisiae* as does overexpression of *YAP1*. Moreover, pSY1 partially suppresses the H_2O_2 sensitivity of a *yap1* disruptant, which *YAP2* overexpression does not achieve (Hirata et al. 1994).

The *KlYAP1* gene encodes a bZip protein

Transposon insertional mutagenesis of plasmid pSY1 was used to localize the region of the clone essential for conferring cadmium resistance. All of the insertions that abolished the phenotypic complementation of Δ*yap1*-Δ*yap2* were located in a 2.5-kb region of the *K. lactis* fragment. Subcloning and sequence analysis of this DNA segment revealed a single ORF designated *KlYAP1* (accession number GenBank AF006499). From the sequence data, we designed *KlYAP1*-specific

Table 1 Pleiotropic drug resistance conferred on *Saccharomyces cerevisiae* by overexpression of *YAP1*, *YAP2*, and *KlYAP1*

Strain[a]	H_2O_2 (mM)		Cyh (μg/ml)		Cd (μM)		Phe (μg/ml)	
	3	4	0.5	1	0.15	0.3	15	20
wt	++	+	–	–	+	+/–	+	–
wt/2μ*YAP1*	++	+	++	++	++	++	++	++
wt/2μ*YAP2*	++	+	++	+	++	++	++	+
wt/pSY1	++	+	++	+	++	+	++	+/–
Δ*yap1*	–	–	–	–	–	–	+	–
Δ*yap1*/2μ*YAP2*	–	–	++	+	++	++	++	+
Δ*yap1*/pSY1	+	–	++	+	++	+	++	+/–
Δ*yap2*	+	–	–	–	+	+/–	+/–	–
Δ*yap1*Δ*yap2*	–	–	–	–	–	–	+/–	–
Δ*yap1*Δ*yap2*/pSY1	+	–	++	+	++	+	++	+/–

[a] Wild-type (*wt*, W303α) and the isogenic Δ*yap1*, Δ*yap2*, and Δ*yap1*Δ*yap2* strains were transformed with *YAP1* or *YAP2* carried by pGM26 (high-copy-number plasmid) and the *KlYAP1*-containing plasmid pSY1. Resistance of the transformants was evaluated after 2–4 days of growth on YPD containing the indicated dose of H_2O_2, cycloheximide (*Cyh*), cadmium (*Cd*) or *o*-phenanthroline (*Phe*). The phenotypes are classified as: ++ normal growth, + intermediate growth, +/– slight growth, – no growth

oligonucleotides and performed PCR amplification experiments on pSY1 and plasmids from the 25 other cadmium-resistant clones isolated from the initial screening. The same PCR product was obtained in each case, indicating that all plasmids tested carried *KlYAP1*, a result consistent with the finding that they conferred the same pleiotropic resistance phenotype on *S. cerevisiae*.

Examination of the *KlYAP1* ORF indicates that it can encode a polypeptide of 583 amino acids with features characteristic of bZip proteins (Fig. 2). The bZip motif is located in the N-terminal region and is classically composed of clusters of basic amino acids separated by alanine residues (Busch and Sassone-Corsi 1990). Immediately adjacent to this putative DNA-binding domain lies a leucine zipper dimerization motif characterized by a repetition of five occurrences of a leucine every seventh residue, with an unusual substitution of the expected third leucine by a serine. A sequence comparison in protein data banks revealed homologies between Klyap1p and several members of the bZip family. The three best alignments were obtained with the *S. cerevisiae* Yap1p and Yap2p (43% and 29.3% identity, respectively) and the *S. pombe* pap1 (28.3% identity; Toda et al. 1991) proteins. The sequence similarity is centered on the bZip domain, and also extends towards the C-terminal portion of the proteins (Fig. 2), which is the region suggested to be required for Yap1p stability (Wemmie et al. 1994a).

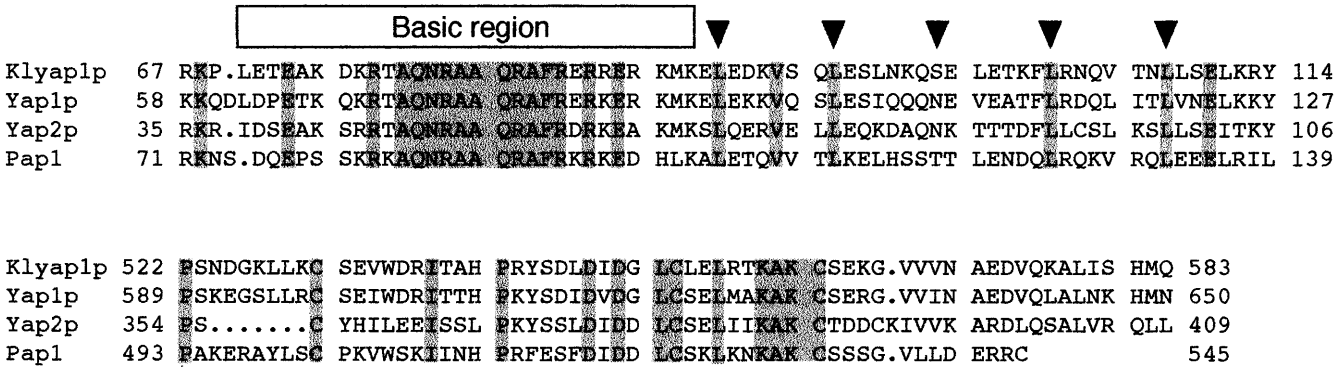

Fig. 2 Sequence similarity between Klyap1p and other bZip proteins. Related regions in Klyap1p, Yap1p, Yap2p, and Pap1 corresponding to the bZip motif (*upper lines*) and the C-terminal region (*lower lines*) were aligned with the aid of the PILEUP program (GCG package). Identical residues are shown by *shading*. The *box* indicates the basic domain, and the leucine residues of the leucine zipper are marked with *triangles*

Stress-induced *KlYAP1*-dependent transcription

It is well established that Yap1p is a DNA-binding protein that can activate transcription in an ARE-dependent manner (Harshman et al. 1988; Moye-Rowley et al. 1989). This activity is stimulated in the presence of oxidants (Kuge and Jones 1994). Considering the structural and functional resemblances between Yap1p and Klyap1p, we tested the possibility that *KlYAP1* encodes an ARE-dependent activator. For this purpose, the *KlYAP1* gene was cloned in a centromeric plasmid and introduced into either wild-type *S. cerevisiae* cells or an isogenic Δ*yap1* strain, both of which contain an ARE–*CYC1*–*lacZ* fusion. The expression of this gene fusion was then determined following growth in the presence or absence of 0.4 mM H_2O_2 (Fig. 3). While

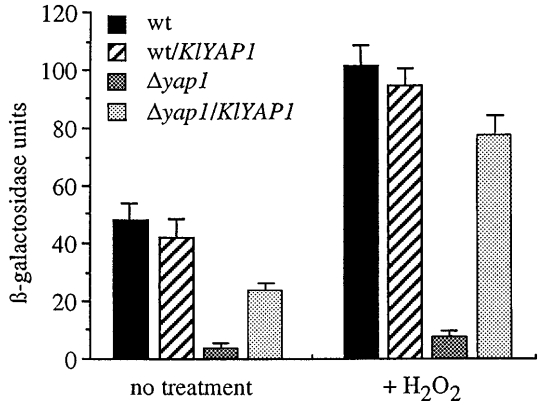

Fig. 3 *KlYAP1*-dependent transcriptional activation of an ARE–*CYC1–lacZ* reporter gene fusion in *S. cerevisiae*. Cultures of W303 and the isogenic Δ*yap1* strains containing the gene fusion plasmid pEPC11 (ARE–*CYC1–lacZ*) were transformed or not with the centromeric plasmid pCY193 bearing *KlYAP1*. Transformants were grown aerobically to exponentially phase (approximate $A_{600} = 1$) and then treated for 1 h with 0.4 mM H_2O_2 or not treated. Cells were harvested and *β*-galactosidase activity was determined. The data shown are the mean of three independent experiments

little or no effect of Klyap1p was observed on *β*-galactosidase activity in a wild-type background, Klyap1p was found to strongly stimulate *lacZ* expression in the Δ*yap1* strain. This level of expression was further increased threefold by H_2O_2 treatment. These results indicate that Klyap1p behavior in *S. cerevisiae* is similar to that of Yap1p and that it can mediate ARE-dependent transcriptional activation induced by H_2O_2 in the heterologous species.

Disruption and overexpression of *KlYAP1*

Since *KlYAP1* is homologous to, and can complement the activity of, the *S. cerevisiae YAP1* gene, we wished to determine whether they performed similar physiological roles in the cell. To investigate this possibility, we constructed a *klyap1*::*LEU2* disruptant and analyzed the phenotype of the mutant. The *klyap1* disruptant was viable and grew normally on both rich and minimal media, indicating that *KlYAP1* is not essential for growth under these conditions. Since the *S. cerevisiae YAP1* gene has been shown to be involved in stress response, particularly in oxidative stress and resistance to heavy metals and ion chelators, we examined the effect of *KlYAP1* gene dosage on the sensitivity to H_2O_2, cadmium, and *o*-phenanthroline. Centromeric and multicopy plasmids bearing *KlYAP1* were introduced into wild-type or Δ*klyap1* strains, and the ability of each transformant to grow in the presence of varying amounts of the toxic compounds was assessed. As shown in Fig. 4, inactivation of *KlYAP1* rendered cells hypersensitive to H_2O_2 and cadmium, while *o*-phenanthroline sensitivity remained unchanged compared to that of the wild-type strain. Conversely, overproduction of Klyap1p from the pSY3 plasmid in a wild-type

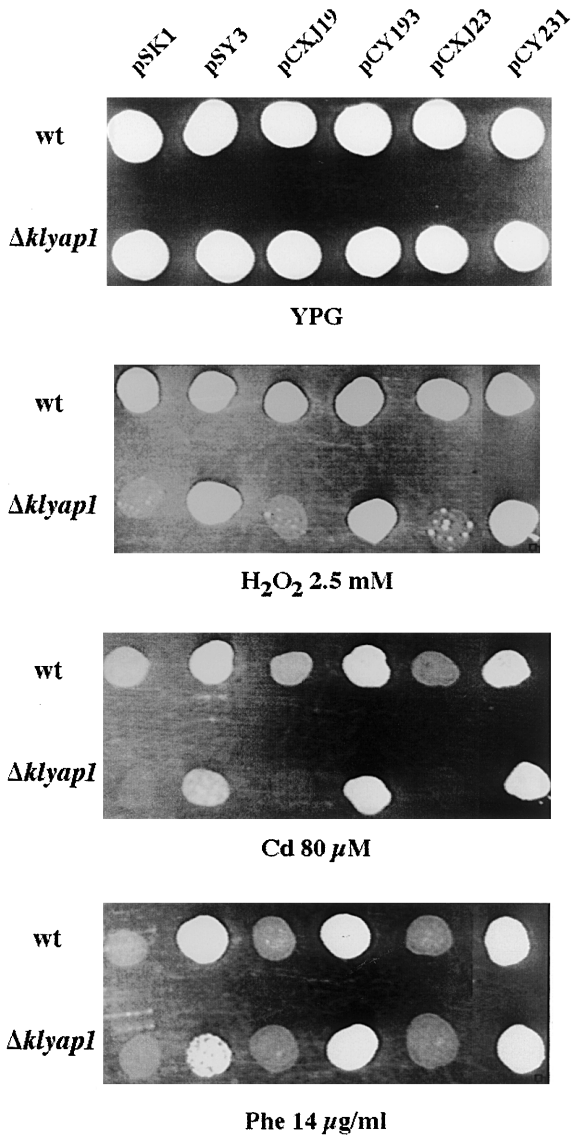

Fig. 4 Effect of the *KlYAP1* gene dosage on drug resistance. Equal amount of cells from exponentially growing cultures ($A_{600} = 1$) of the wild-type strain MW179-1D (*wt*) and the isogenic Δ*klyap1* strain (Δ*klyap1*) transformed with centromeric and multicopy plasmids with or without the *KlYAP1* gene were spotted on YPG plates containing no drug (*YPG*), 2.5 mM H_2O_2 (*H_2O_2*), 80 μM cadmium (Cd) or 14 μg/ml *o*-phenanthroline (*Phe*)

background led to the acquisition of elevated levels of cadmium and *o*-phenanthroline resistance, but had no effect on H_2O_2 resistance. A similar effect was observed in a Δ*klyap1* background. Moreover, expression of the *KlYAP1* gene containing a 400-bp promoter region from a centromeric plasmid efficiently complements all the phenotypes associated with the *KlYAP1* deletion.

To assess the possibility of transcriptional regulation of the *KlYAP1* gene, Northern blot analysis was performed on RNA isolated from unexposed strains and strains pretreated with 0.4 mM H_2O_2. This analysis indicated that *KlYAP1* is transcribed as a 2.4-kb mRNA

that is not induced by H_2O_2. As expected, this mRNA was not detected in a Δ*klyap1* strain (data not shown).

Effect of the *KlYAP1* gene on the response to oxidative stress

Having found that a Δ*klyap1* strain is highly sensitive to H_2O_2, we examined whether the mutation also affected sensitivity to other oxidants. Cultures of the wild-type strain and the Δ*klyap1* mutant were plated out and challenged with either H_2O_2 or the superoxide anion-generating agent menadione. As shown in Table 2, both H_2O_2 and menadione inhibited growth of the Δ*klyap1* strain more severely than that of the wild type. These plate tests confirmed that the *KlYAP1* gene is implicated in resistance to oxidative compounds.

To gain further insight into the relationship between *KlYAP1* and oxidative stress, we examined whether or not a H_2O_2 and menadione adaptive response exists in *K. lactis*, and the possible role of Klyap1p in this response. Exponentially growing cultures of the wild-type and Δ*klyap1* strains were first exposed to various concentrations of either H_2O_2 or menadione. As expected from the test plate observations, the Δ*klyap1* mutant was clearly more sensitive than the wild type to both H_2O_2 and menadione (Fig. 5A). However, hypersensitivity of Δ*klyap1* was observed only for concentrations of between 0.2 and 2 mM H_2O_2, whereas it was diminished at all levels of menadione used. Also, we noticed that *K. lactis* shows much greater resistance to superoxide anions than does *S. cerevisiae* (Jamieson 1992; Flattery-O'Brien et al. 1993). Strains were also treated with non-lethal doses of H_2O_2 (0.2 mM) or menadione (1 mM) and subsequently challenged with higher levels of the same oxidants. Such pretreatments rendered wild-type cells highly resistant to the lethal stress (Fig. 5A), thus demonstrating the existence of both H_2O_2 and menadione adaptive stress responses in *K. lactis*. However, the protective effect provided by the H_2O_2 adaptive stress response was completely abolished in a Δ*klyap1* mutant, while the Δ*klyap1* mutation had no detectable effect on the inducibility of the adaptive protective stress response towards superoxide anions.

The role of mitochondrial respiratory functions in induction of the response to oxidants was also investigated by determining whether cells grown on glucose in the presence of antimycin could still adapt to H_2O_2 and menadione. Although antimycin treatment drastically reduced the level of resistance to both oxidants, cells were still able to show an adaptive response (data not shown). This suggests that respiratory functions contribute to the protection against oxidants, but are not absolutely necessary to its induction.

We further examined the resistance of stationary-phase cultures towards both H_2O_2 and superoxide anions. Wild-type and Δ*klyap1* strains were grown on complete glucose medium to saturation and survival was measured following exposure to lethal doses of each oxidant. Under these conditions, wild-type cells became extremely resistant to both H_2O_2 and menadione (Fig. 5B). Surprisingly, a similar increased resistance was observed in the Δ*klyap1* strain, indicating that *KlYAP1* does not play a significant role in stationary phase-mediated resistance against oxidative stress.

Table 2 Influence of the *KlYAP1* gene on sensitivity to oxidant agents

Strain	Diameter of zone of growth inhibition (cm)[a]	
	H_2O_2	Menadione
wt	2.8	3.0
Δ*klyap1*	5.5	4.0

[a] Zone-inhibition assays were performed on the wild type (*wt*) MW179-1D and the isogenic *klyap1* mutant (Δ*klyap1*) strains

Discussion

It is widely accepted that the yeasts *K. lactis* and *S. cerevisiae* are closely related to one another, especially considering the sequence similarity of many of their genes so far analyzed. However, each displays differences in lifestyle and metabolism that suggest the existence of differences in their regulation of cellular physiology. Strikingly, despite the presence of sometimes low sequence similarity, most of the genes of *K. lactis* encoding transcriptional regulators have been successfully cloned by in vivo complementation of *S. cerevisiae* mutants: *GAL4* (Salmeron and Johnston 1986), *ABF1* (Goncalvés et al. 1992), transcription factor IIB (Na and Hampsey 1993), *CPF1* (Mulder et al. 1994a), *HAP3* (Mulder et al. 1994b), *HAP2* (N'Guyen et al. 1995). The apparent ability of homologous *K. lactis* and *S. cerevisiae* genes to cross-complement may therefore be exploited to isolate and study new regulatory mechanisms in *K. lactis*.

In this work, we have identified a gene of *K. lactis*, designated *KlYAP1*, able to complement, at least in part when expressed from a multicopy plasmid, the phenotypes of a *S. cerevisiae* Δ*yap1*Δ*yap2* mutant: hypersensitivity to both cadmium and H_2O_2. Such phenotypes have been attributed to the requirement of the Yap1p protein for the transcription of a set of stress-protective genes (see Ruis and Schüller 1995; Moradas-Ferreira et al. 1996 and references herein). We have also found that overexpression of the *KlYAP1* gene in a wild-type strain improved the tolerance of the cells towards cycloheximide and *o*-phenanthroline, a phenotype reminiscent of the pleiotropic drug resistance conferred by overexpression of either *YAP1* or *YAP2*. These results, together with the structural similarities observed between the *YAP1* and *KlYAP1* gene products, suggest that Klyap1p fulfills in *S. cerevisiae* the same physiological role as Yap1p and consequently activates expression of at least some of the same target genes. The possibility that Klyap1p might replace Yap2p rather

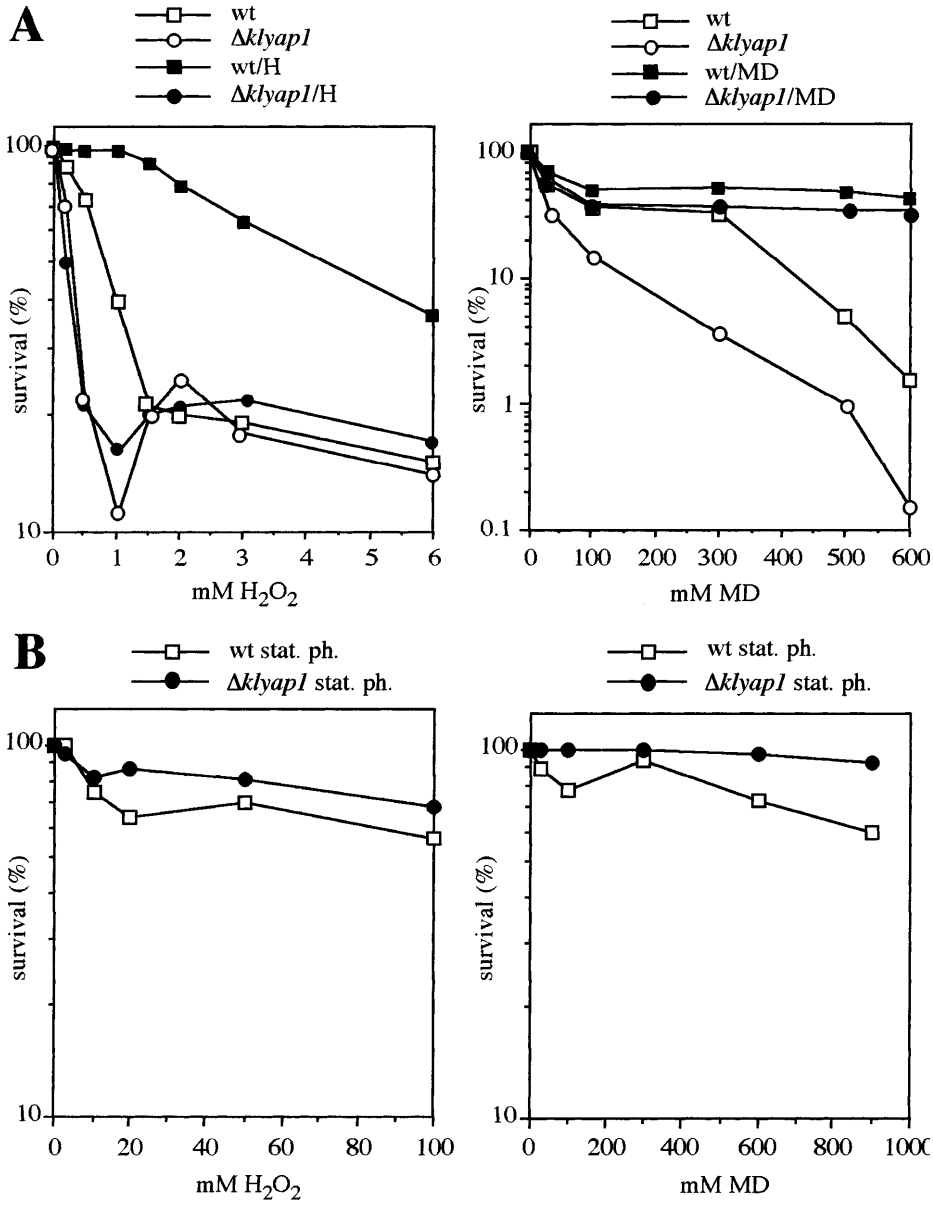

Fig. 5 Sensitivity to H_2O_2 and menadione of adapted or non-adapted exponential cultures **(A)** and stationary-phase cultures **(B)** of strain MW179-1D and the isogenic Δ*klyap1* mutant. **A** Exponentially growing cells at an A_{600} of 0.15 were not pretreated (*open symbols*) or were pretreated (*closed symbols*) with 0.2 mM H_2O_2 (*/H*) or 1 mM menadione (*/MD*) and then exposed to increasing levels of either H_2O_2 or menadione. **B** Cells were grown aerobically to saturation and diluted aliquots were directly challenged with increasing levels of either H_2O_2 or menadione. The data shown are the means of two independent experiments, standard deviation is less than 10%

than Yap1p was ruled out by its ability to suppress the H_2O_2 sensitivity of a *yap1* disruptant, suppression that does not occur when *YAP2* is overexpressed.

The *KlYAP1* gene product displays all the structural features of the Jun-type family of proteins in yeasts, composed of the *S. cerevisiae* Yap1p and Yap2p and *S. pombe* pap1 proteins. All of them possess almost identical bZip motifs localized in the N-terminal regions, together with another conserved domain at the C-terminus which has been proposed to stabilize the Yap1p protein (Wemmie et al. 1994a). An interesting feature of the leucine zipper found in these proteins is the replacement of the canonical third leucine by either a threonine, an asparagine or a serine residue in pap1, Yap1p and Yap2p, and Klyap1p, respectively. The functional significance of such a replacement is, at present, unknown.

Evidence that *KlYAP1* encodes the functional homolog of Yap1p was provided by its ability to promote the transcriptional activation of an ARE–*CYC1*–*lacZ* fusion in *S. cerevisiae*. The level of activation is quite similar to that mediated by the genomic copy of YAP1 a result that can explain the heterologous complementation of the Δ*yap1*Δ*yap2* mutant strain. Importantly, we have shown that, even in a heterologous context, Klyap1p-mediated ARE-dependent transcription was increased threefold in the presence of H_2O_2. Such stress-induced activation has been reported for both Yap1p and Yap2p, but the mechanism of activation is still unclear, although phosphorylation is likely to be involved (Hirata et al. 1994; Kuge and Jones 1994). We show here that the regulation of Klyap1p activity in *K. lactis* does not appear to occur at the transcriptional level. We also examined the 5′-untranslated region of

KlYAP1 for the presence of small ORFs (uORF). Both *YAP1* and *YAP2* genes possess a small uORF in their 5′regions (Moye-Rowley et al. 1989; Bossier et al. 1993), suggesting that their expression might be controlled at the translational level, as is the case with the *GCN4* gene (Hinnebusch 1984). However, no such uORF was found in the 5′-untranslated region of *KlYAP1*. It is therefore likely that H_2O_2-induced activation of *KlYAP1* occurs by a post-transcriptional modification such as phosphorylation, or by direct modification of the redox status of Klyap1p, as is the case for the transcriptional activator oxyR of *E. coli* (Farr and Kogoma 1991). Attempts to demonstrate the *trans*-acting activity of Klyap1p in *K. lactis* using an ARE–*RAG1*–*lacZ* fusion (see Materials and methods) were unsuccessful, and will require the identification of a target gene in order for further conclusions to be drawn.

Variation in *KlYAP1* gene dosage produced essentially similar phenotypes to those described for *YAP1* gene dosage in *S. cerevisiae*. A Δ*klyap1* strain became highly sensitive to H_2O_2, menadione, and cadmium, whereas overproduction of Klyap1p led to the acquisition of elevated levels of cadmium and *o*-phenanthroline resistance. Furthermore, investigations revealed the existence in *K. lactis* of an adaptive response to both H_2O_2 and menadione – not surprisingly in view of the number of aerobic organisms in which such adaptive mechanisms have been described. Here we show that Klyap1p plays an essential role in the H_2O_2 adaptive stress response but does not affect the menadione-mediated adaptive stress response, a situation reminiscent of that observed with both the *YAP1* and *YAP2* genes in *S. cerevisiae*. However, the two systems appear to be somewhat different. First, hypersensitivity of a Δ*klyap1* strain was observed only for concentrations between 0.2 and 2 mM H_2O_2. This result suggests the existence of at least one Klyap1p-independent mechanism of protection against H_2O_2 that may be induced in conditions of high oxidative stress. In *S. cerevisiae*, glutathione reductase activity has recently been demonstrated to increase upon entry into stationary phase, and a strain deleted for *GLR1*, encoding glutathione reductase, becomes highly sensitive to H_2O_2 during stationary phase. The stationary phase induction of *GLR1* expression was found to be entirely dependent upon Yap1p (Grant et al. 1996b). The situation in *K. lactis* differs, in that Klyap1p does not appear to markedly affect stationary phase-mediated resistance against H_2O_2 stress, indicating a potentially different glutathione metabolism in this yeast or the existence of some other protective mechanism(s). Obviously, this does not exclude the possibility that stationary-phase induction of specific genes is Klyap1 dependent.

Altogether, our results suggest that the *KlYAP1* gene plays a similar, but not identical, role in *K. lactis* to that filled by *YAP1* in *S. cerevisiae*. Although a direct demonstration is presently lacking, our current hypothesis is that the phenotypes produced by variation of *KlYAP1* gene dosage in the cell are a direct consequence of the Klyap1p-dependent transcriptional regulation of a set of stress genes in the cell. Whether the target genes are the same as in *S. cerevisiae* remains to be established.

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PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression

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Summary

A novel member of the poly(ADP-ribose) polymerase (PARP) family, hPARP-3, is identified here as a core component of the centrosome. hPARP-3 is preferentially localized to the daughter centriole throughout the cell cycle. The N-terminal domain (54 amino acids) of hPARP-3 is responsible for its centrosomal localization. Full-length hPAPR-3 (540 amino acids, with an apparent mass of 67 kDa) synthesizes ADP-ribose polymers during its automodification. Overexpression of hPARP-3 or its N-terminal domain does not influence centrosomal duplication or amplification but interferes with the G1/S cell cycle progression. PARP-1 also resides for part of the cell cycle in the centrosome and interacts with hPARP-3. The presence of both PARP-1 and PARP-3 at the centrosome may link the DNA damage surveillance network to the mitotic fidelity checkpoint.

Key words: Centrosome, NAD^+ metabolism, DNA damage, G1/S cell cycle control, Midbody

Introduction

Poly(ADP-ribosyl)ation is an immediate post-translational modification of nuclear proteins induced by DNA damaging agents. At a site of DNA breakage, the enzymes poly(ADP-ribose) polymerase-1 (PARP-1, 113 kDa) and PARP-2 (62 kDa) catalyze the transfer of the ADP-ribose moiety from the respiratory co-enzyme NAD^+ to a limited number of acceptor proteins involved in chromatin architecture (histones H1, H2B, HMG proteins, lamin B and nucleolar proteins such as B23) and in DNA metabolism (DNA replication factors and topoisomerases including PARP-1) (D'Amours et al., 1999; de Murcia and Ménissier de Murcia, 1994; de Murcia and Shall, 2000). Poly(ADP-ribosyl)ation of these proteins establishes de facto a molecular link between DNA damage and chromatin modification and appears as an obligatory step in a detection/signaling pathway leading ultimately to the resolution of strand interruptions (Shall and de Murcia, 2000).

A superfamily of PARP-domain-containing proteins has recently emerged (Amé et al., 1999; Jacobson and Jacobson, 1999; Smith, 2001). From this family, PARP-1 and PARP-2 were until now the only characterized enzymes whose activity has been shown to be stimulated by DNA strand breaks (Amé et al., 1999; Schreiber et al., 2002). Acting as survival factors in mammalian cells under genotoxic stress, they are localized in the nucleus and possibly function as homo and/or heterodimers. Murine fibroblasts carrying a targeted disruption of either the *mPARP-1* or *mPARP-2* gene are defective in base excision repair (BER), indicating that PARP-1 and PARP-2 can reciprocally, but partially, compensate for the absence of each other (Schreiber et al., 2002).

VPARP (vault-PARP, PARP-4) was characterized through its interaction with the MVP (major vault protein) in yeast in a two-hybrid screen. Vault particles are large ribonucleoprotein complexes found in the cytoplasm of mammalian cells (Kickhoefer et al., 1999), which may have a transport function. VPARP poly(ADP-ribosyl)ates MVP in purified vaults, but the consequences of this modification on vaults properties remain elusive. Tankyrase-1 (PARP-5a) was initially identified through its interaction with the telomeric protein TRF1, a negative regulator of telomere length (Smith et al., 1998b). In vitro poly(ADP-ribosyl)ation by tankyrase-1 inhibits TRF1 binding to telomeric DNA, suggesting a role for tankyrase-1 in telomere function (Smith and de Lange, 2000). Overexpression of tankyrase-1 has recently been found to release TRF1 from telomeres, thus inducing their elongation (Smith and de Lange, 2000). Tankyrase-2 (PARP-5b) appears to interact with many partners at discrete subcellular locations, including the Golgi complex (Chi and Lodish, 2000) and endosomes (Lyons et al.,

2001). It also displays telomeric functions while interacting with tankyrase-1 (Cook et al., 2002). During mitosis VPARP and tankyrase-1 are located at the mitotic spindle (Kickhoefer et al., 1999) and in the pericentriolar region, respectively (Smith and de Lange, 1999).

A sequence encoding a 60 kDa protein with homology to PARP-1 and 2, called PARP-3, was previously discovered in an EST library screening using the catalytic domain sequence of hPARP-1 (Johansson, 1999). Using the same approach, we cloned a different human cDNA encoding a version of PARP-3 that was seven amino acids longer. Here we report that recombinant hPARP-3 is endowed with PARP activity in vitro. During the entire cell cycle, hPARP-3 is localized to the centrosome, the microtubule organising centre of animal cells, and resides preferentially in the daughter centriole. Given the potential role of centrosomes in cell cycle regulation, we tested whether hPARP-3 participates in the regulation of cell cycle checkpoints. Our results argue that hPARP-3 negatively influences the G1/S cell cycle progression without interfering with centrosome duplication. Moreover, we found that hPARP-3 interacts with hPARP-1, which was previously shown to be present at the centrosome as well (Kanai et al., 2000).

Materials and Methods

EST searches and isolation of cDNA clones

A human PARP-3 EST (#1889095) was identified in the LIFESEQ™ database of Incyte Pharmaceuticals using the human PARP-1 catalytic domain sequence for a database search. This EST was used to screen a human frontal cortex cDNA library cloned in UNI ZAP XR (Stratagene). The selected clone used in this study contained an insert of 2108 bp starting 94 nucleotides upstream of the ATG codon and covering the complete 1599 bp open reading frame encoding hPARP-3.

Fluorescence in situ hybridization (FISH) analysis

Human chromosomes were prepared from human peripheral blood lymphocyte cultures after BrdU incorporation during the last 7 hours before harvesting. Mouse chromosomes were prepared from normal mouse fibroblast cultures without BrdU incorporation. The full-length cDNA sequence encoding the *hPARP-3* gene and a 2.8 kb mouse *PARP-3* genomic clone (to be described elsewhere) were labelled by nick-translation with biotin-11-dUTP. 20 ng/μl hPARP-3 probe was hybridized to human chromosomes in hybridization buffer; the mouse PARP-3 probe was hybridized to mouse chromosomes at a concentration of 15 ng/μl, as previously described (Apiou et al., 1996). Detection of hybridization was performed using a goat anti-biotin antibody (Vector Laboratories, Peterborough, UK) and a rabbit anti-goat fluorescein isothiocyanate-conjugated antibody (P.A.R.I.S., Compiègne, France). Direct banding of 5-BrdU-substituted chromosomes stained with propidium iodide was obtained by incubation in an alkaline solution of p-phenylenediamine (PPD11) (Lemieux et al., 1992). Mouse chromosomes were stained with DAPI and identified by computer-generated reverse-DAPI banding. Metaphases were observed under a fluorescent microscope (DMRB, Leica, Germany). Images were captured using a cooled Photometrics CCD camera and Quips-smart capture software (Vysis).

Overproduction and purification of h PARP-3

hPARP-3 cDNA was cloned into the baculovirus transfer vector pFASTBAC1 (Life Technologies, Invitrogen, Cergy Pontoise, France). Sf9 cell propagation and protein production was performed according to Miranda et al. (Miranda et al., 1997). Purification of hPARP-3 was performed by affinity chromatography on Affigel-3-Aminobenzamide as previously described (Amé et al., 1999; Giner et al., 1992).

Plasmids

cDNA encoding hPARP-3 or its N-terminal domain (54 amino-acids) were cloned either into the eukaryotic expression vectors pBC (Chatton et al., 1995) in frame with GST, giving raise to pBC hPARP-3 and pBC N-ter hPARP-3, or into the pEGFP vector (Clontech), giving raise to pEGFP-hPARP-3 and pEGFP N-ter hPARP-3.

Poly(ADP-ribosyl)ation assay, western blot, south-western blot and GST pulldown

900 ng of purified hPARP-3 were incubated in 20 μl of buffer containing 100 mM Tris-HCl, pH 8.0, 10 μM NAD^+ and 10 μCi [^{32}P] NAD^+ (3 Ci/mmoles). After 15 minutes of incubation at 25°C, the reaction was stopped by dilution in the Laemmli buffer. Samples were analyzed on a 10% SDS-PAGE and blotted as described previously (Mazen et al., 1989) and autoradiographed on Kodak BiMax MS film. South-western blotting using [^{32}P]-labelled nick-translated activated DNA was performed as described previously (Mazen et al., 1989).

Two peptides matching the hPARP-3 N-terminal region (amino-acid 25-37 and 8-22) were used to elicit hPARP-3 polyclonal antibodies in rabbits (Ab 1650) and in mice (TJ56), respectively. Potential partners of hPARP-3 were isolated using the GST-pulldown technique described previously (Masson et al., 1998).

Cell culture and cell cycle analysis

Mammalian cell cultures were maintained at 37°C in 5% CO_2. The human lymphoblastic KE 37 cell line (Moudjou and Bornens, 1994) was grown in suspension in RPMI 1640 medium supplemented with 7% foetal calf serum (FCS) (GIBCO) and gentamicin. HeLa, CHO, 3T3 and HeLa HC1 cells stably expressing the fusion GFP-centrin (Piel et al., 2000) were cultured in DMEM containing 10% FCS and gentamicin. For cell cycle analysis, HeLa cells were transiently transfected by plasmids expressing GST alone or in fusion with hPARP-3 or N-ter hPARP-3. After 24 hours, cells were mock-treated or treated with 1 mM N-methyl N-nitrosourea (MNU) for 20 hours and trypsinized. Cell suspensions were washed twice in PBS containing 1% glucose, 1 mM EDTA and fixed with cold 70% ethanol in PBS for 2 hours. The fixed cells were then washed once with PBS, 1% glucose, 1 mM EDTA, once with PBS, 1% glucose, 1 mM EDTA, 0.1% Triton X100 and incubated for 45 minutes at room temperature with a monoclonal anti-GST antibody diluted 1:400. Cells were washed twice with PBS, 1% glucose, 1 mM EDTA, 0.1% Triton X100 and incubated with FITC-conjugated anti-mouse antiserum (Cappel) for 45 minutes at room temperature. After a last wash with PBS, 1% glucose, 1 mM EDTA, cells were incubated with 100 μg/ml RNaseA for 30 minutes at room temperature, stained with propidium iodide and analyzed with a FACScan flow cytometer using a gate on FITC-positive cells.

Isolation of centrosomes

Purified centrosomes were isolated from KE 37 cells according to the method described by Moudjou and Bornens (Moudjou and Bornens, 1994). The centrosome fractions were analyzed by immunofluorescence as described previously (Bornens and Moudjou, 1999).

Indirect immunofluorescence

Cells (5×10^4) grown on coverslips were transfected or not with the plasmids expressing GST- or GFP-fusion proteins and subsequently untreated or treated with various DNA-damaging agents, using either

γ-irradiation (10 Grays, 1.0 Gy/minute delivered by a ^{60}Co source) or a treatment with 1 mM N-methyl-N-nitrosourea (MNU) for 30 minutes or 1 mM H_2O_2 for 10 minutes. Amplification of centrosomes was evaluated in CHO cells treated with 4 mM Hydroxyurea (HU) for 48 hours prior to processing for immunochemistry (Meraldi et al., 2002; Meraldi et al., 1999). Following fixation with 100% methanol for 5 minutes at –20°C, cells were washed three times with PBS supplemented with 0.1% Tween (v/v). Cells were incubated overnight at 4°C or for 2 hours at room temperature, with a primary antibody – the polyclonal antibody anti-hPARP-3 (1650) (1:100), a monoclonal IgG2a antibody anti-p34^{cdc2} (1:200, Sigma), a monoclonal IgG2b antibody anti-acetylated α-tubulin (1:1000, Sigma) or a monoclonal antibody anti-glutamylated tubulin (Gt 335) (1:2000) or a monoclonal antibody anti-hPARP-1 (F1-23, 1:100). After washing, cells were incubated for 2 hours at room temperature with the appropriate conjugated secondary antibody: a Texas-Red-conjugated anti-rabbit antiserum (1:400, Sigma), a sheep FITC-conjugated anti-mouse antiserum (1:400, Sigma) or an Alexa Fluor (568 or 488) goat-anti-mouse IgG (1:1000, Molecular Probes). DNA was counterstained with DAPI. Immunofluorescence microscopy was performed using a Zeiss Axioplan equipped with a DP50 chilled CCD camera (Olympus) and the capture software ViewFinder Lite (Olympus). Alternatively, observations were made with a confocal microscope equipped with an argon/krypton laser and suitable barrier filters (Leica TCS4D, Heidelberg, Germany).

Results

hPARP-3 is a new member of the PARP family

A human PARP-3 EST was isolated using the sequence encoding the human PARP-1 catalytic domain. The human PARP-3 cDNA sequence encodes a 540 amino acid protein that encompasses a 54 amino acid N-terminal domain and a catalytic domain of 489 amino acids that has 39% identity (61% similarity) with the human PARP-1 catalytic domain. The sequence of hPARP-3 can be aligned with the other members of the PARP family (Fig. 1A). All of them contain the PARP signature that characterizes the ADP-ribose donor site (NAD^+) and the essential residues forming the poly(ADP-ribose) acceptor site including a catalytic glutamate (E522). In the hPARP-3 sequence the acceptor site is much less conserved, although it constitutes a part of the sequence that has strongly conserved secondary structure (Fig. 1B).

Analysis of the 5′ end of the human *PARP-3* gene (accession number: GenBank AY126341) revealed the presence of two potential splicing acceptor (AS) sites, AS1 and AS2 (Fig. 1C), which give rise to two proteins differing by seven amino acids at the N-terminus. A sequence of hPARP-3 has been previously published by Johansson (Johansson, 1999) that lacks the first seven N-terminal amino acids. In order to assess the real occurrence of the longest version, we used a PCR strategy to detect the 5′ end of hPARP-3 reverse-transcribed mRNA from normal human lung tissue (Fig. 1D). Primers were designed to match the nucleotide sequence encoding the longest form of hPARP-3 (PCR1). Under high stringency PCR conditions, the sense primer can hybridize only when AS2 is selected. As a PCR control, we used a second sense primer (PCR2) that matches hPARP-3 mRNA downstream of the splicing site whatever the type of mRNA in the cell. As shown in Fig. 1D, the PCR2 control product was 141 bp, as expected, and a unique band of 162 bp was detected in PCR1, which is in good agreement with the presence of the longest *PARP-3* encoding mRNA. Therefore, we concluded that a human PARP-3, which is seven residues longer than the amino-acid sequence published by Johansson (Johansson, 1999), is expressed in human cells. Nevertheless we cannot exclude the possibility that both sequences are present in cells, depending upon physiological conditions.

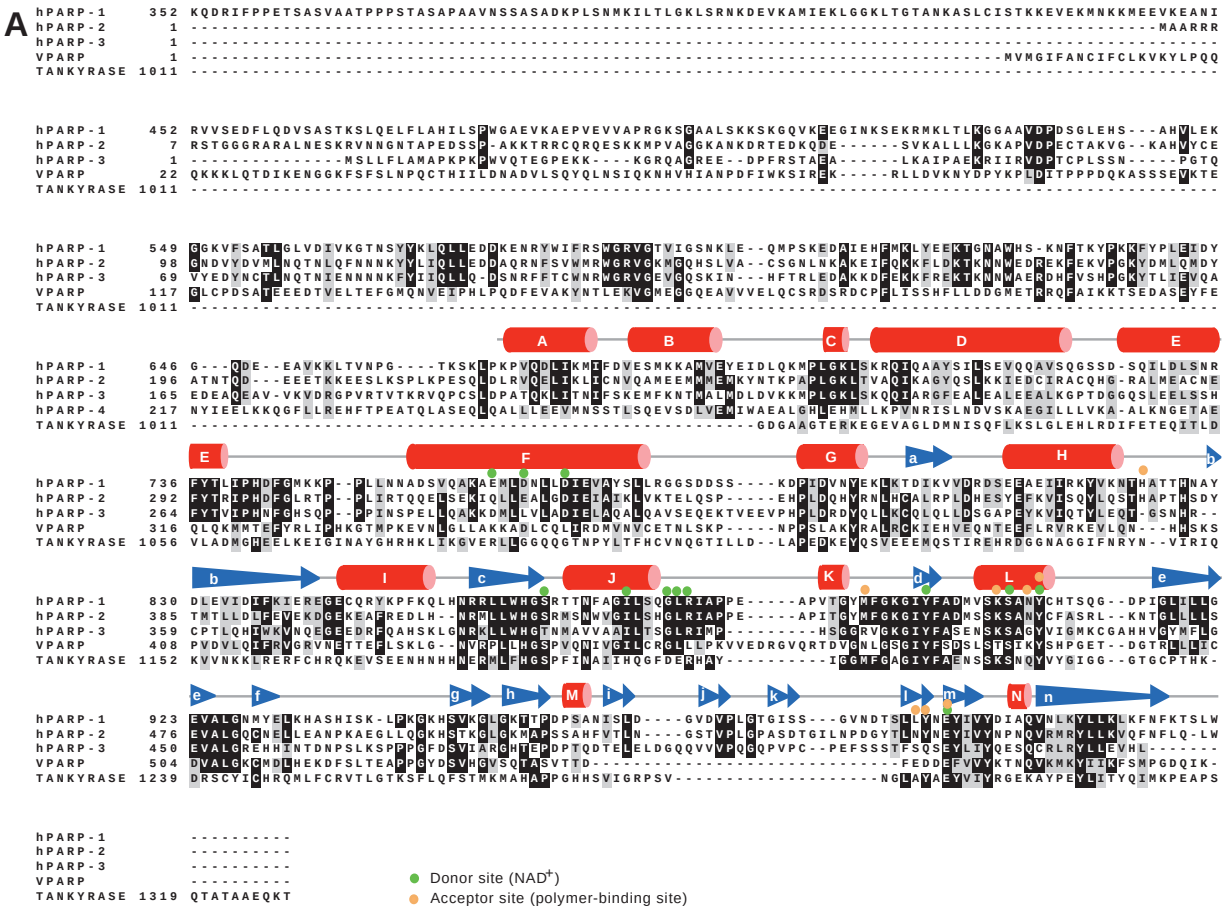
The chromosomal localization of the *hPARP-3* gene was identified by using FISH on human chromosomes using a human cDNA PARP-3 probe. Consistent signals on chromosome 3 band 3p21.1 to 3p21.31 were identified (Fig. 1E) and on chromosome 9, band F1-F2 in mouse (Fig. 1F-G), which confirms that hPARP-3 is a novel member of the PARP family encoded by a specific gene. As it is already the case for the *PARP-1* and *PARP-2* genes, a synteny was noticed between the human and mouse chromosomal regions coding for *PARP-3* genes.

Full-length hPARP-3 was overexpressed in the baculovirus/Sf9 system and purified by affinity chromatography on Affigel-3AB (Giner et al., 1992), yielding a polypeptide with an apparent molecular mass of 67 kDa (Fig. 2A, lane b). We took advantage of the unique N-terminal domain of hPARP-3, which has no counterpart in PARP-1 and PARP-2 to generate two polyclonal antibodies 1650 and TJ56. As shown in Fig. 2B (lanes f to j), anti-PARP-3 antibody 1650 recognizes the recombinant hPARP-3 as well as hPARP-3 in HeLa cell extracts. As expected, these antibodies did not crossreact with either hPARP-1 or hPARP-2 (data not shown).

The ability of hPARP-3 to synthesize ADP-ribose polymers in an autopoly(ADP-ribosyl)ation reaction was examined in an in vitro PARP activity assay using [^{32}P] NAD^+ as a substrate; the optimal concentration of 10 μM NAD^+ was determined. As shown in Fig. 2A (lane d), PARP-3 automodification occurs and is specifically inhibited by the competitive inhibitor 3-Aminobenzamide (3-AB) (lane e). Interestingly, a slight increase in enzymatic activity (two-fold) was repeatedly observed in the presence of nicked DNA, in accord with the capacity of the N-terminal domain to bind DNA in a south-western assay (Fig. 2A, lane c). Altogether, these results clearly indicate that PARP-3 is a bona fide poly(ADP-ribose) polymerase.

hPARP-3 localizes preferentially to the daughter centriole throughout the cell cycle

To establish the subcellular localization of hPARP-3, exponentially growing HeLa cells were stained with the purified hPARP-3 antibody 1650. hPARP-3 localized to two closely spaced dots resembling centrosomes, usually located close to the nuclear envelope. As a control, we performed a double immunofluorescence experiment using the anti-hPARP-3 antibody 1650 and an antibody raised against p34^{cdc2}, as p34^{cdc2} has been previously shown to localize to centrosomes (Bailly et al., 1989; Pockwinse et al., 1997). As shown in Fig. 3A, the immunostaining of hPARP-3 was clearly associated with the centrosomal staining of p34^{cdc2}. To verify this observation, HeLa HC1 cells constitutively expressing the centrosomal protein centrin in fusion with GFP (Piel et al., 2000; White et al., 2000) were immunostained with the anti-hPARP-3 antibody 1650. The colocalization of hPARP-3 with GFP-centrin is clearly visible in Fig. 3B and confirms the association of PARP-3 with the centrosome. A similar result was obtained with the mouse polyclonal TJ56 antibody (data not shown). Staining of centrosomes was independent of the fixation method, as it was observed following procedures based


A
hPARP-1
hPARP-2
hPARP-3
VPARP
TANKYRASE
A
B
C
D
E
F
G
H
a
b
I
c
J
K
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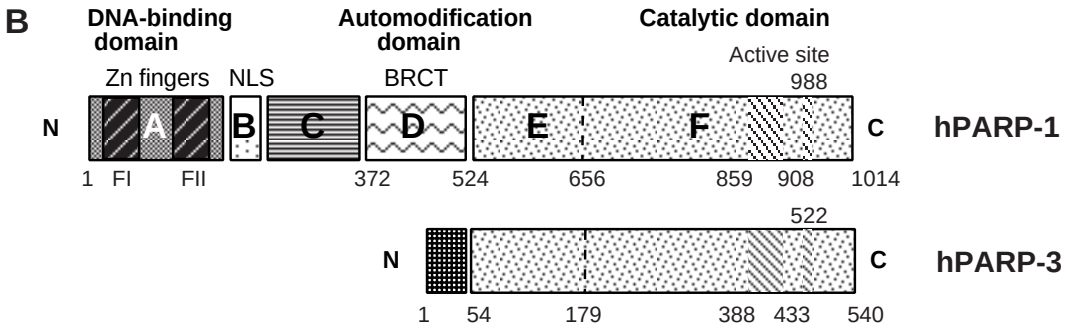
N
n
Donor site (NAD+)
Acceptor site (polymer-binding site)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Zn fingers
NLS
Automodification domain
BRCT
Catalytic domain
Active site
988
N
A
B
C
D
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C
hPARP-1
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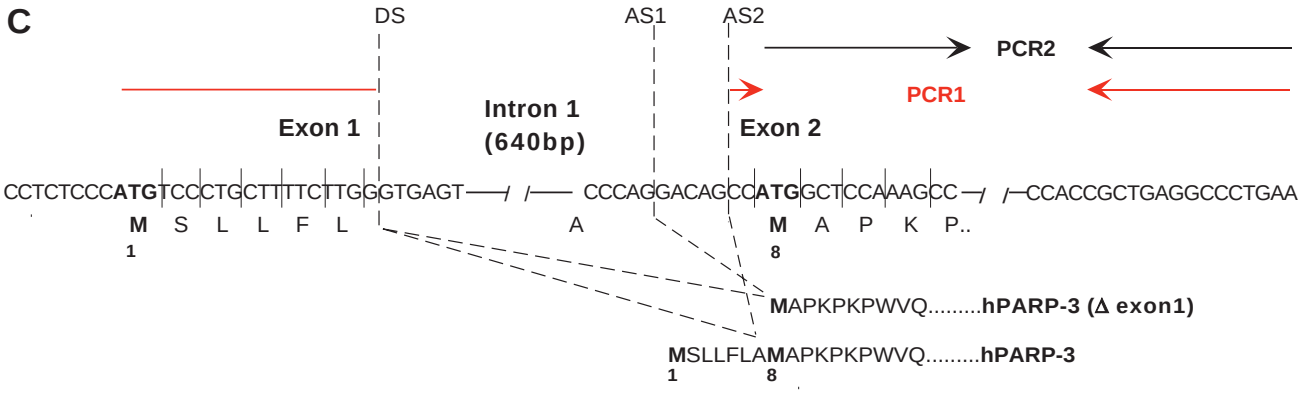
C
hPARP-3
1
54
179
388
433
540


C
DS
AS1
AS2
PCR2
PCR1
Exon 1
Intron 1
(640bp)
Exon 2
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CCACCGCTGAGGCCCTGAA
M S L L F L
A
M A P K P..
1
8
MAPKPKPWVQ.....hPARP-3 (Δ exon1)
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1
8

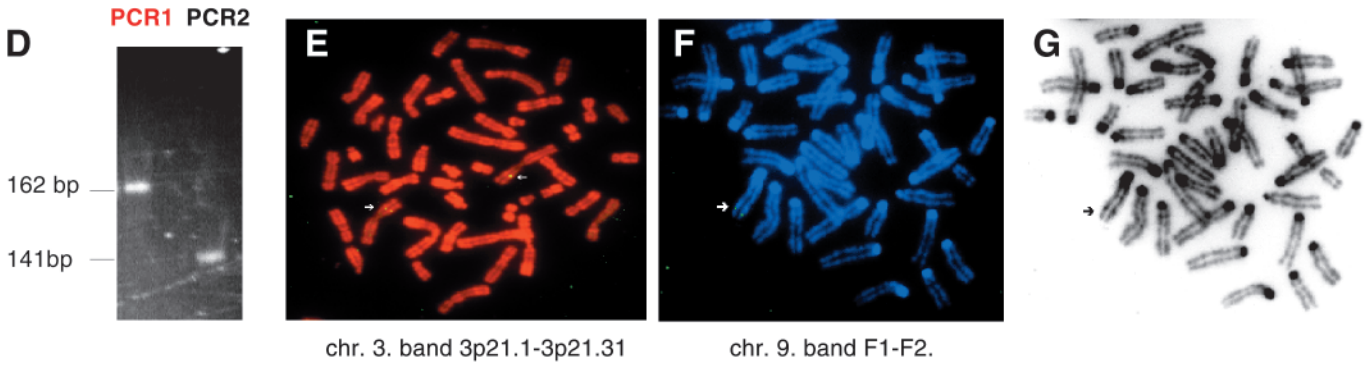

Fig. 1. (A) Sequence alignment of the five first members of the PARP family. Sequence alignment of amino acids 352 to 923 of human PARP-1 [hPARP-1, accession number P09874 (Cherney et al., 1987; Kurosaki et al., 1987; Uchida et al., 1987)], human PARP-2 [hPARP-2, AJ236912, (Ame et al., 1999)], human PARP-3 [hPARP-3, accession number NM_005485 (Johansson, 1999)], vault-particle-associated PARP [VPARP, accession number AF057160 (Kickhoefer et al., 1999)] and tankyrase [accession number AF082556 (Smith et al., 1998b)]. Cylinders and arrows schematically represent α helices and β-strands, respectively, as previously shown in the chicken PARP-1 structure (Ruf et al., 1996). (B) Schematic representation of the functional domains of hPARP-1 and hPARP-3. (C) Structure of the two possible versions of the human PARP-3 gene product by alternative splicing of the first exon. AS, acceptor site; DS, donor site. (D) PCR products loaded on 6% polyacrylamide gel. (E) Chromosomal mapping of *hPARP-3*: FISH of the *hPARP-3* gene on a human lymphocyte chromosome spread (arrows). Chromosomes are counterstained with propidium iodide. (F,G) Chromosomal mapping of mouse PARP-3: FISH of mPARP-3 on a mouse fibroblasts chromosome spread. Chromosomes are counterstained with DAPI. The sequence data of hPARP-3 is available from GenBank/EMBL/DDBJ under accession number AY126341.

on either aldehyde or organic solvent fixation. Finally, cell treatment with a microtubule-depolymerizing drug such as nocodazole or with a microtubule-stabilizing drug such as taxol did not displace the centrosomal signal emerging from the anti-PARP-3 antibodies (data not shown), demonstrating that the localization of hPARP-3 to the centrosome is independent of microtubule dynamics.

Strikingly, a preferential colocalization of hPARP-3 with one of the two centrioles was repeatedly noticed (Fig. 3C). The nature of the two centrioles can be distinguished by using immunocytochemistry in mouse 3T3 cells (Chang and Stearns, 2000; Lange and Gull, 1995). In G1 phase, the mother centriole of these cells grows a primary cilium, which is partially made of an acetylated form of α-tubulin. As shown in Fig. 3D, the signal corresponding to hPARP-3 antibodies cannot be superimposed on the signal from anti-acetylated α-tubulin antibodies, therefore suggesting that hPARP-3 colocalizes preferentially with the daughter centriole. A similar conclusion could be reached from colocalization experiments using HeLa HC1 cells, where the reproducible association of hPARP-3 with the 'smaller' (daughter) centriole was clearly visible (Fig. 3C).

We next examined the stage of the cell cycle that hPARP-3 associates with the centrosome. Following immunostaining of exponentially growing HeLa HC1 cells, hPARP-3 could unequivocally be identified at the centrosome in >90% of cells, independently of the antibody used. Moreover, confocal microscopy revealed a colocalization of hPARP-3, mostly with one of the two centrioles, most probably the daughter one, in G2 and throughout mitosis from early prophase to telophase (Fig. 3E). Taken together, these data demonstrate that hPARP-3 is a core component of the centrosome and is preferentially associated with the daughter centriole at all stages of the cell cycle.

Purified centrosomes are enriched in hPARP-3

To further substantiate the cellular distribution of hPARP-3,

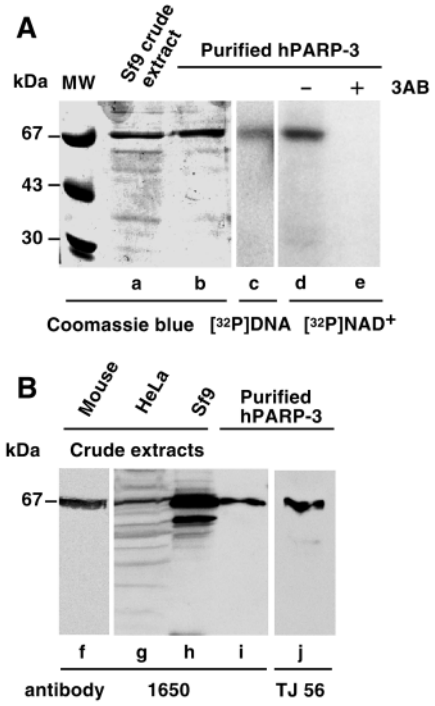

Fig. 2. (A) Purification and characterization of recombinant hPARP-3 overexpressed in the Sf9/baculovirus system. Crude extract from infected Sf9 cells (lane a); Purified recombinant hPARP-3 (lanes b-e); DNA-binding activity of hPARP-3 detected by south-western blotting (lane c); autopoly(ADP-ribosyl)ation of purified hPARP-3 incubated with [α-^{32}P] NAD^+ (lane d); inhibition of hPARP-3 autopoly(ADP-ribosyl)ation by 2 mM 3-Aminobenzamide (lane e). (B) Western blot detection of hPARP-3 in crude extracts from mouse lung (lane f), HeLa cells (lane g) or infected Sf9 cells (lane h) and purified recombinant hPARP-3 (lanes i,j) using two different anti hPARP-3 antibodies (see Materials and Methods).

centrosomes were isolated from KE 37 cells (Moudjou and Bornens, 1994) and examined by indirect immunofluorescence (Fig. 4A). Centrosomes were spun down on coverslips and co-stained with the anti-p34^{cdc2} antibody, to label centriole doublets, and with the purified polyclonal anti-PARP-3 antibody 1650. Again, a specific staining of one of the two centrioles was observed, in agreement with the above results obtained with whole cells.

The presence of hPARP-3 in the centrosome was also confirmed biochemically (Fig. 4B). Low-speed Triton X-100 soluble and insoluble fractions of unsynchronized KE 37 cell lysates were prepared as described previously (Tassin and Bornens, 1999) and submitted to western blot analysis together with centrosome sucrose gradient preparations (Moudjou and Bornens, 1994) and recombinant hPARP-3. Proteins were probed with the affinity-purified anti-hPARP-3 antibody 1650 (Fig. 4B). A band at 67 kDa is observed in enriched centrosomes, as well as in the Triton-insoluble fraction and to a lesser extent in the Triton-soluble fraction of KE 37 cells.

DNA damage does not affect hPARP-3 localization

Sato et al. have previously

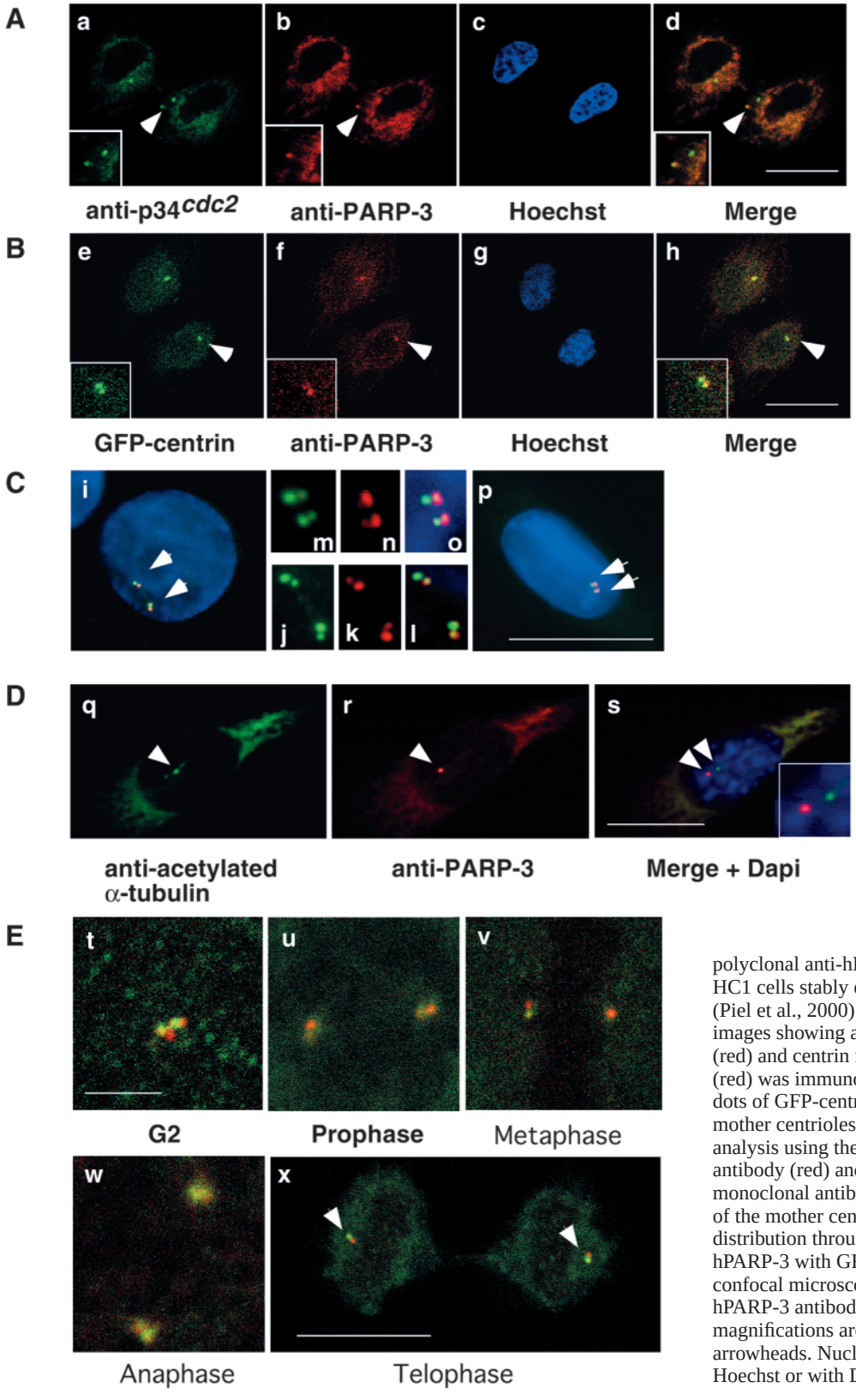

Fig. 3. hPARP-3 preferentially localizes to the daughter centriole throughout the cell cycle. (A) Confocal imaging of the subcellular distribution of hPARP-3 (red) at the centrosome marked with the anti-p34^{cdc2} antibody (green) in HeLa cells; d is a merge of a and b; (B) Colocalization of hPARP-3 immunostained with the polyclonal anti-hPARP-3 antibody 1650 (red) with HeLa HC1 cells stably expressing centrin (green) fused to GFP (Piel et al., 2000). h is a merge of e and f. (C) Merged images showing an asymmetric distribution of hPARP-3 (red) and centrin fused to GFP in S/G2 cells. hPARP-3 (red) was immunostained as in B. Strongly fluorescent dots of GFP-centrin (insets m and j) are attributed to the mother centrioles. (D) Immunofluorescence microscopy analysis using the 1650 polyclonal anti-hPARP-3 antibody (red) and the anti-acetylated α-tubulin monoclonal antibody (green) showing the primary cilium of the mother centriole. (E) hPARP-3 subcellular distribution throughout the cell cycle. Colocalization of hPARP-3 with GFP-centrin in HeLa HC1 cells by confocal microscopy analysis using the polyclonal anti-hPARP-3 antibody 1650 (red). For all pictures, the magnifications are details of the area surrounding the arrowheads. Nuclei in c, g, i, p and s are stained with Hoechst or with DAPI in S phase. Bars, 10 μm.

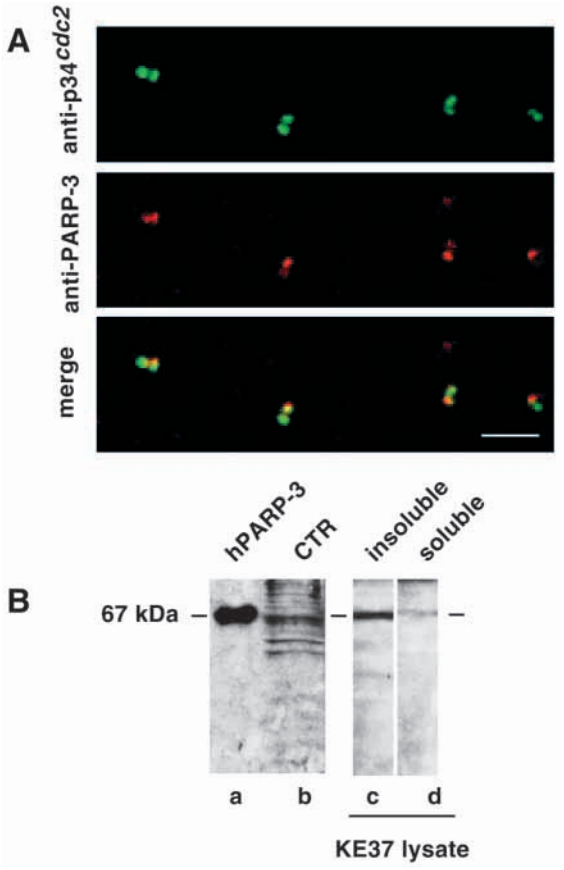

Fig. 4. hPARP-3 is detected in purified centrosomes. (A) Immunostaining of centrosome preparations from KE 37 cells. Immunolabelling was carried out using an anti-p34^{cdc2} antibody (green) and the polyclonal anti-hPARP-3 antibody 1650 (red). Bar, 2 μm. (B) Western blot of purified recombinant hPARP-3 (lane a), a sample of purified centrosomes (10^8) (lane b) and a KE 37 lysate containing a highly enriched centrosome preparation present in both the Triton-insoluble (lane c) and Triton-soluble fractions (lane d).

reported that γ-irradiation of U2-OS osteosarcoma cells or HeLa cells results in centrosome overduplication (Sato et al., 2000). Moreover, we and others have demonstrated that radiation-induced DNA strand-breaks activate PARP-1 and PARP-2 in the nucleus. We thus examined the hPARP-3 localization in HeLa HC1 cells exposed to various DNA-damaging agents including γ-radiation, N-methyl-N-nitrosourea (MNU) or H_2O_2 treatment. Asynchronous HeLa HC1 cells were irradiated at a single dose of 10 Gy or treated with 1 mM MNU or 1 mM H_2O_2; the centrosome number and hPARP-3 subcellular localization were subsequently determined by immunofluorescence at various time points following DNA damage (Fig. 5 and data not shown). Although the abnormal cells with more than two centrioles were less than 5% of the population in untreated cells, this population increased up to 70% by 120 hours post-irradiation or 72 hours post-MNU treatment as previously (Sato et al., 2000). Whatever the type of DNA injury, multipolar spindles (Fig. 5A-C) and coalescence of centrosomes (Fig. 5D-F) were frequently observed, as already described (Brinkley, 2001). However, co-staining of treated cells with the affinity-purified anti-hPARP-3 antibody and the anti-p34cdc2 antibody revealed that hPARP-3 was always present in the centrosome even under DNA damage conditions. Therefore, the localization of hPARP-3 is not affected by centrosome dynamics in response to DNA-damaging agents.

Overexpression of hPARP-3 or its N-terminal domain interferes with the cell cycle progression at the G1/S transition

Given the tight link between centrosome homeostasis and cell cycle regulation, we tested whether hPARP-3 participates in cell cycle regulation in mock or DNA-damage-exposed cells. We transiently expressed in HeLa cells the full-length hPARP-3 or its N-terminal domain as a glutathione S-transferase (GST)-fusion protein, and the cell cycle distribution of the GST-expressing cells was analyzed. As displayed in Fig. 6A, in untreated cells the expression of both hPARP-3 or its N-terminal domain caused an imbalance in normal cell cycle distribution characterized by an increase in the fraction of cells in G1/S compared to cells expressing GST alone.

DNA damage checkpoints arrest the cell cycle at the G2/M boundary to allow DNA repair, thus preventing progression of cells into mitosis. Following DNA base damage induced by a treatment with MNU, HeLa cells expressing only GST showed, as expected, a prominent

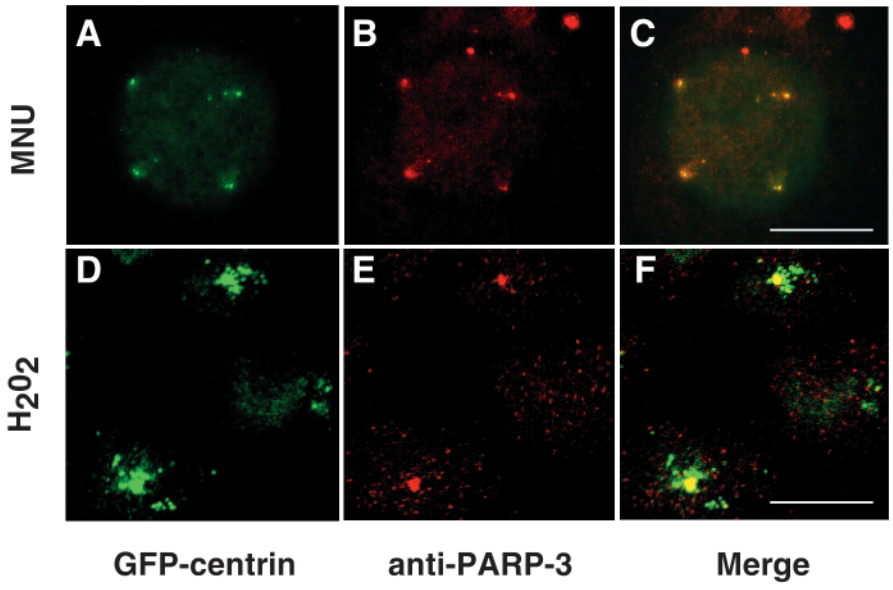

Fig. 5. DNA damage induces centrosome amplification but does not relocate hPARP-3. HeLa HC1 cells expressing GFP-centrin (green) were immunostained with an anti-hPARP-3 (red) antibody 120 hours after treatment with 1 mM MNU (A-C) or with 1 mM hydrogen peroxide (D-F). Examples of monopolar and multipolar spindles are shown. Bars, 10 μm.

accumulation at the G2/M boundary (39%). In contrast, the proportion of cells at the G2/M boundary was markedly decreased in cells expressing GST-N-ter hPARP-3 or GST-hPARP-3 (13% and 22% respectively); instead the GST fusion protein accumulated at the G1/S boundary. Together, these results imply that hPARP-3 acts at the G1/S cell cycle transition and that this function is carried out by its N-terminal domain but not by its catalytic domain.

To better correlate the biological function of hPARP-3 with its localization at the centrosome, we transiently expressed in HeLa cells the full-length hPARP-3 or its N-terminal domain as GFP fusions and analyzed their subcellular distribution using anti-γ-tubulin antibodies as centrosome markers. As shown in Fig. 6B, hPARP-3 (panel d-f), and more precisely its 54 amino-acid N-terminal domain (panel a-c), contains a motif responsible for centrosomal retention. This centrosomal localisation paralleled the G1/S cell cycle accumulation, as the exon-1-deleted version of the hPARP-3 N-terminal domain in fusion with GST did not target to the centrosome and did not

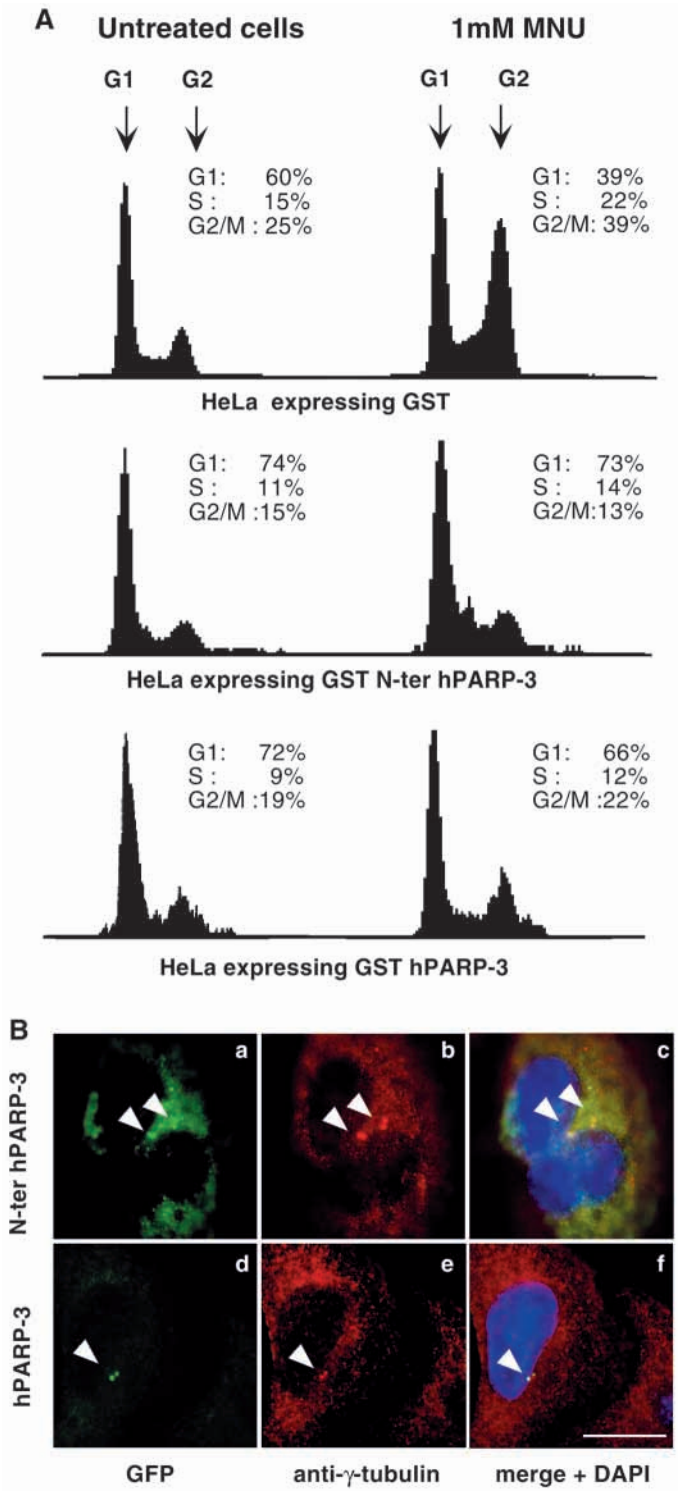

Fig. 6. (A) hPARP-3 or N-ter hPARP-3 overexpression leads to G1/S cell cycle arrest. FACS analysis on undamaged (left column) and MNU-treated (right column) HeLa cells expressed by GST, GST N-ter hPARP-3 or GST-hPARP-3. (B) hPARP-3 (d-f) or its N-terminal domain (a-c) target the GFP fusion protein to the centrosome, which is immunostained with an anti γ-tubulin antibody (red). Bar, 10 μm.

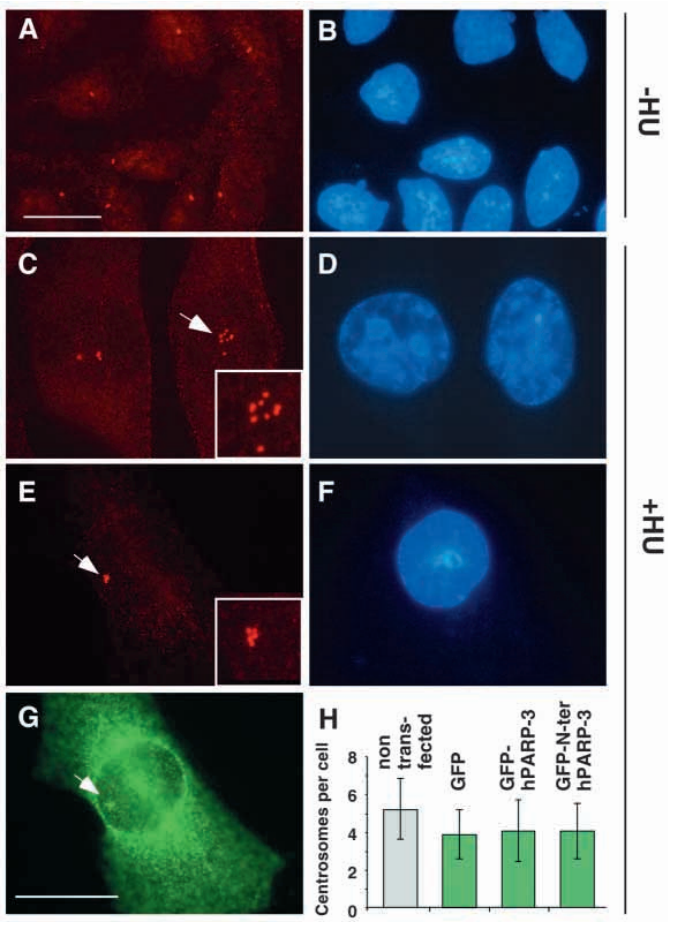

Fig. 7. hPARP-3 overexpression does not prevent centrosome amplification induced by hydroxyurea (HU) in CHO cells. Cells were transfected for 48 hours to express full-length or N-ter hPARP-3 as GFP-fusion proteins and cultured in the presence (C-F) or absence (A-B) of HU. Transfected cells were identified by fluorescence microscopy, and the number of centrosomes quantified using the antibody anti-glutamylated tubulin Gt335 (red). (C,D) Non-tranfected cells. (E-G) Transfected cells expressing GFP-hPARP-3. (H) Histogram showing the mean number of centrosomes±s.d. counted in non-transfected cells or in cells expressing GFP alone, GFP-hPARP-3 or GFP-N-ter hPARP-3 following HU treatment. The arrows point to centrosome amplification. Bars, 10 μm.

induce a G1/S accumulation when overexpressed in HeLa cells (data not shown).

hPARP-3 overexpression does not interfere with centrosome overduplication induced by hydroxyurea treatment

We considered the possibility that the G1/S block observed in cells expressing GST-N-ter hPARP-3 or GST-hPARP-3 is caused by an inhibition of centrosome duplication. To test this hypothesis, the GFP-hPARP-3 or GFP-N-ter hPARP-3 constructs were tested for their ability to interfere with centrosome duplication in an assay developed by Balczon et al. (Balczon et al., 1995). This assay is based on the observation that hydroxyurea (HU) treatment of CHO cells blocks DNA replication but allows multiple rounds of centrosome replication to occur. Following 40 hours of treatment, more than 50% of cells contain more than two centrosomes (Balczon et al., 1995; Matsumoto et al., 1999; Meraldi et al., 1999). As displayed in Fig. 7E-G, CHO cells expressing GFP-hPARP-3 were not affected by centrosome overamplification (Fig. 7C,D). Similar results were obtained with CHO cells expressing the GFP-N-ter hPARP-3.

To quantify the amplification of centrosomes, the number of spots obtained after anti-γ-tubulin staining were counted, and the mean values±s.d. for at least 50 cells were calculated and plotted on a histogram (Fig. 7H). For cells expressing GFP in fusion with hPARP-3 or its N-terminal domain, we detected, respectively, 4.3±1.6 (range 1 to 9) and 4.2±1.5 (range 2 to 7) centrosomes. This number is slightly decreased compared with non-transfected cells, which display 5.4±1.6 (range 2 to 10) centrosomes, but quite similar to the number (4.1±1.3, range 2 to 7) of centrosomes detected in cells expressing GFP alone. Overall, these results suggest that the G1/S cell cycle block mediated by hPARP-3 overexpression is not due to direct inhibition of centrosome duplication.

PARP-3 interacts with PARP-1 at the centrosome

Both the previously demonstrated localization of hPARP-1 to the centrosome (Kanai et al., 2000) and the ability of different PARPs to interact with each other (Sbodio et al., 2002; Schreiber et al., 2002) prompted us to investigate the possibility of a contact between hPARP-3 and hPARP-1. To this end, hPARP-3 or its N-terminal domain was transiently

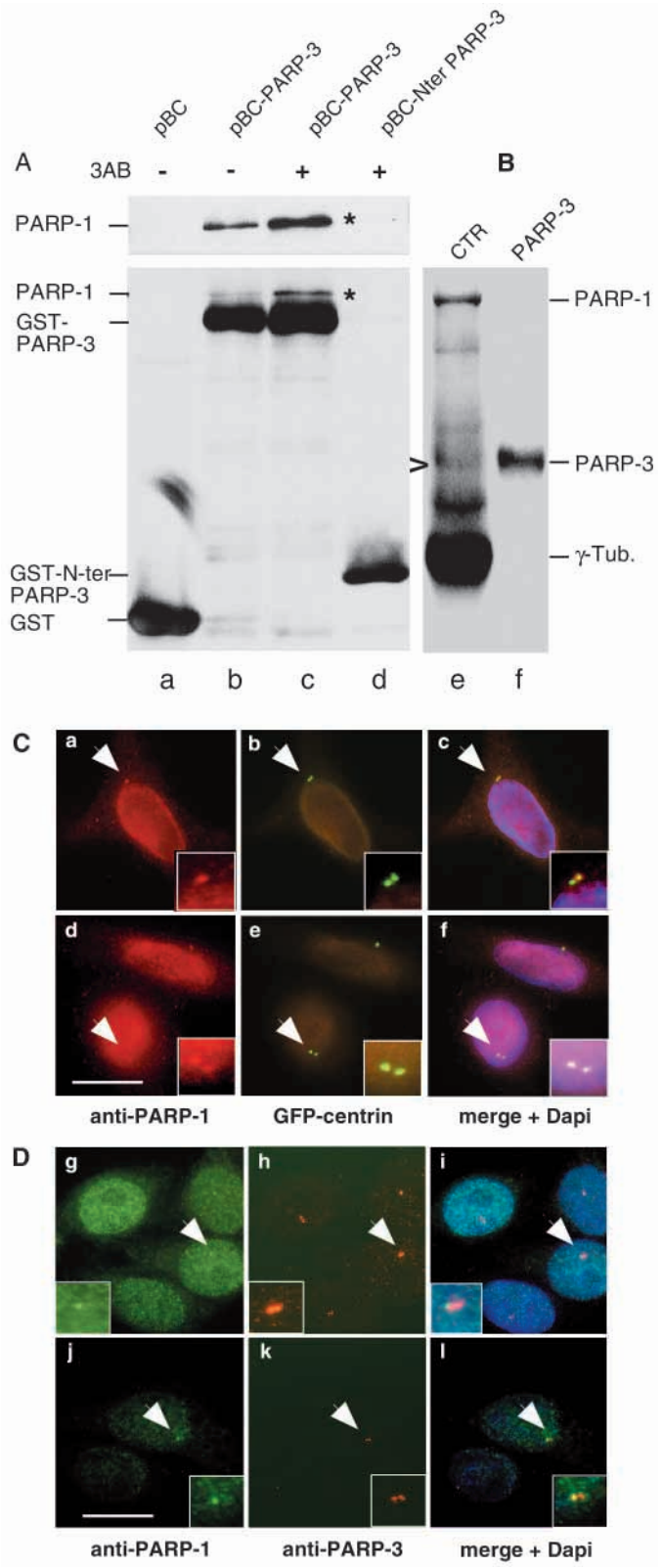

Fig. 8. hPARP-3 interacts with hPARP-1 at the centrosome. (A) Extracts from HeLa cells expressing either GST (lane a), GST-hPARP-3 (lanes b and c) or GST-N-ter hPARP-3 (lane d) were submitted to GST pull-down experiments, and the interacting proteins were analyzed by western blotting. When indicated, a treatment with 2 mM 3AB was applied for 2 hours before harvesting cell extracts. The blot was firstly probed with the mouse anti-hPARP-1 antibody (EGT69) (asterisks in upper panel) and subsequently with a polyclonal anti-GST antibody to reveal the proper expression of the fusion proteins (lower panel). (B) Sample of purified centrosomes (10^7) separated on SDS-PAGE and analyzed by western blotting using successively antibodies against hPARP-1, hPARP-3 and γ-tubulin (lane e); purified recombinant hPARP-3 (50 ng) (lane f). (C) Subcellular localization of hPARP-1 in GFP-centrin expressing HeLa HC1 cells. hPARP-1 was detected using a monoclonal antibody (F1-23) followed by the anti-mouse fluor Alexa 568 conjugate (red). (D) Both hPARP-1 and hPARP-3, immunostained with their respective specific antibodies, are detected at the centrosome in HeLa cells. For all pictures, the magnifications are details of the area surrounding the arrowheads. Bars, 10 μm.

overexpressed as a GST-fusion proteins in HeLa cells, and the interacting proteins were analyzed by western blotting. As shown in Fig. 8A, hPARP-1 was captured by the GST-hPARP-3 fusion protein but not by GST alone or GST fused to the N-terminal domain of hPARP-3. Moreover, the contact between hPARP-1 and hPARP-3 could be substantially enhanced when poly(ADP-ribose) synthesis was inhibited in the presence of the PARP inhibitor 3-aminobenzamide (Fig. 8A, compare lanes b and c), demonstrating an increased affinity of hPARP-1 and hPARP-3 for their respective unmodified form.

Proof of this interaction was further strengthened by the detection of both hPARP-1 and hPARP-3 in enriched fractions of purified centrosomes, which were characterized by the presence of γ-tubulin, a major component of centrosomes (Fig. 8B). Thus, the probability that the two PARPs interact is thought to be highest in this subcellular compartment. Indirect immunofluorescence experiments confirmed that hPARP-1 also resides in the centrosome. Indeed, hPARP-1 colocalizes with GFP-centrin in HeLa HC1 cells stably expressing this typically centrosomal protein (Fig. 8C). Despite the large nuclear staining of hPARP-1 and the close proximity of centrosomes to the nucleus, which makes it harder to affirm the centrosomal localization of hPARP-1, the colocalization of hPARP-1 and hPARP-3 could be performed using their respective antibodies (Fig. 8D). Taken together, these results indicate that hPARP-1 is present in both centrioles and interacts with hPARP-3, most probably at the daughter centriole. The enzymatic activity of both enzymes at the centrosome could be visualized in purified centrosome spreadings following incubation with NAD^+ and immunostaining with the anti-poly ADP-ribose antibody (data not shown). Finally, using PARP-1 knockout cell lines, we could demonstrate that the location of mPARP-3 at the centrosome was independent of the presence of mPARP-1 (data not shown).

Discussion

PARP-3 is a bona fide poly(ADP-ribose) polymerase

In this work, we have characterized a novel member of the PARP family, PARP-3. hPARP-3 catalyzes the synthesis of poly(ADP-ribose) in vitro and in purified centrosome preparations, presumably through an automodification mechanism. Like most of the other novel PARPs, with the exception of VPARP, the catalytic domain of hPARP-3 is located at its C-terminus. The N-terminal region, which is particularly short in the case of hPARP-3 (54 residues), apparently contains a targeting motif that is sufficient to localize the enzyme or the reporter protein GFP to the centrosome. No significant sequence homologous to this part has yet been found in the data banks. Moreover, the presence of basic residues in its N-terminus, giving a pI of 10 over the 54 first N-terminal residues, may explain the unexpected property of hPARP-3 to bind to and be moderately activated by DNA; however, up to now, no DNA has been detected in the centrosome.

The gene encoding hPARP-3 has been localized to the short arm of chromosome 3. Interestingly, allele loss involving the 3p arm is one of the most frequent and earliest known genetic events in lung cancer (Wistuba et al., 2000). Moreover, in the particular region 3p21.1 to 3p21.31 containing the *hPARP-3* gene a 600 kb region is most frequently undergoing allelic loss in the bronchial epithelia of smokers. It is therefore important to test whether hPARP-3 is present in lung epithelia, particularly in lung cancers.

PARP-3 localizes to the daughter centriole throughout the cell cycle

Indirect immunofluorescence experiments and subcellular fractionation concur to demonstrate that hPARP-3 is a core component of the centrosome. This particular localization, throughout the cell cycle, is independent of the microtubule polymerization status. Our data show that hPARP-3 is preferentially associated with the daughter centriole. Two independent lines of evidence support this idea: (i) in HeLa cells expressing GFP-centrin, PARP-3 immunostaining coincides with the smaller and less bright labelling already identified as the daughter centriole (White et al., 2000); and (ii) in mouse 3T3 cells, PARP-3 is clearly not associated with the primary cilium that identifies specifically the mature (mother) centriole during G1 phase (Chang and Stearns, 2000; Lange and Gull, 1995). Therefore, hPARP-3 appears to be the first known marker of the daughter centriole. What function does it exert here?

The mammalian centrosome is composed of two barrel-shaped centrioles, each formed by nine triplets of short microtubules, surrounded by a fibrous pericentriolar material. It is a vital organelle in animal cells as it directs the nucleation and organization of microtubules (Lange and Gull, 1996; Tassin and Bornens, 1999; Urbani and Stearns, 1999). As a consequence, the centrosome is essential during interphase for intracellular organelle transport, cell migration and the establishment of cell shape and polarity. The interphase centrosome then duplicates only once per cell cycle, thereby ensuring a strictly bipolar mitotic spindle axis (Mazia, 1984). Therefore, it plays a crucial role during mitosis in the equal and correct segregation of chromosomes as well as in the exit of cytokinesis (Piel et al., 2000). Indeed, many human tumor cells, including those lacking the tumor supressor p53 (Fukasawa et al., 1996), have abnormally high number of centrosomes (Pihan et al., 1998; Winey, 1996), and it has long been proposed that such aberrations may cause aneuploidy and contribute to cancer development (Brinkley, 2001; Doxsey, 1998). More recently, Nigg and collaborators (Meraldi et al., 2002; Meraldi and Nigg, 2001) have put forward a different mechanism where centrosome anomalies arise through failures in cell division, which lead to tetraploidization and subsequent propagation in tumor tissues, especially when the p53 pathway is abrogated or deregulated. Whatever the molecular scenario for the origin of centrosome aberrations may be, a strong correlation between centrosome amplification and aneuploidy exists and probably contributes to the selection of rare survival daughter cells that have acquired a mutator phenotype (Salisbury, 2001; Salisbury et al., 1999).

Overexpression of hPARP-3 or its N-terminal domain in HeLa cells interfered with the G1/S cell cycle transition, perhaps by titrating out a key regulator normally required at this critical stage when the decision to divide is taken. The lack of effect on HU-induced centrosome overduplication in CHO cells suggests that hPARP-3 does not interfere directly with

centrosome duplication. Our results rather point to a possible role for hPARP-3 in cell cycle progression at the G1/S transition. What is the connection between the influence of hPARP-3 at the G1/S transition and its preferential localization to the daughter centriole? The respective role of the two centrioles, mother and daughter, has been recently documented (Piel et al., 2000); they separate and function as independent structures at two stages of the cell division: (i) after the formation of the cleavage furrow, the mother centriole nucleates a microtubule aster, whereas the daughter centriole exhibits a considerable mobility, which progressively slows down from the onset of centrosome duplication at the G1/S border, up to late G2. (ii) At the end of telophase, just before abscission, the mother centriole moves to the intercellular bridge. Its repositioning back to the cell centre seems to provoke the completion of the cell division characterized by the narrowing of the intracellular bridge and abscission. On the basis of these observations, which clearly indicate that each centriole plays a specific role, it is tempting to speculate that hPARP-3, associated to the daughter centriole, may control its maturation until the G1/S restriction point is past. The regulating function of centrosome in the G1 to S transition has been recently studied using microsurgery and laser ablation. Indeed, when centrosomes were removed from somatic vertebrate cells, a proportion of cells completed cell division but failed to undergo the next round of DNA synthesis, suggesting a critical role of centrosomes in cell cycle progression (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Piel et al., 2000).

Alternatively, the presence of both hPARP-1 and hPARP-3 at the centrosome may be a part of a detection/signalling pathway aimed at monitoring the eventual presence, in the midbody, of broken DNA that originate from tension forces between two daughter cells experiencing unbalanced chromosome segregation. Indeed, both DNA and poly(ADP-ribose) can be easily detected in some arrested daughter cells as a long thin filament in the midbody (C.S. and G.dM., unpublished). Thus, DNA-binding enyzmes PARP-1 and PARP-3 might contribute to an ultimate cell division checkpoint linking the mitotic fidelity to the DNA damage surveillance network.

In conclusion, hPARP-3 and hPARP-1 add to the growing number of proteins that have been recently found, transiently or constitutively, associated with the centrosome. Conversely, the centrosomal protein centrin-2 has been recently identified as a constituent of the XPC (xeroderma pigmentosum) complex, a key component of global genome nucleotide excision repair acting as the initial damage detector (Araki et al., 2001), which points to an even more general link between DNA damage and repair and cell division (Su and Vidwans, 2000).

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Evidence for a Key Role of Leptin in Osteoarthritis

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Objective. **To evaluate the contribution of leptin (an adipose tissue–derived hormone) to the pathophysiology of osteoarthritis (OA), by determining the level of leptin in both synovial fluid (SF) and cartilage specimens obtained from human joints. We also investigated the effect of leptin on cartilage, using intraarticular injections of leptin in rats.**

Methods. **Leptin levels in SF samples obtained from OA patients undergoing either knee replacement surgery or knee arthroscopy were measured by enzyme-linked immunosorbent assay. In addition, histologic sections of articular cartilage and osteophytes obtained during surgery for total knee replacement were graded using the Mankin score, and were immunostained using antibodies to leptin, transforming growth factor β (TGFβ), and insulin-like growth factor 1 (IGF-1). For experimental studies, various doses of leptin (10, 30, 100, and 300 μg) were injected into the knee joints of rats. Tibial plateaus were collected and processed for proteoglycan synthesis by radiolabeled sulfate incorporation, and for expression of leptin, its receptor (Ob-Rb), and growth factors by reverse transcriptase–polymerase chain reaction and immunohistochemical analysis.**

Results. **Leptin was observed in SF obtained from human OA–affected joints, and leptin concentrations correlated with the body mass index. Marked expression of the protein was observed in OA cartilage and in osteophytes, while in normal cartilage, few chondrocytes produced leptin. Furthermore, the pattern and level of leptin expression were related to the grade of cartilage destruction and paralleled those of growth factors (IGF-1 and TGFβ1). Animal studies showed that leptin strongly stimulated anabolic functions of chondrocytes and induced the synthesis of IGF-1 and TGFβ1 in cartilage at both the messenger RNA and the protein levels.**

Conclusion. **These findings suggest a new peripheral function of leptin as a key regulator of chondrocyte metabolism, and indicate that leptin may play an important role in the pathophysiology of OA.**

Leptin, a small (16 kd) polypeptide encoded by the *obese* (*ob*) gene, is produced predominantly in white adipose tissue and regulates food intake and energy expenditure at the hypothalamic level (1–5). Leptin-deficient mice (*ob*/*ob*) fail to produce the functional protein and exhibit severe obesity (6). This hormone achieves its biologic effects by interacting with specific receptors (Ob-R) that result from the alternative splicing of the *db* gene transcript (7). Only Ob-Rb (the long isoform of the leptin receptor) contains intracellular motifs required for the signal transduction pathway through activation of signal transducers and activators of transcription (8,9). Because both leptin and its receptor share common structural and functional properties with the family of interleukin-6 (IL-6) cytokines (10,11), this adipose-derived protein has been classified as an adipocytokine.

Because of the wide pattern of Ob-R expression in peripheral tissues (12), leptin may be considered a pleiotropic hormone involved in the control of various physiologic processes, such as lipid homeostasis (13), insulin secretion (3), reproductive functions (14), thermogenesis (3,15), or angiogenesis (16). In addition, this

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adipocytokine plays an essential role in immune functions (17). The dysregulation of cytokine production in leptin-deficient mice leads to an increased susceptibility to infection and inflammatory stimuli (18,19). Recently, leptin was described as a new regulator of bone growth, acting either through a neuronal network to release an unidentified antiosteogenic factor (20,21) or directly by inducing osteoblast proliferation, collagen synthesis, and bone mineralization (22,23). Leptin is also a skeletal growth factor that stimulates endochondral ossification (24). Because functional leptin receptor has been shown to be expressed in normal human cartilage (25), it can be speculated that leptin could also act on this articular connective tissue, especially during osteoarthritis (OA), a disease in which great metabolic changes of chondrocytes are observed.

OA is a degenerative joint disorder and represents one of the most frequent and disabling diseases encountered in elderly individuals. In the early stages of the disease, the cartilage surface undergoes fibrillations before full-thickness matrix loss occurs. OA changes are not limited to cartilage, because remodeling of the underlying bone and development of osteophytes are also observed in osteoarthritic joints. Several inflammatory components such as tumor necrosis factor α and IL-1 have been detected in the synovial fluid (SF) of patients with OA and have been implicated in the degenerative process by inhibiting extracellular matrix synthesis and increasing catabolic activities of metalloproteinases (26–29). In response to cartilage damage, various growth factors, including insulin-like growth factor 1 (IGF-1) or transforming growth factor β (TGFβ), are activated and stimulate chondrocytes to repair the damaged extracellular matrix by forming cell clusters and increasing their anabolic activity (30–33). In fact, TGFβ has a dual role in OA, having a beneficial effect on cartilage repair but inducing osteophyte formation (34,35). Despite the increased synthetic response induced by growth factors, the enhanced synthesis of degrading enzymes ultimately results in cartilage erosion. Although the role of these factors in OA has been clearly demonstrated, the pathologic processes involved in this degenerative articular disease remain unclear.

The potential role of leptin in OA is supported by the relationship between high body mass index (BMI) and an increased risk of OA (36). This positive association is observed not only for knee joints but also for non–weight-bearing joints such as the hands (37,38), suggesting that OA may be initiated by metabolic disorders, with progression being worsened by high mechanical stress on abnormal cartilage. In order to better understand the molecular mechanisms involved in OA, the present study was undertaken to evaluate the contribution of leptin to cartilage changes associated with this joint disease.

This study is the first to show that leptin was detected in SF obtained from patients with OA, and that levels of leptin correlated with the BMI. Moreover, leptin was strongly overexpressed in human OA cartilage and in osteophytes. Human OA chondrocytes also produced growth factors, the topographic localization and staining intensity of which varied according to the histologic grade of cartilage destruction and paralleled those of leptin. Results of animal studies indicated that this adipocytokine stimulated anabolic functions of chondrocytes and induced the synthesis of growth factors in cartilage. Taken together, results of the current study provide evidence for a key role of leptin in the pathophysiology of OA.

PATIENTS AND METHODS

Patients and samples. Specimens of hyaline cartilage and osteophytes were obtained from 11 patients (8 women and 3 men, ages 56–80 years [mean age 69.3 years]) who had OA according to the American College of Rheumatology criteria (39). Tibial plateaus and osteophytes from the condyles were collected during total knee replacement surgery. For comparison with diseased tissues, normal knee joint cartilage was obtained from 2 transplant donors (a 56-year-old man and a 41-year-old woman) through an agreement with the Etablissement Français des Greffes.

SF samples were obtained from OA patients who either were undergoing knee replacement surgery (n = 11; 4 women and 7 men, ages 56–80 years [mean age 70.5 years]) or required knee arthroscopy (n = 9; 2 women and 7 men, ages 45–72 years [mean age 62.1 years]). This human study was conducted in conformity with the declaration of Helsinki principles, and written informed consent was obtained from all participants.

Leptin measurement in SF. To determine whether leptin is present within the joint, SF samples were analyzed for leptin, using specific enzyme-linked immunosorbent assays (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Leptin concentrations were determined in duplicate, after 10-fold dilution with a solution supplied by the manufacturer.

Histologic assessment. Tibial plateaus and osteophytes were fixed for 24 hours in 4% paraformaldehyde immediately after removal, decalcified in rapid bone decalcifier (RDO; Apex Engineering Products, Plainfield, NJ) for 8 hours, and further fixed in 4% paraformaldehyde. For each lateral and

medial plateau, 5 full-depth cartilage biopsy specimens were sampled using a biopsy punch (6 mm diameter) according to a standardized diagram. Cartilage biopsy specimens and osteophytes were then dehydrated in a graded series of alcohol and embedded in paraffin.

For all cartilage samples, hematoxylin–eosin–saffron and toluidine blue stainings were performed on 5-μm serial sections to determine histologic grading. For each biopsy, the severity of OA cartilage lesions was evaluated by 2 independent observers and was graded using the Mankin score (40). Forty-nine specimens representative of all grades were selected for the immunohistochemical study.

Animal studies. Male Wistar rats (150–175 gm) were obtained from Iffa Credo (L'Arbresle, France). The animals were housed under controlled temperature and lighting conditions, and received food and water ad libitum. Animals were acclimatized to the laboratory environment for 1 week before the experiment. The local Animal Care and Use Committee approved the experimental protocols, and guidelines for laboratory procedures were followed at all times.

In order to investigate the in vivo effects of leptin on articular cartilage, rat recombinant leptin (endotoxin $<0.01\%$) (R&D Systems), in a volume of 50 μl sterile saline, was injected into the joint cavity of the right knee of rats. Rats injected with saline served as controls. The tibial plateaus from rats killed 24 hours after intraarticular administration of leptin (30 and 100 μg) were collected for reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. The tissue samples were decalcified overnight in 150 m*M* EDTA at 4°C, and cartilage was isolated from the underlying bone before total RNA extraction. Tibial plateaus, which were removed 48 hours following leptin injection (10, 30, 100, and 300 μg), were processed for proteoglycan synthesis determination. For immunohistochemical analysis, total knee joints were dissected 48 hours after leptin administration (100 μg), fixed in 4% paraformaldehyde for 24 hours, decalcified in RDO for 4 hours, further fixed in paraformaldehyde for 24 hours, and then embedded in paraffin.

Immunohistochemical analysis. Paraffin sections (5 μm) from human and rat cartilage specimens were deparaffinized in Tissue Clear (Bayer Diagnostics, Puteaux, France) and rehydrated in a graded series of ethanol. After blocking nonspecific sites for at least 1.5 hours with phosphate buffered saline (PBS) containing bovine serum albumin (BSA, 4% weight/volume), sections were incubated overnight at 4°C with the different primary polyclonal antibodies diluted in BSA. For human cartilage tissues, antibodies for leptin and TGFβ1 (sc-842 and sc-146, respectively; Santa Cruz Biotechnology, Le Perray en Yvelines, France) were both used at a final concentration of 4 μg/ml, and antibody for IGF-1 (sc-7144; Santa Cruz Biotechnology) was used at a final concentration of 7 μg/ml. For rat specimens, antibodies for leptin, TGFβ1, and IGF-1 were used at final concentrations of 1 μg/ml, 0.7 μg/ml, and 2 μg/ml, respectively. Tissues obtained from normal rats were also analyzed for the expression of the long isoform of the leptin receptor (Ob-Rb) in articular cartilage, by using a specific antibody (sc-1834; Santa Cruz Biotechnology) diluted at 7 μg/ml.

After washing twice in PBS, biotinylated secondary antibodies (1 μg/ml) (goat anti-rabbit IgG [Vectastain ABC kit; Novocastra, Le Perray en Yvelines, France] and bovine anti-goat IgG [sc-2347; Santa Cruz Biotechnology]) were applied for 45 minutes at room temperature. Slices were then treated with hydrogen peroxide (0.3%) to quench endogenous peroxidase. The signal was amplified with preformed avidin–biotinylated horseradish peroxidase complexes for 45 minutes at room temperature (Vectastain ABC kit; Novocastra), and staining was developed with 3,3′-diaminobenzidine (0.05% in hydrogen peroxide) (Liquid DAB Substrate Kit; Novocastra). Counterstaining of nuclei was performed with hematoxylin, and slices were mounted in Eukitt (CML, Nemours, France). Sections incubated without primary antibody served as controls.

The entire surface of human cartilage specimens was examined by 2 independent observers. The percentage of positively stained cells was rated on a scale of 0 (negative) to +++, with + representing 5–20%, ++ representing 21–50%, and +++ representing >50% staining.

Polymerase chain reaction. For the semiquantitative RT-PCR, total RNA was isolated from cartilage using a commercially available phenol–chloroform solution (TRIzol). Following precipitation with isopropanol, one-fifth of the RNA samples were reverse transcribed to complementary DNA (cDNA) using oligo(dT) primers (100 pmoles) and Moloney murine leukemia virus reverse transcriptase (200 units) (Gibco BRL, Cergy-Pontoise, France). PCR amplification was then performed on one-tenth of the RT products with *Taq* polymerase (2.5 units) (Gibco BRL). PCR conditions were 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds, unless otherwise indicated. As an internal control, the same cDNA was subjected to PCR analysis for the L27 gene (melting temperature = 61°C), encoding for a ribosomal protein.

PCR primers were selected to amplify a 373-bp fragment for leptin (forward 5′-CCT-CAT-CAA-GAC-CAT-TGT-CAC-C-3′; reverse 5′-AGA-ATG-TCC-TGC-AGA-GAG-CCC-3′), a 453-bp fragment for TGFβ (forward 5′-CTA-CGC-CAA-AGA-AGT-CAC-CC-3′; reverse 5′-GTT-CAT-GTC-ATG-GAT-GGT-GC-3′), a 356-bp fragment for IGF-1 (forward 5′-TCA-CAT-CTC-TTC-TAC-CTG-GC-3′; reverse 5′-TCC-TGC-ACT-TCC-TCT-ACT-TG-3′), and a 225-bp fragment for L27 (forward 5′-TCC-TGG-CTG-GAC-GCT-ACT-C-3′; reverse 5′-CCA-CAG-AGT-ACC-TTG-TGG-GC-3′) (MWG Biotech, Ebersberg, Germany). The PCR products were separated on 1.2% agarose gels, and bands were semiquantitatively analyzed with a GelDoc digital imaging system (Bio-Rad, Marne la Coquette, France).

Assessment of proteoglycan synthesis in rat cartilage. Tibial plateaus were dissected with a minimal amount of adjacent tissue and incubated for 3 hours at 37°C in RPMI-HEPES 1640 medium supplemented with 2 m*M* L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.6 μCi/ml of $Na_2{}^{35}SO_4$. After 5 washing steps in saline, tibial plateaus were fixed overnight at room temperature in 0.5% cetylpyridinium chloride dissolved in 10% formalin buffer. Plateaus were then decalcified for 6 hours in 5% formic acid, and 4 specimens were obtained from each lateral and medial plateau. Each specimen was dissolved in 0.5 ml Soluene overnight at

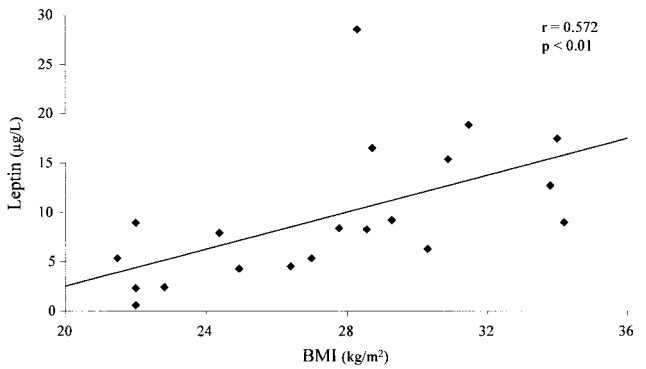

Figure 1. Correlation between body mass index (BMI) and the concentration of leptin in synovial fluid, in 20 patients with osteoarthritis.

42°C. The ^{35}S content was measured by liquid scintillation counting.

Statistical analysis. For human studies, the correlation between SF leptin levels and BMI was determined by linear regression analysis, using StatView for Windows, version 5.0 (SAS, Cary, NC). For experimental studies, each value is the mean ± SEM of 4 samples for RT-PCR analysis, and of 6 samples for the determination of proteoglycan synthesis analyzed from 3 independent experiments. The different groups were compared using a 2-way analysis of variance test. *P* values less than 0.05 were considered significant.

RESULTS

Correlation between SF leptin levels and BMI. Synovial fluid samples were obtained from 11 patients undergoing knee replacement surgery and from 9 patients requiring knee arthroscopy. In the 14 male patients, the BMI ranged from 22 kg/m^2 to 34.2 kg/m^2 (mean 28.15 ± 4.26 kg/m^2), and in the 6 female patients, the range was 22 kg/m^2 to 31.5 kg/m^2 (mean 26.04 ± 4.03 kg/m^2). This study is the first to show that leptin was detected in SF obtained from OA patients, at concentrations of 0.60–17.40 μg/liter (mean 8.16 ± 5.50 μg/liter) in men and 5.38–28.50 μg/liter (mean 12.95 ± 8.92 μg/liter) in women. Leptin levels in SF were significantly correlated with BMI ($r = 0.572$, $P < 0.01$) (Figure 1).

All OA grades exhibited in cartilage biopsy specimens. Normal cartilage slices showed very few surface irregularities and intense toluidine blue staining in all areas (score = 0). For each lateral and medial part of the tibial plateaus derived from OA patients, 5 cartilage biopsy specimens were removed according to a standardized scheme, except for regions with subchondral bone exposure. Cartilage samples exhibited variability in their histologic appearance, ranging from surface irregularities to marked fibrillations, cluster formation, and clefts. Proteoglycan loss was evident histologically in samples of moderately damaged OA cartilage, but the overall thickness of the cartilage was preserved. In contrast, specimens showing advanced cartilage destruction exhibited severe proteoglycan loss and cartilage erosion. Overall, 49 biopsy specimens obtained from 11 knees were selected and divided into 3 groups for further immunohistochemical analysis, as follows: Mankin scores of 1–4, 5–9, and 10–14 were considered to represent mild, moderate, and severe OA, respectively (Table 1).

Relationship between leptin immunostaining in human articular cartilage and OA grade. In biopsy specimens of normal cartilage, few positive chondrocytes

Table 1. Frequency of cells positive for leptin, TGFβ1, and IGF-1 in human OA cartilage specimens of various histologic grades*

	Leptin			TGFβ1			IGF-1		
OA group	+	++	+++	+	++	+++	+	++	+++
Mild (n = 13)	10 (77)	0	3 (23)	11 (85)	2 (15)	0	12 (92)	0	1 (8)
Moderate (n = 26)	15 (58)	5 (19)	6 (23)	17 (65)	4 (16)	5 (19)	20 (77)	4 (15)	2 (8)
Severe (n = 10)	3 (30)	2 (20)	5 (50)	3 (30)	3 (30)	4 (40)	3 (30)	2 (20)	5 (50)

* Values are the number (percentage) of cartilage biopsy specimens in each group stained for leptin, transforming growth factor β1 (TGFβ1), or insulin-like growth factor 1 (IGF-1). Histologic grades of cartilage destruction were determined using the Mankin score after examining cartilage sections stained with hematoxylin–eosin–saffron and toluidine blue. Scores of 1–4, 5–9, and 10–14 were considered mild, moderate, and severe osteoarthritis (OA), respectively. The percentage of positive chondrocytes throughout the entire biopsy specimen is rated as follows: + = 5–20%, ++ = 20–50%, and +++ = >50%. In normal samples, <5% of total cells exhibited positive staining for leptin, and no expression of growth factors was detected.

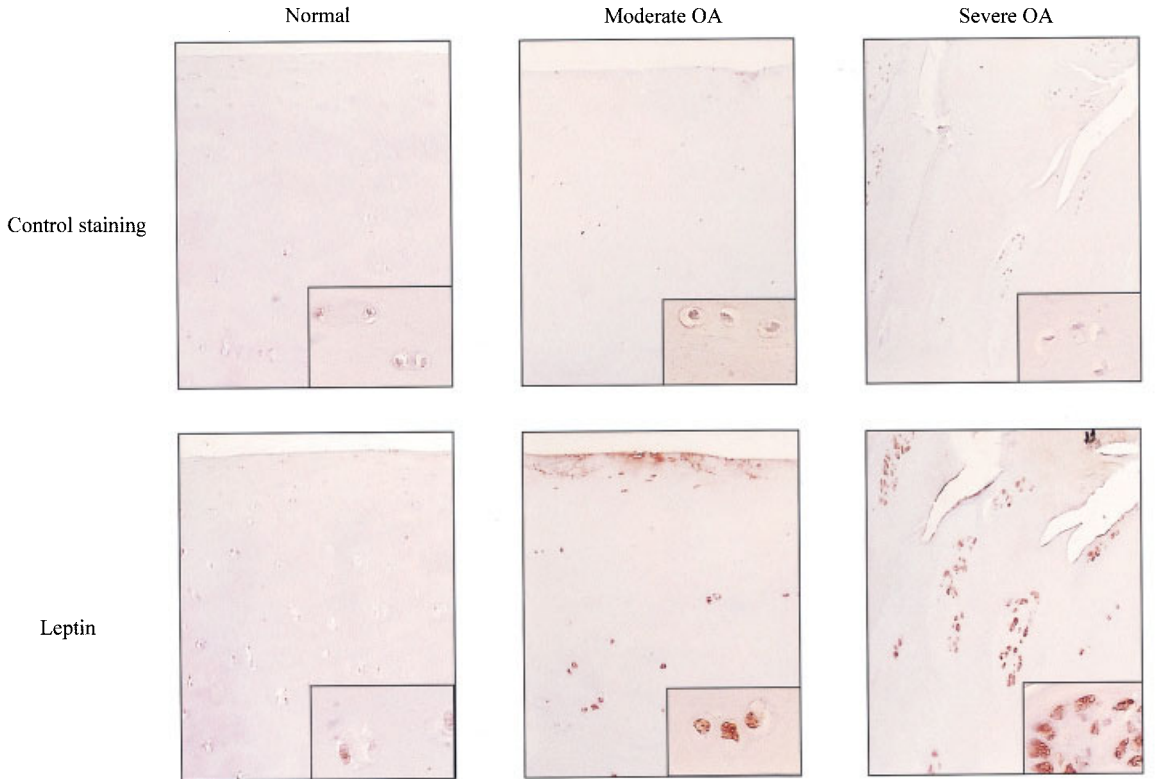

Figure 2. Expression of leptin in representative serial sections of human cartilage. Control staining was performed without primary antibody. Positive cells are brown; nuclei are counterstained with hematoxylin. OA = osteoarthritis (original magnification × 40; × 160 in **insets**).

were detected in the middle cartilage layer (Figure 2). Our immunohistochemical experiments indicated that leptin expression was up-regulated in human OA chondrocytes. No background staining was observed in negative control studies when primary antibody was omitted (Figure 2). Leptin immunostaining was scored according to the percentage of positive chondrocytes over the entire biopsy specimen (+, ++, +++). The frequency of cells positive for leptin in each specimen was related to the histologic OA score (Table 1). In fact, 77% of cartilage biopsy specimens showing mild OA had low leptin staining, and 50% of cartilage specimens showing severe OA exhibited strong staining.

In addition, the distribution of leptin was associated with the severity of the lesions. Leptin immunostaining was detected in the middle zone of cartilage samples showing mild OA. In cartilage specimens showing moderate OA, superficial cells and more cells of the middle layer were positive (Figure 2). Leptin expression was most prominent in the superficial and middle layers of severely damaged cartilage and was especially associated with areas of matrix depletion, fibrillations, and chondrocyte clusters. In contrast, leptin immunostaining was rarely detected in the deep layer of cartilage, except in a few chondrocyte clusters. Finally, osteophytes obtained from condyles exhibited strong leptin expression throughout the entire thickness of the slice (Figure 3). Increased immunoreactivity was observed in the transition area between the fibrous tissue and the underlying cartilage.

Distribution pattern of leptin, TGFβ1, and IGF-1 in human tibial cartilage. Biopsy specimens of normal cartilage showed no staining for TGFβ1 or

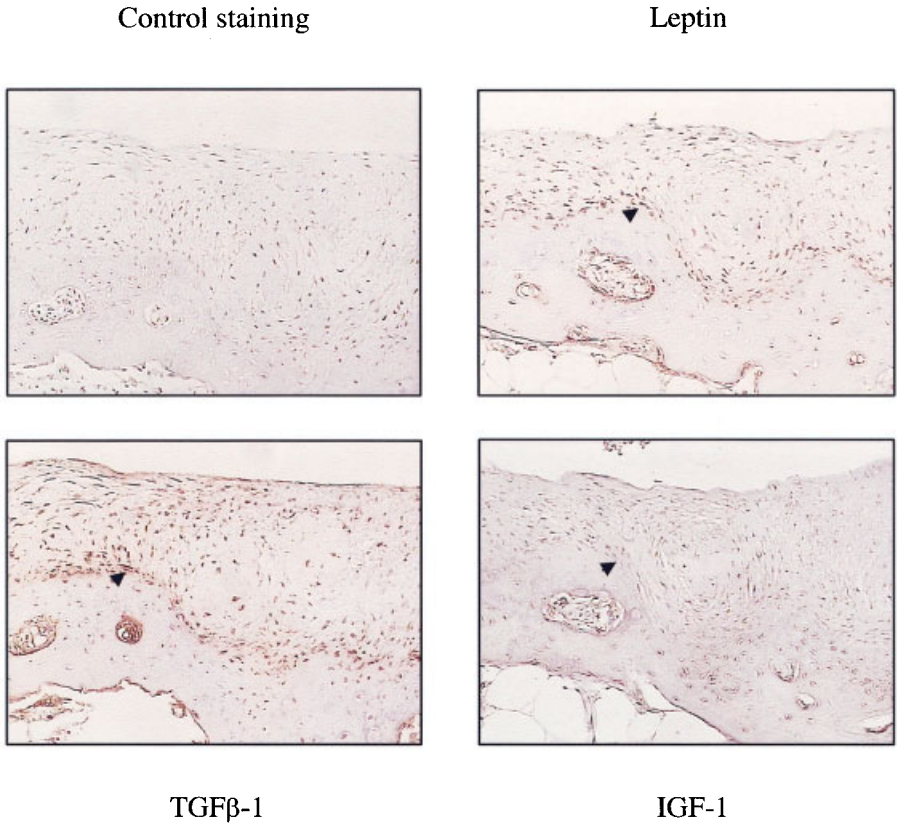

Figure 3. Expression of leptin, transforming growth factor β1 (TGFβ1), and insulin-like growth factor 1 (IGF-1) in representative serial sections of osteophytes. Control staining was performed without primary antibody. Positive cells are brown; nuclei are counterstained with hematoxylin. **Arrowheads** indicate strong staining in the transitional layer between cartilage and fibrous tissues (original magnification $\times$ 40).

IGF-1 in any cartilage layer. In contrast, growth factors were expressed in OA cartilage (Figure 4). Interestingly, the topographic distribution and staining intensity for these growth factors varied according to the grade of cartilage destruction and paralleled those of leptin. As shown in Table 1, 85% and 92% of cartilage biopsy specimens showing mild OA exhibited weak expression of TGFβ1 and IGF-1, respectively. Conversely, 40% and 50% of specimens showing severely damaged cartilage had high levels of TGFβ1 and IGF-1, respectively (Table 1). Furthermore, as was observed for leptin, strong TGFβ1 expression was demonstrated in osteophytes obtained from patients with OA (Figure 3). In contrast, expression of IGF-1 was barely detectable in these marginal osteophytes.

Leptin stimulation of proteoglycan synthesis in rat cartilage. We investigated the effects of leptin on proteoglycan anabolism by measuring ^{35}S incorporation in 4 areas of the tibial plateaus (medial and lateral) after intraarticular injection of leptin (10, 30, 100, and 300 μg/50 μl) into the right knee joint (Figure 5A). After a 30-μg injection, a marked increase in proteoglycan synthesis was observed in all cartilage areas of the plateau (Figure 5B). This stimulatory effect of leptin decreased at the highest dose (300 μg). Variation in this anabolic response was observed in different sites of the plateaus, as follows: the increase in proteoglycan synthesis following a 30-μg dose of leptin was significant in the medial plateau only (+65% in area 3 [$P = 0.043$] and +94% in area 4 [$P = 0.015$]), whereas the lateral plateau

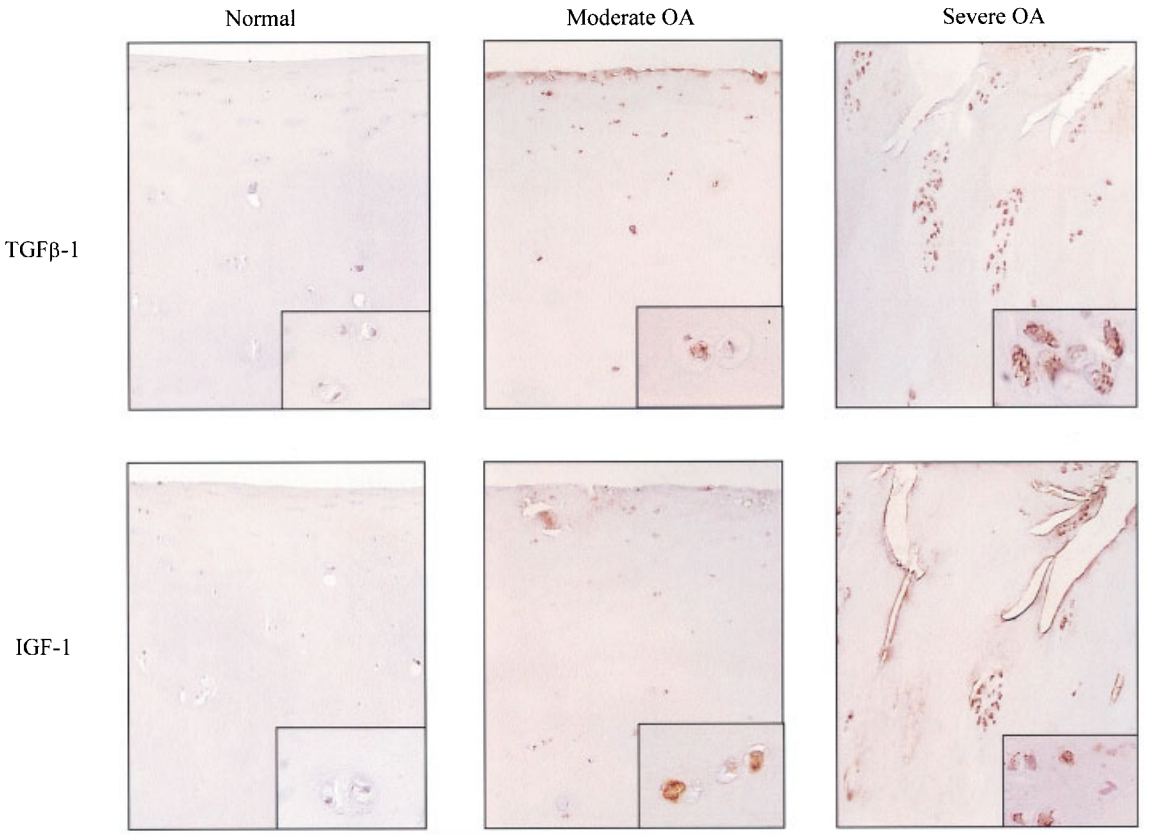

Figure 4. Expression of TGFβ1 and IGF-1 in representative serial sections of human cartilage. Control staining was performed without primary antibody. Positive cells are brown; nuclei are counterstained with hematoxylin. OA = osteoarthritis (see Figure 3 for other definitions). (Original magnification $\times$ 40; $\times$ 160 in **insets**.)

responded to higher doses of leptin (+76% in area 1 [$P = 0.015$] and +131% in area 2 [$P = 0.003$] after a 100-μg dose; and +65% in area 1 [$P = 0.036$] and +87% in area 2 [$P = 0.034$] after a 300-μg dose).

Leptin-induced expression of TGFβ1, IGF-1, and leptin messenger RNA (mRNA) in rat cartilage. RT-PCR analysis was performed to evaluate the effects of intraarticular injections of leptin (30 μg and 100 μg) on the expression of IGF-1, TGFβ1, and leptin mRNA in rat cartilage plateaus. The results showed that the low dose of leptin induced leptin mRNA expression in rat tibial plateaus (Figure 6). Regardless of which dose was administered, leptin up-regulated the expression of TGFβ1 and IGF-1 transcripts in tibial cartilage (Figure 6). The stimulating effect of leptin on growth factor expression was dose-related, with a significant effect at the highest dose used in this experiment (100 μg) ($P < 0.05$).

Leptin-induced stimulation of leptin, IGF-1, and TGFβ1 protein expression in rat cartilage. This is the first study to demonstrate expression of leptin and its receptor (Ob-Rb) in rat cartilage (Figure 7). Injection of 100 μg of leptin into the knee joint induced overexpression of leptin in each cartilage layer (Figure 7). Growth factor expression in tibial-plate cartilage was also up-regulated after administration of 100 μg of leptin. Negative control studies without primary antibody demonstrated no staining for any of the proteins studied.

DISCUSSION

The present study was designed to investigate the contribution of leptin to cartilage changes in human OA. This is the first study to demonstrate the presence of leptin in SF obtained from patients with OA. In addition, this adipose-derived cytokine was expressed in

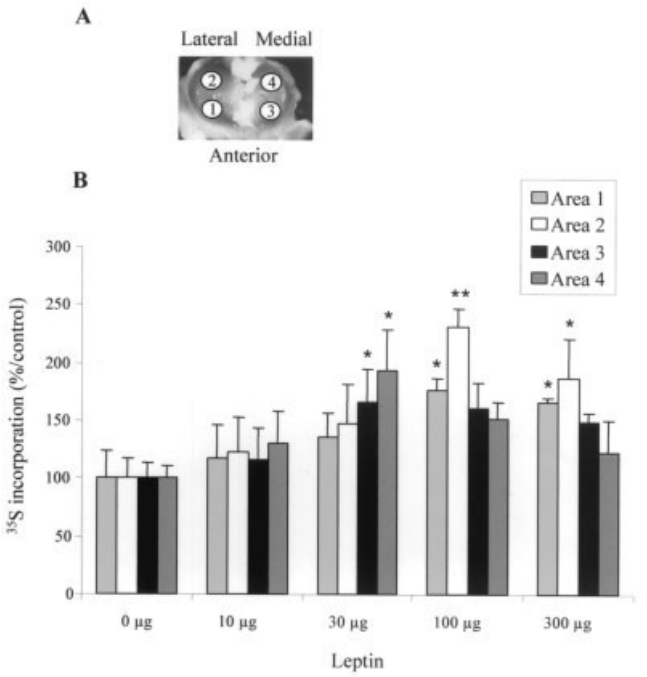

Figure 5. Effect of leptin on proteoglycan synthesis in rat cartilage. **A,** Localization of biopsy specimen punched out from the tibial plateau. **B,** Proteoglycan synthesis in cartilage specimens was measured by $Na_2{}^{35}SO_4$ incorporation, 48 hours following intraarticular injection of 10, 30, 100, or 300 μg of rat recombinant leptin. Results (mean and SEM) are expressed as the percentage of ^{35}S incorporation in leptin-injected rats compared with that in saline-injected rats, and represent 1 of 3 experiments (n = 6 per group). $* = P < 0.05$; $** = P < 0.01$.

human chondrocytes, and its synthesis was up-regulated in OA, in both cartilage and osteophytes. Interestingly, the pattern of leptin expression was related to the grade of cartilage destruction and was shown to be similar to that of growth factors. The stimulatory effect of leptin on both proteoglycan synthesis and growth factor expression in rat cartilage suggested a new peripheral function of this adipocytokine as a key regulator of chondrocyte metabolism. Taken together, these findings indicate that leptin may play an important role in the pathogenesis of OA by modulating chondrocyte functions and by contributing to osteophyte formation.

The mean levels of leptin in SF obtained from OA patients were in the same range as those previously reported in serum, and the leptin level in women was higher than that in men (41). The significant correlation between BMI and the concentration of leptin in SF suggests that this circulating adipocytokine with low molecular weight reaches the joint by diffusion through the synovial membrane.

The expression of leptin in cartilage obtained from OA patients was examined and compared with that observed in normal tissue. In order to take into account the focal character of the degenerative disease, cartilage specimens were removed from various areas of the tibial plateaus and further analyzed for histologic grading as well as immunostaining for leptin. In normal cartilage, few chondrocytes in the middle zone exhibited positive staining for leptin. This immunoreactive staining strongly increased in OA cartilage, especially in specimens showing severe damage, in which leptin was also observed in chondrocyte clusters and in cells located in

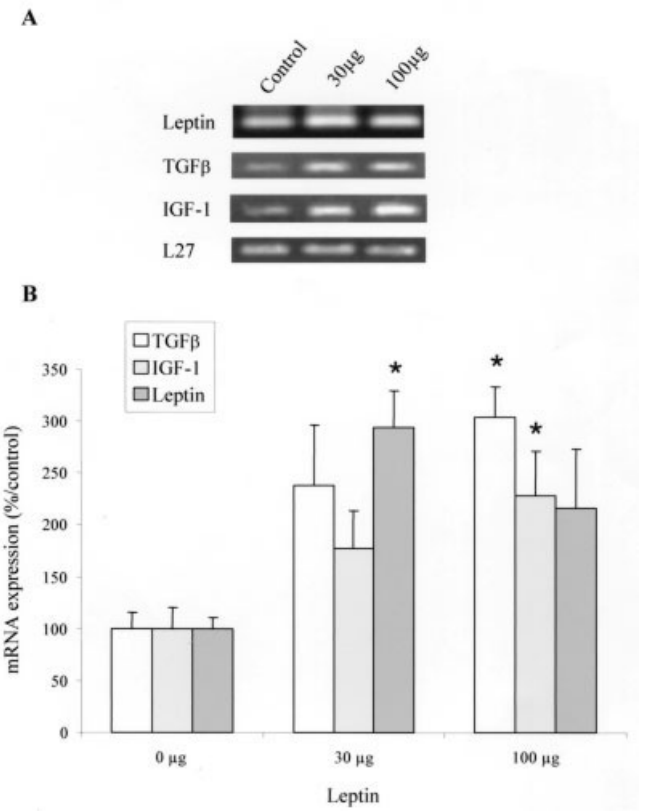

Figure 6. Effect of leptin on mRNA expression for leptin, IGF-1 and TGFβ in rat cartilage. **A,** Total RNA was isolated from cartilage of tibial plateaus collected 24 hours following intraarticular injection of 30 μg or 100 μg of leptin into the knee joint, and was subjected to reverse transcriptase–polymerase chain reaction (PCR) analysis using specific primers for each gene. PCR amplification of L27 was performed on the same RNA preparation, as an internal control for the relative amounts of template. **B,** Semiquantitative analysis of expression levels. Results (mean and SEM) are expressed as the percentage of gene expression in leptin-injected rats versus that in saline-injected rats. Assays were performed in triplicate, using at least 4 samples at each time point. $* = P < 0.05$. See Figure 3 for other definitions.

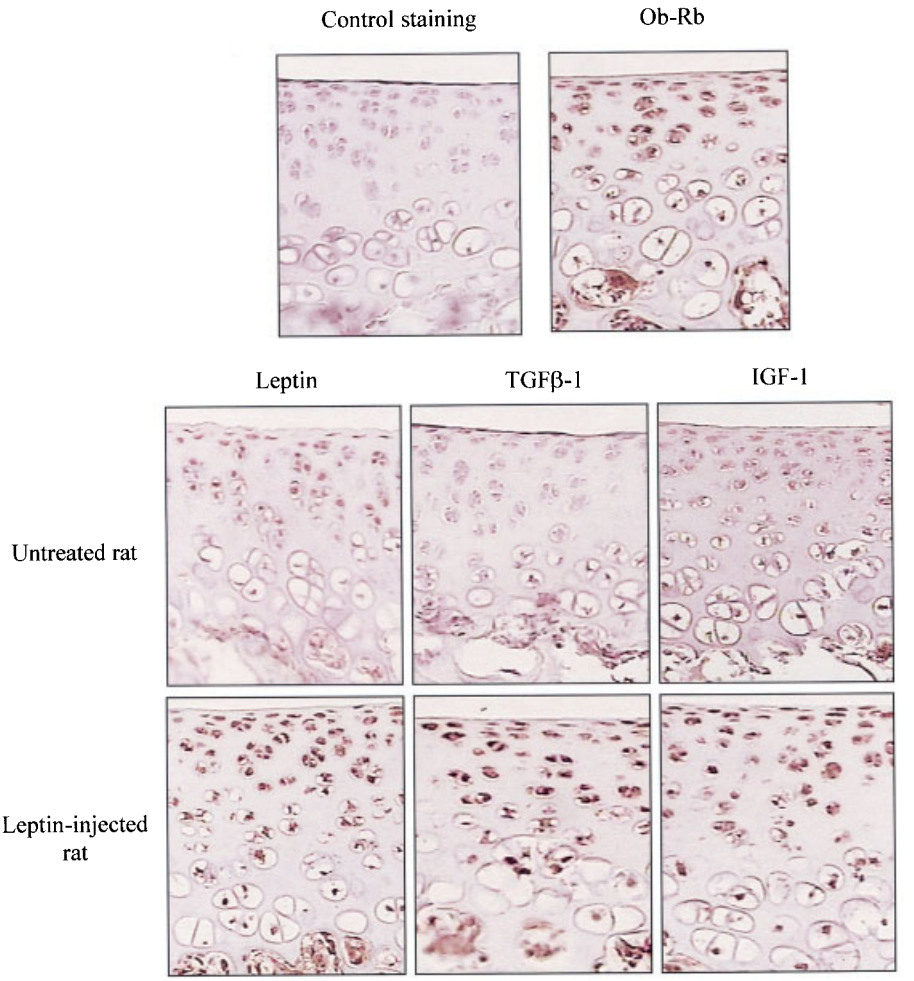

Figure 7. Immunohistochemical analysis of the expression of leptin and its receptor (Ob-Rb) in rat cartilage (top), and effect of leptin on leptin, TGFβ1, and IGF-1 synthesis in representative sections of tibial plateaus obtained from rats killed 48 hours following intraarticular injection of 100 μg of rat recombinant leptin (bottom). Sections from untreated rats and from leptin-injected rats were stained for leptin, TGFβ1, and IGF-1. Control staining was performed without primary antibody. Positive cells are brown; nuclei are counterstained with hematoxylin. See Figure 3 for definitions (original magnification $\times$ 40).

the superficial zone. Data obtained from animal studies showed that rat chondrocytes also produced leptin and expressed the long form of the leptin receptor. Leptin synthesis was up-regulated at both the mRNA and protein levels when rats received injections of leptin into the joint. Because leptin was detected in SF from patients with OA, it can be speculated that the protein may penetrate into cartilage and may target the recently identified leptin receptors on human chondrocytes (25).

Taken together, these findings suggest a direct stimulatory effect of this adipocytokine on its own synthesis by chondrocytes. However, adjacent cartilage specimens with various levels of OA severity exhibited different staining patterns for leptin, in terms of both topographic distribution and intensity. This focal response may be explained either by different levels of leptin receptor expression depending on the disease state of cartilage samples or by better access to chondrocytes of leptin derived from the SF due to fissuring or damage in the extracellular matrix. The strong staining observed around clefts in the damaged cartilage further supports the latter hypothesis. Finally, chondrocytes

themselves may act through a paracrine and/or autocrine pathway to induce leptin synthesis.

Results of the present study indicate that in specimens of human cartilage showing moderate OA, the positive immunoreactive staining for leptin was located mainly in chondrocytes in the middle layer. Interestingly, OA chondrocytes in this zone were shown to be hyperactive toward matrix synthesis, while cells in the upper zone down-regulated their expression of matrix components (42). The stimulatory effect of leptin on proteoglycan synthesis observed in rats suggests that this protein may be involved in the up-regulated anabolic activity observed in OA chondrocytes. This contribution may also occur in the early stages of the disease, which are characterized by enhanced chondrocyte synthetic activity (43). This observed stimulatory effect of leptin on cartilage anabolism is consistent with recent studies in which leptin was shown to promote bone growth by targeting osteoblasts directly and therefore has been considered to be a new hormonal regulator of bone growth (23,44). The similar anabolic response of osteoblasts and chondrocytes to leptin is not very surprising, because both cell types are derived from mesenchymal precursors and may share common regulatory mechanisms to produce their respective extracellular matrix.

Human OA chondrocytes were shown to produce both leptin and growth factors, in the same topographic distribution and to the same extent, depending on lesion severity. To determine whether an increase in leptin synthesis in pathologic tissues may be responsible for high levels of growth factors, rats received injections of the recombinant protein into the knee joint. Intraarticular administration of leptin induced expression of IGF-1 and TGFβ in rat chondrocytes at both the mRNA and protein levels. This stimulating effect may explain the enhanced proteoglycan synthesis observed in leptin-injected rats, which may be further increased by the up-regulation of growth factor receptors, as was previously shown for IGF-1 receptor in leptin-stimulated mandibular condyles (45).

All of these findings support the concept that leptin may have a role in OA. High levels of IGF-1 and TGFβ were indeed measured in the SF from patients with OA (46,47), and synthesis of these growth factors was increased in human OA cartilage (48–50) as well as in animal models of OA (33,51). These growth factors are believed to have a beneficial function in the cartilage repair process that may occur during OA (51–53). However, besides their protective role against cartilage damage, they may also trigger degeneration of this connective tissue. Excessive and/or prolonged exposure to TGFβ leads to development of lesions similar to those observed in mice with spontaneous OA (43,54). Because of the dual effect of growth factors (especially TFGβ) on cartilage, it would be interesting to identify any agent that is able to modulate their production. Although several compounds, including IL-1 or prostaglandin E_2, have been shown to promote the release of growth factors (55,56), no locally produced factors that might stimulate their synthesis in chondrocytes have been characterized. Because leptin induced IGF-1 and TGFβ expression at both the mRNA and protein levels, this study demonstrated, for the first time, that this adipocytokine is a key regulator of growth factors synthesis, and thus implied that leptin may contribute to the pathologic process of OA.

In addition to their effects on chondrocyte functions, growth factors are also involved in osteophyte formation, which is a prominent feature of OA-affected joints (51). TGFβ and IGF-1 have been detected in osteophytes (57–59), and repeated injections or overexpression of TGFβ in mice knee joints led to osteophyte generation (43,60). In the current study, leptin (as well as TGFβ) was strongly produced in osteophytes from OA patients, which is similar to what was observed in cartilage. This adipose-derived protein may contribute to osteophyte formation either indirectly by stimulating TGFβ expression or directly by inducing endochondral ossification (24). In addition, leptin was strongly expressed within the fibrous mesenchymal tissue in the upper zones of osteophytes, where a sequential process of differentiation of pluripotent cells may result in the formation of new cartilaginous outgrowths that ultimately may ossify.

After examining various osteophytes, Aigner et al suggested that different cell and tissue types in osteophytes were similar to those observed in normally developing fetal epiphyses (61). Interestingly, several studies ascribed a role for leptin in the early stages of fetal bone and/or cartilage development (62,63). The high levels of leptin detected in osteophytes indicated that this adipocytokine may contribute to the formation of these new bone structures by promoting fetal-like skeletal development processes, with synthesis of new extracellular matrix.

In conclusion, our data demonstrate that leptin is overexpressed in the human OA knee joint and provide evidence that leptin contributes to the pathogenesis of OA through stimulation of growth factor synthesis. Therefore, this adipocytokine may have dual effects on

the joint: variations in the levels of systemic and/or locally produced leptin might regulate chondrocyte proliferation and anabolic function, and these variations might induce osteophyte formation during OA. Results of the current study further support the hypothesis that OA is a systemic disorder in which disturbances of lipid homeostasis might be involved (64). Moreover, the effects of leptin on articular cartilage may explain the relationship between BMI and the increased risk of OA. Further investigation, throughout the course of OA, is required to characterize the mechanisms implicated in the regulation of leptin expression in articular cartilage. In addition, the use of leptin-deficient mice would be helpful in defining the overall contribution of leptin to the pathophysiology of OA.

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SPECIAL ISSUE RESEARCH ARTICLE

Evidence for a Conserved Role of Retinoic Acid in Urodele Amphibian Meiosis Onset

Angelina Wallacides, Amand Chesnel, Dominique Chardard, Stéphane Flament, and Hélène Dumond*

***Pleurodeles waltl* is a urodele amphibian displaying a ZZ/ZW genetic mode of sex determination. Gonad differentiation can later be modulated by hormone treatment. To investigate germ cell differentiation, we analyzed the expression of the meiosis marker PwDmc1 and show that germ cells enter meiosis in late larval life in females, and 2 months after metamorphosis in males. Organotypic cultures of gonad–mesonephros complexes demonstrated that retinoic acid triggers meiosis entry in *P. waltl*. In vivo analyses of both PwRaldh2 and PwCyp26b1 expressions, the enzymes required for RA synthesis and degradation respectively, indicate that meiosis onset depends on PwCyp26b1 repression in the gonad during normal or steroid-induced sex-reversed development. Taken together, our results show that RA-dependent meiosis entry could be a conserved mechanism of germ cell differentiation in vertebrates and provide evidence for crosstalk between steroid and RA signaling in the course of sex differentiation. *Developmental Dynamics 238:1389–1397, 2009*. © 2009 Wiley-Liss, Inc.**

Key words: *Pleurodeles waltl*; meiosis entry; sex differentiation; steroids; germ cells

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INTRODUCTION

The newt *Pleurodeles waltl* is a urodele amphibian that obeys female ZW heterogamety. Z and W sex chromosomes cannot be distinguished by conventional karyotypes but the genotype can be deduced at early larval stage from the analysis of a sex-linked marker, peptidase 1 (Ferrier et al., 1980). Of interest, ZW but not ZZ sex determination can be reversed by rearing the animals at 32°C instead of 20°C (reviewed in Dumond et al., 2008a).

When compared with other vertebrates, gonad differentiation is a slow process in amphibians and especially in *Pleurodeles*: it takes place mainly during larval life but goes on after metamorphosis and even in adulthood with differentiation of multiple testes (Dournon et al., 1990; Dumond et al., 2008a). Although the somatic compartment of the gonad and the role of steroid hormones have been studied extensively in this species (Kuntz et al., 2003a), little is known about germline differentiation due to the lack of molecular markers up to now. Using embryonic surgery experiments, Maufroid and Capuron (1985) showed that germ cells are induced by endodermal cells from the presumptive mesoderm. However, germ cells resulting from this induction cannot be identified until much later, just before hatching (stage 35) by using morphological features. From stage 35 to 41, primordial germ cells (PGCs) enter a resting period in both sexes while migrating to colonize the somatopleure, leading to the formation of genital ridges (Dournon et al., 1989, 1990). Then, proliferation of both germ cells (stage 42 in ZZ larvae and stage 44 in ZW larvae) and somatic cells starts in the undifferentiated gonad. Beyond stage 53, ovarian differentiation in ZW larvae is characterized by the development of the cortical part, which includes large follicles while medulla

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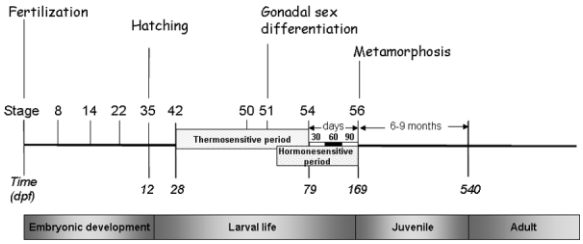


Fig. 1. Timetable of *Pleurodeles waltl* development. Until stage 54, larvae are staged according to Gallien and Durocher (1957). Timing of development is expressed as "days postfertilization" (dpf). Sex differentiation process is first observed at stage 51 through induction of aromatase activity in females and is detected histologically at stage 53. Metamorphosis occurs at stage 56, approximately 90 days after the beginning of stage 54. During the thermosensitive period (from stage 42 to stage 54), females can be reversed to fertile males if reared at 32°C. During the hormonesensitive period (stage 52 to stage 56), males treated with estradiol are reversed into females, while females treated with DHT are reversed into males.

regression leaves a cavity. In contrast, testis differentiation results from a strong development of the medulla. In *Pleurodeles*, stage 53 is the first stage at which gonadal sex may be ascertained by histological examination. Vitellogenic oocytes can be detected at metamorphosis and spermatogenesis begins during juvenile stage (see Fig. 1). Nevertheless, the precise time course of germ cell meiosis entry in both sexes and the factors triggering this process are still completely unknown.

As observed in other nonmammalian vertebrates, steroid hormones play a key role in *Pleurodeles waltl* sex differentiation wherein the P450-aromatase enzyme (P450arom) responsible for estrogen synthesis, is a major player (Kuntz et al., 2003b). Measurements of P450arom activity in gonad–mesonephros (GM) complexes reveal a higher activity in ZW larvae from stage 52 than in their ZZ counterparts (Chardard et al., 1995) and treatment of ZW larvae with an aromatase inhibitor induces sex reversal (Chardard and Dournon, 1999). These results indicate that elevation of P450arom expression is the actual first sign of ovarian determination. The role of steroids in sex differentiation is also evidenced by the fact that, when added to the rearing water of ZZ larvae from stage 52 until metamorphosis, estradiol induces a male-to-female sex reversal whereas nonaromatizable androgens drive ZW larvae to differentiate as males (Chardard et al., 2003). Although these experimental data clearly demonstrate the effect of exogenous steroids on gonad differentiation, we have no knowledge about their potential effect on germline.

In mammals, meiosis entry followed by prophase I arrest is initiated during fetal development in females, whereas in males, spermatogenesis begins only after birth. Until recently, it was commonly believed that female germ cells have the intrinsic property to enter meiosis, whereas this process was thought to be delayed in males by an unknown factor. However, two studies from Koubova et al. (2006) and Bowles et al. (2006) demonstrated the involvement of retinoic acid (RA) in determining germ cell fate in mouse. They showed that RA is released from the mesonephros of both sexes following Aldh1A2 (also called Raldh2) activity from 10.5 days post-conception (dpc), while Cyp26b1, a P450-cytochrome enzyme that degrades RA, is expressed in the gonad. At 12.5 dpc, by the time of meiosis entry in females, Cyp26b1 is no more expressed in females, leading to high levels of RA in the developing ovary, and becomes strikingly male-specific. Moreover, inhibition of Cyp26b1 in the testis induces expression of specific premeiotic (Stra8) and meiotic (Dmc1, Scp3) markers, whereas blockade of RA signaling pathway in the ovary delays meiosis entry. Therefore, the authors conclude that Cyp26b1 is the "meiosis-inhibiting factor" in male mice.

More recently, the picture became more complex: Best et al. (2008) showed that changes in membrane trafficking in Sertoli cells of embryonic mouse testis induces male-to-female sex reversal and concluded to the existence of a secreted meiosis preventing substance (MPS). By using an organotypic culture system, Trautmann et al. (2008) further demonstrated that RA could in turn inhibit this MPS which is also necessary for mitotic arrest at 13.5 dpc and, therefore, induce germ cell death probably due to misunderstanding between somatic and germinal compartments in the embryonic testes. Finally, results from Suzuki and Saga (2008) indicated that Nanos2 is part of this germ cell sex-determining pathway because from 13.5 dpc, it is (1) required to maintain the suppression of meiosis by preventing Stra8 expression, (2) shown to force male differentiation when expressed in female germ cells, and (3) down-regulated by RA. Altogether, these data emphasize the complex relationship between soma and germ during gonad development as well as the key role of RA in this process but also strongly suggest the involvement of other factors. Finally, a recent molecular analysis of meiosis onset in avian embryo further suggested that a regulatory role of retinoic acid may be conserved in higher vertebrates (Smith et al., 2008).

In the present study, we analyzed meiosis onset in the amphibian *Pleurodeles waltl*. To determine at which developmental stage meiosis entry takes place in standard male (ZZ) and female (ZW) animals, we first analyzed the expression of the meiosis marker PwDmc1 (Dumond et al., 2008b). Then, we designed a protocol for organotypic cultures of GM complexes to evaluate the involvement of retinoid and steroid hormones in meiosis initiation. Altogether, our results demonstrate for the first time the conservation of RA-dependent mechanisms for germ cell differentiation in a urodele amphibian.

RESULTS

Meiosis Entry Occurs During Larval Life in Females and After Metamorphosis in Males

The first aim of the study was to follow germ cell differentiation during development in vivo. Therefore, partial cDNA of *P. waltl* Dmc1 ortholog (PwDmc1), which encodes a Rad51-

like protein in the synaptonemal complex, was cloned by degenerated reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends-PCR (RACE-PCR). Deduced amino acid sequence is 98% identical to the urodele amphibian *Cynops*, 92% to human and chicken. Expression of PwDmc1 mRNA was examined from stage 48 to adulthood as a marker of meiosis entry in male and female gonads (Fig. 2). At each stage, PwVasa transcript expression was measured to verify the presence of a detectable amount of germ cells. In females, the earliest expression of PwDmc1 mRNA was observed at stage 54 + 45 days (Fig. 2A) whereas in males, PwDmc1 mRNA was not detected until 8 weeks after metamorphosis (Fig. 2B). These results indicating an earlier meiosis entry in females were confirmed by a histological analysis (Fig. 3). Germ cells that have not yet entered meiosis are characterized by a very large polylobular nucleus whereas meiotic germ cell nuclei are circular and contain condensed chromatin. Once initiated, meiosis seems to be maintained all through the juvenile stage.

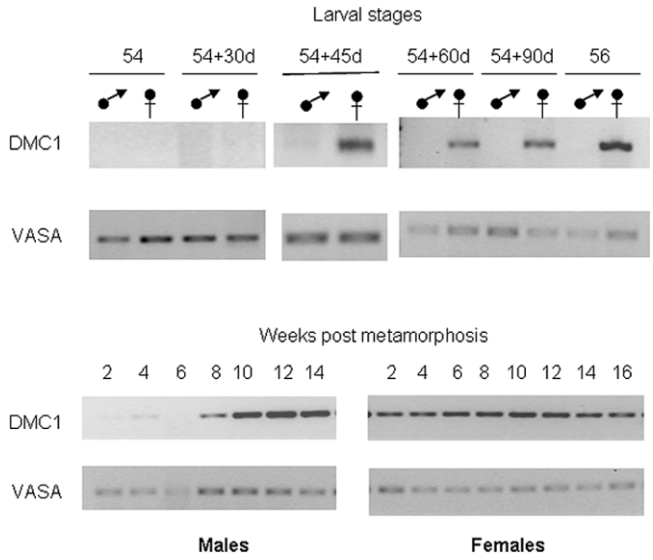


Fig. 2. Onset of meiosis during development of *Pleurodeles waltl*. Gonad–mesonephros (GM) complexes (stage 54) or isolated gonads (from stage 54 + 30 days to stage 56) were harvested from males or females. Before metamorphosis, analyses were performed on pools from 3 larvae whereas after metamorphosis, isolated gonads were sampled. Total RNA was reverse transcribed and amplification of the meiotic marker PwDmc1 was performed as described in the Experimental Procedures section. PwVasa amplification served as a control for relative germ cell amounts. Analyses were repeated twice on two RNA samples independently. The "54+xd" indicates stage 54 plus x days.

Exogenous RA Induces Meiosis Entry In Vitro

To understand the physiological process initiating meiosis in *Pleurodeles waltl* and based on recent data highlighting the role of RA in mouse and chicken, we investigated the effect of all-*trans* RA on germ cells in vitro. GM complexes were carefully dissected at different stages from males or females and individually grown in presence or absence of 1 or 2 μM RA (see the Experimental Procedures section for further details). After 2 days of culture, 3 complexes were pooled for RNA extraction and PwDmc1 mRNA expression was checked by qualitative RT-PCR (Fig. 4A). Such analyses performed either in females at stage 50 or in males at stage 54 (that is before meiosis entry in vivo) revealed that 2 μM RA treatment for 2 days induces PwDmc1 transcription.

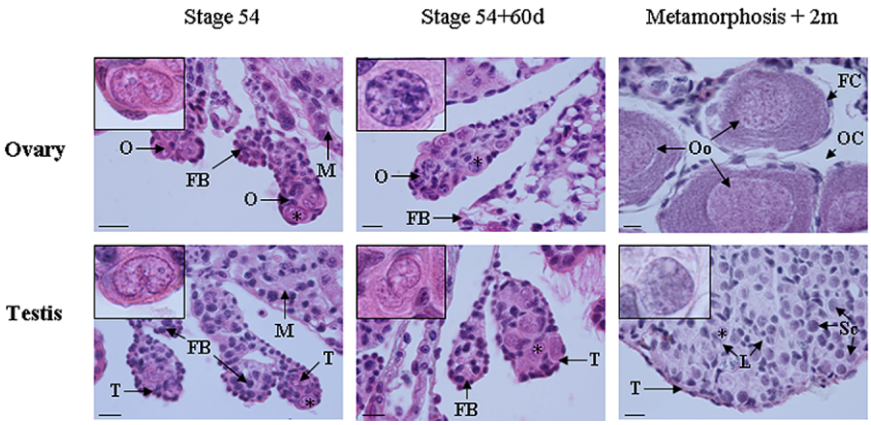
Furthermore, we repeated the culture experiment on stage 54 male GM complexes for 2 or 5 days in presence of 2 μM RA and measured PwVasa and PwDmc1 mRNA expressions by quantitative RT-PCR. Such analyses revealed that: (1) PwVasa expression is unaffected by 5 days organ culture; (2) PwDmc1 is transiently expressed; (3) PwDmc1 mRNA is no more detectable after 5 days in presence of RA (Fig. 4B).

These results suggest that, like in mouse and chicken, exogenous RA can trigger meiosis entry in *Pleurodeles waltl*. Nevertheless, in our hands, RA may be necessary but not sufficient to promote complete meiosis and/or endogenous factors rapidly degrade RA in the cultured gonads. Indeed, we cloned the RA degrading enzyme encoding gene PwCyp26b1 (GenBank accession no. EU213038) and observed a strong induction of its transcription under a 48h treatment in the presence of 2μM RA (data not shown).

Endogenous RA Induces Meiosis Entry In Vitro

To demonstrate the involvement of endogenous RA metabolism, we cloned PwRaldh2 (GenBank accession no. EU213037), the enzyme that synthesizes RA. RT-PCR analyses showed that this enzyme was expressed in organotypic cultures of GM complexes at all the stages studied. Then we tried to modulate the activity of either PwCyp26b1 or PwRaldh2. GM complexes from males at stage 54 were treated for 2 days with 2 μM R115866, an inhibitor of PwCYP26 enzyme. RT-PCR revealed a high level of PwDmc1 mRNA in response to R115866 (Fig. 5A), demonstrating that the amount of RA produced endogenously in male GM complex at stage 54 is sufficient to induce meiosis entry.

Moreover, this finding suggests that an in vivo PwCYP26-dependent degradation of RA could be responsible for delaying meiosis entry in male. The latter hypothesis was further supported by results from another organotypic culture experiment. We treated GM complexes from male at stage 54 with 1 nM Am580, a nondegradable analogue of RA that specifically binds to RAR-alpha receptors, and observed both the induction of PwDmc1 expression after 2 days (Fig. 5A) and the typical condensed chromatin of meiotic nuclei after 3 days (Fig. 5B). However, after 5 days, we were not able to perform RT-PCR analysis because cultured GM complexes displayed many dying germ cells with picnotic nuclei and altered structure of mesonephros. Finally, we treated female GM complexes, harvested at stage 54 + 90d (after natural meiosis initia-



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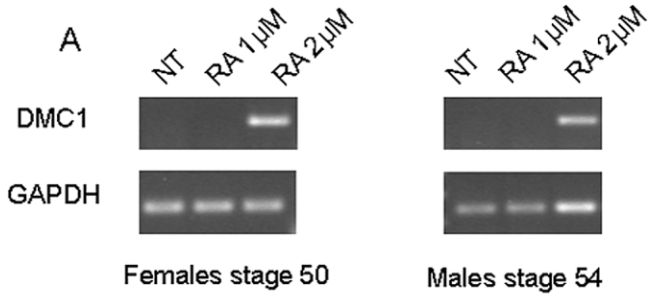

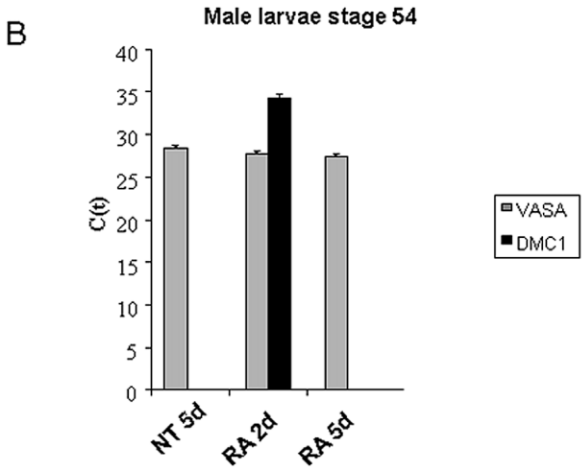

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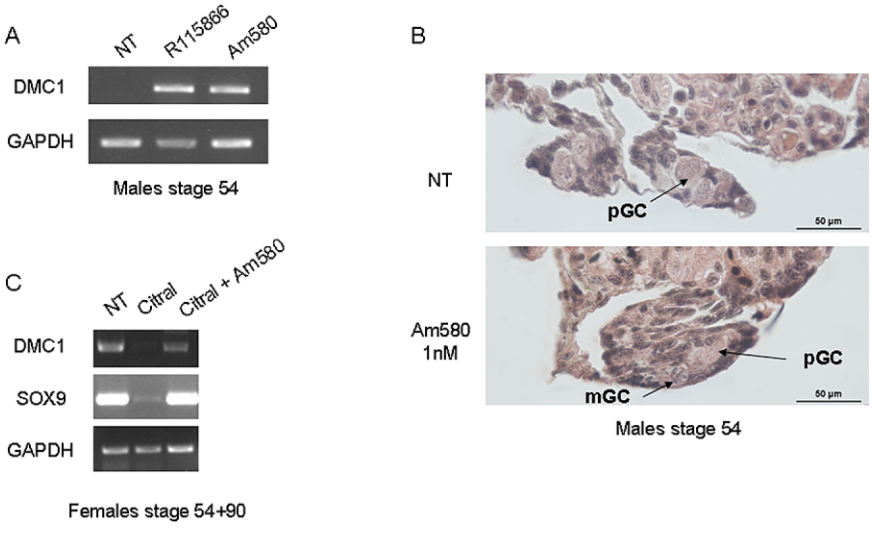

Fig. 5.

tion), with 60 µM Citral, an inhibitor of retinoic acid synthesizing aldehyde dehydrogenase (RALDH), and observed an inhibition of PwDmc1 expression, which can be reversed by a cotreatment with Am580 (Fig. 5C). In the latter experiment, endogenous PwSox9 expression was used as a control of RA signaling pathway inhibition since Afonja et al. (2002) described SOX9 up-regulation by RARalpha-selective (like Am580) but not RXR-selective agonists in breast cancer cell lines.

Fig. 3. Histology of ovary and testis at stage 54, 54 + 2 months, and metamorphosis + 2 months. FB, fat body; L, testis lobe; M, mesonephros; O, ovary; OC, ovarian cavity; Oo, ovocyte; Sc, Spermatocytes; T, testis. Asterisks indicate typical germ cells from each stage presented with higher magnification. At stage 54, neither female nor male gonads contain meiotic germ cells. Such cells are observed at stage 54 + 2 months in the ovary but only 2 months after metamorphosis in the testes. Scale bars = 20 µm.

Fig. 4. Exogenous retinoic acid (RA) triggers premature meiosis entry in male and female gonad–mesonephros (GM) complexes cultured ex vivo. **A:** Three GM from females at stage 50 or males at stage 54 were exposed to 1 or 2 µM RA for 2 days. One microgram of total RNA was reverse transcribed and qualitative amplification of PwDmc1 was performed as previously described. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) amplification served as a control for relative RNA amounts. **B:** Real-time polymerase chain reaction (PCR) analyses of PwVasa and PwDmc1 expression were performed on cDNA samples obtained from male GM complexes cultured in 2 µM all-*trans* RA containing medium for 2 or 5 days. Results are expressed as the mean of C(t) ratios from triplicates. PwVasa amplification served as a control for relative gem cell amounts.

Fig. 5. PwCyp26b1 activity modulates meiosis entry in male and female gonad–mesonephros (GM) complexes cultured ex vivo. **A:** Three GM from males at stage 54 were exposed for 2 days to 2 µM of the Cyp26b1 inhibitor R11866 or to 1 nM of the nondegradable analogue of RA Am580. One microgram of total RNA was reverse transcribed and qualitative amplification of PwDmc1 and PwGapdh were performed as previously described. **B:** Histology of GM complexes cultured for 2 days in 1 nM Am580 containing medium. pGC, primordial germ cell; mGC, meiotic germ cell with condensed chromatin. **C:** Three GM from females at stage 54 + 90 days (after onset of meiosis) were exposed for 2 days to 60 µM of the PwRaldh2 inhibitor Citral alone or with 1 nM Am580. One microgram of total RNA was reverse transcribed and qualitative amplification of PwDmc1 and PwGapdh were performed as previously described. PwSox9 expression analysis serves as control for Citral treatment efficiency.

PwCyp26b1 Expression Is Repressed in Female Gonad at Meiotic Stage In Vivo

Because RA seems to play a key role in meiosis entry in organotypic cultures, we underwent in vivo experiments wherein GM complexes were dissected from males and females at stage 54 and stage 54 + 60d, *id est* before or after female meiosis onset, respectively. Western-blotting analyses of PwCYP26 and PwRALDH expressions were performed in both sexes and stages using specific polyclonal antibodies prepared and characterized in the laboratory (see the Experimental Procedures section for further details). The results presented in Figure 6A indicate that PwRALDH is expressed at the same level in males and females at both stages, whereas PwCYP26 expression is diminished in female gonads at the time of meiosis onset (54 + 60d). These results were further confirmed by quantification of the two enzyme expression levels in both sexes from stage 50 to adult stage (Fig. 6B). Before metamorphosis, protein expression was measured in GM complexes because dissection of individual gonads does not give sufficient material. Immunohistological analyses performed at stage 56 with the same antibodies confirmed a higher level of PwCYP26b1 expression in male mesonephros and gonad than in female ones, which correlates with the presence of meiotic germ cells in the ovaries but not in the testes. Such analysis also provided further information on PwRALDH and PwCYP26 localization in male or female GM complexes at the time of metamorphosis. Both enzymes are expressed in mesonephric ductules, with a higher level of CYP26b1 expression observed in male ones. RALDH2 expression is also observed into somatic and germ cells of the gonads, whereas CYP26b1 appears to be mainly expressed into testicular germ cells (Fig. 6C).

After metamorphosis, Western blots were performed on gonads separated from adjacent mesonephroi. Quantification of enzyme expression levels clearly show that, before male meiosis onset occurs, PwCYP26b1 is still expressed at a higher level in male gonads than in female ones, whereas in the adult stage, this difference is no more observed. The same sex-dependent differential expression level was also obtained in kidney, but to a lesser extent (data not shown).

Hormonal Status of the Gonad May Alter Germ Cell Differentiation

Steroid hormones play a major role in sex differentiation in urodele amphibians and can even lead to complete functional sex reversal (Kuntz et al., 2003a). In *Pleurodeles waltl*, aromatase activity that leads to estradiol (E_2) production increases in female gonads from stage 52 (Chardard et al., 1995; Kuntz et al., 2003a,b). This induction is also observed even if delayed until late stage 54, in males feminized with E_2 treatment. Therefore, we investigated if the hormonal status of the gonad could modify endogenous production/degradation of RA and alter germ cell differentiation.

We analyzed PwDmc1 expression in ZZ larvae treated with E_2 (100 μg/L from stage 50), or ZW larvae grown in presence of the nonaromatizable androgen DHT (400 μg/L from stage 50). No expression was detected until stage 54 + 60d (not shown). At the time of metamorphosis (stage 56), PwDmc1 expression was observed in E_2-treated ZZ larvae but not in DHT-treated ZW ones (Fig. 7A). This result was correlated to the morphology of the gonad (Fig. 7B). Indeed, E_2-treated ZZ animals were sex-reversed and possessed an ovary with an ovarian cavity and germ cells (meiotic or not) localized in the cortex, whereas the gonad from DHT-treated ZW larvae showed testes containing a few primordial germ cells with a polylobular nucleus in the medulla, surrounded by nonorganized somatic cells.

In an attempt to correlate germ cell phenotype and the presence of RA in hormone-treated or standard animals, we measured the mRNA expression levels of PwRaldh2 and PwCyp26b1 in vivo. At stages 54 + 90d and 56 (metamorphosis), high level of PwCyp26b1 expression was detected in gonads from standard males but not from standard females or E_2-treated males. Similar levels of PwRaldh2 transcripts were observed in all samples (Fig. 7C). Therefore, the low level or absence of PwCyp26b1 expression seems to correlate with meiosis entry in sex-reversed animals.

DISCUSSION

In this study, we investigated the mechanisms that trigger germ cell differentiation in the urodele amphibian *Pleurodeles waltl*. We show that female germ cells enter meiosis at the end of larval life (54 + 60d), whereas in male germ cells, meiosis is delayed until juvenile stage (metamorphosis + 2 months). This result is correlated with morphological changes in germ cell nuclei. Using an organ culture system, we demonstrate for the first time in an amphibian model that RA determines germ cell fate in male gonad because addition of exogenous all-*trans* RA is sufficient to trigger premature meiosis entry. The same situation was described in mouse where exposure of embryonic testis to RA induces the expression of the meiotic markers Stra8, Dmc1, and Scp3 (Bowles et al., 2006; Koubova et al., 2006). Recently, a key role of RA in regulating the premeiotic marker Stra8 was also demonstrated in avian embryos through a comprehensive analysis of genes involved in RA metabolism during development (Smith et al., 2008).

To test if PwCyp26b1, the enzyme degrading RA in vivo, could be a meiosis inhibiting factor in male *Pleurodeles*, GM complexes were explanted before meiosis entry and grown in the presence of the specific Cyp26b1 inhibitor, R115866. This experiment demonstrated that inhibition of PwCyp26b1 (i.e., actual raise of endogenous RA level) was also able to induce PwDmc1 mRNA expression and chromatin condensation in larval male germ cells. However, prolonged exposure to RA through a 5-day treatment with Am580 is associated with nuclei picnosis. This may reflect a more general feature of Cyp26b1 activity, which was recently demonstrated in the mouse model to be not only necessary for germ cell differentiation in ovaries but also for their survival in testes (Maclean et al., 2007). Moreover, it was shown that mouse embryonic testes exposed to RA on 13.5 dpc in organotypic culture display an inhibition of mitotic arrest,

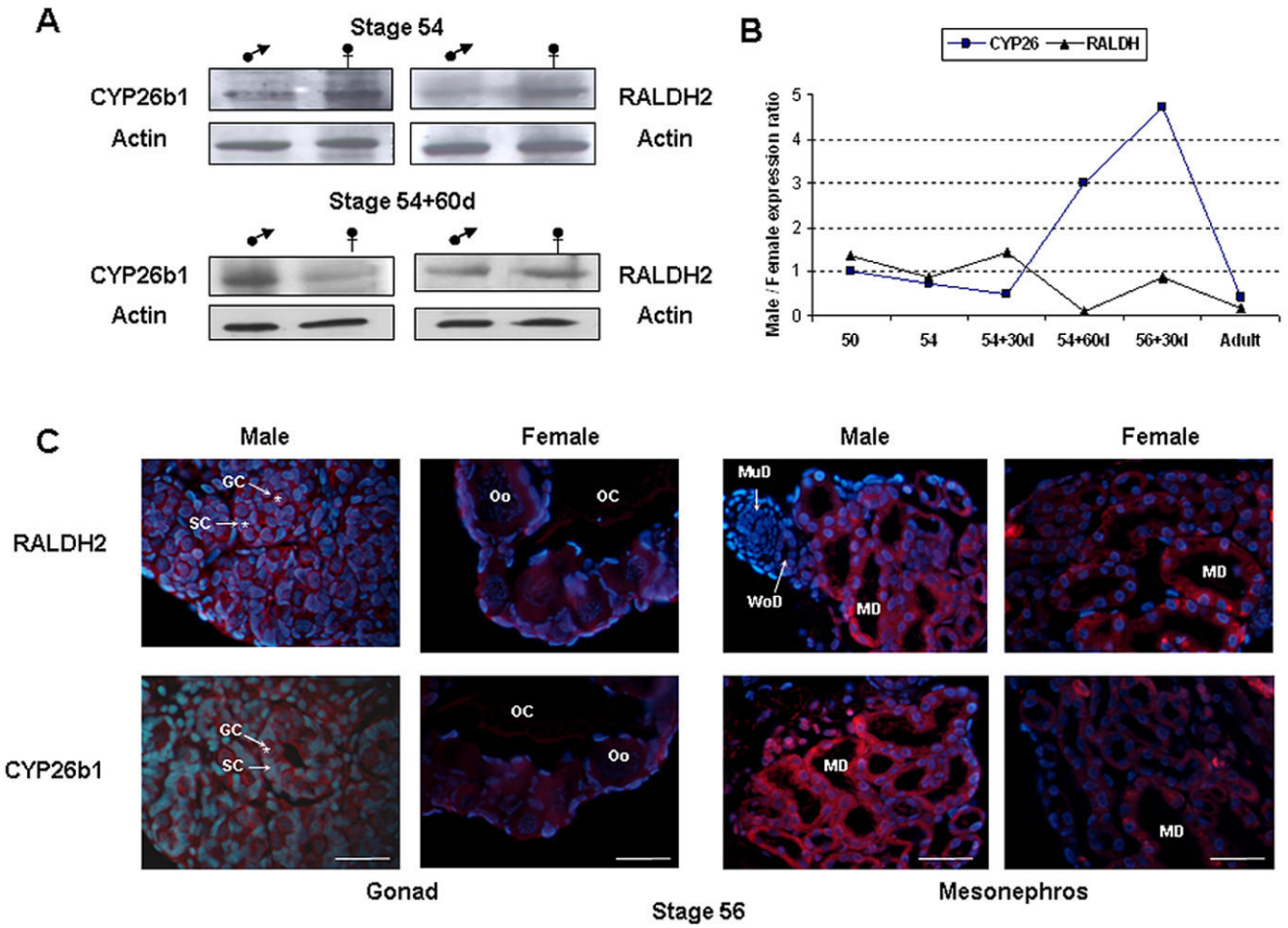

Fig. 6.

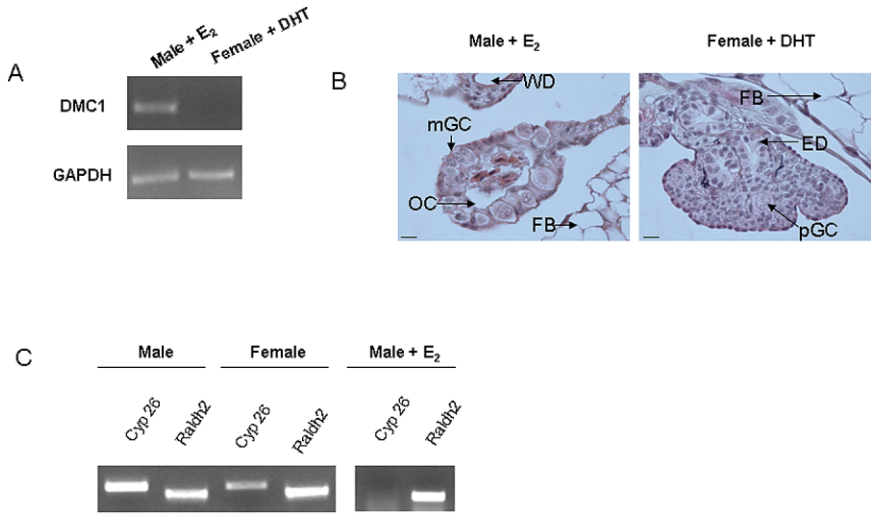

Fig. 7.

which in turn, induces germ cell death with typical picnotic nuclei (Trautmann et al., 2008).

To identify the source of RA in vivo and the time course of its synthesis/degradation, we measured the expression of both PwCyp26b1 and PwRaldh2 at the protein level. In *P. waltl*, both enzymes are expressed in both gonad and mesonephros. Until stage 54, PwRaldh2 and PwCyp26b1 are expressed in GM complexes of both sexes at a similar level, as described in mouse and chicken before female meiosis onset (Bowles et al., 2006; Smith et al., 2008). At stage 54 + 60 days, by the time of female meiosis entry, we observed a reduction of PwCyp26b1 protein level in female complexes, suggesting that an elevation of RA level at least contributes to meiosis entry in vivo. In fact, when the same experiments are performed after metamorphosis, PwCyp26b1 transcript and protein levels also appear to be correlated to meiotic status because standard females as well as sex-reversed males display low level of PwCyp26b1 transcripts in their gonads which contain meiotic germ cells, whereas standard males or neomales (sex-reversed ZW larvae) display high levels of

PwCyp26b1 transcripts but no meiotic cells. Therefore, *Pleurodeles waltl* germline may follow an RA-dependent differentiation process as in mouse and chicken.

In mammals, steroid hormones control genital ducts differentiation and secondary sex characteristics development, whereas in lower vertebrates like urodele amphibians, steroids act at primary steps of sex differentiation (Jost, 1953; Hayes, 1998). Indeed, treatments of *P. waltl* larvae with hormones, anti-hormones, or steroidogenesis enzyme inhibitors can lead to sex reversal (Kuntz et al., 2003a). For example, E_2-treated males differentiate fertile ovaries, while treatments with androgens lead to various results: testosterone-treated males give rise to functional neofemales due to aromatization of the androgen by P450-aromatase while vestigial testes are observed in females treated with the nonaromatizable DHT (Chardard et al., 2003). Therefore, we finally addressed the question of steroid effects on meiosis entry and RA synthesis/degradation pathway in vitro and in vivo.

First, we observed that seasonal variation appeared in PwDmc1 expression in adults (from 18 months old), with male meiosis occurring all year long, whereas females seem to renew their oocyte stock only during summer (May to September) after the reproduction period (data not shown). These seasonal fluctuations of detectable number of germ cells in meiotic prophase in females are in agreement with an elevated ratio of [E_2-17 beta]/[Androgen] which is stable (ratio = 0.2 to 0.3) during the major part of the year, except in July and August, when it reaches the value of 1.0 (Garnier, 1985a). However, no relationships were found between the presence of meiotic germ cells that are observed along the reproductive season in males (September to May) and the two peaks of androgen plasmatic levels (October–November and March) (Garnier, 1985b).

Second, we showed that meiosis entry occurs in males treated with E_2 at the same time as in standard females, whereas it is delayed until after metamorphosis in DHT-treated females. Therefore, hormone-dependent sex reversal cannot only promote the reorganization of the gonad somatic compartment but also affect the germ cell differentiation process, at least in part, through the modulation of RA metabolism. Moreover, our results indicate that the transcription level of PwCyp26b1 is higher in male than in female and E_2-treated neofemale, respectively, suggesting that a high level of E_2 in gonad environment is associated with a high repression level of PwCyp26b1 expression.

Connections between RA signaling and E_2 transduction pathways appear to be also relevant at the molecular level and seem to be a general feature of cells from mesodermic origin that undergo epithelium formation. From 13.5 dpc, mouse male germ cells stop mitotic proliferation and associate to somatic cells to form testicular cords. In fact, RA was demonstrated to block this process through PI3-kinase signaling (Trautmann et al., 2008). In addition, Giretti and Simoncini (2008) pointed out the regulatory actions of E_2 in regulating, focal adhesion assembly involved in epithelial–mesenchyme transition (EMT), namely through the PI3-kinase pathway. Therefore, it would be interesting to address the potential effects of PI3K inhibitors on male gonad differentiation in the course of normal development or during E_2-mediated sex reversal in *Pleurodeles waltl*.

Here, we currently bring the first data about the regulation of RA biosynthesis by steroids in the gonads of an amphibian species. Because it was demonstrated that the gonadic promoter of aromatase, which also drives gene expression in breast cancer, can be negatively regulated by retinoid receptors RAR and RXR (Chen et al., 2002; Rubin et al., 2002), the molecular basis of the crosstalk between RA and steroids during sex differentiation might be more complex than expected up to here and remain to be determined.

EXPERIMENTAL PROCEDURES

Animals

Embryos and larvae were staged (Fig. 1) according to the development timetable stages described by Gallien and Durocher (1957). During larval life, animals were analyzed from stage 48 to stage 56 at which metamorphosis takes place. Juveniles were also harvested every 2 weeks to study meiotic process. Before manipulation, animals were anesthetized with 0.03% benzocaine. The local Animal Care and Use

Fig. 6. Expression of the enzymes synthesizing (PwRaldh2) and degrading (PwCyp26b1) retinoic acid (RA) in larval gonad–mesonephros (GM) complexes of both sexes. **A:** Western blotting analysis of PwCYP26 and PwRALDH expression. A total of 30–50 μg of total protein were probed with 1:1,000 polyclonal-specific antibodies obtained and purified in our laboratory as described in the Experimental Procedures section. **B:** Male/female expression ratios of CYP26b1 and RALDH2 during Pleurodeles development. At stage 50, 54, 54 + 30, and 54 + 60, analyses were performed on pools of either 10 male or female gonad-mesonephros complexes; at juvenile and adult stages, expression level was determined on pools of 3 isolated gonads. Measurements were performed once at stage 50, on 2 pools at stage 54, 54 + 30, and 54 + 60 and juvenile and three times in adults. The graph shows the results of a representative experiment where all stages were analyzed on the same blot. **C:** Immunolocalization of the cells expressing both enzymes in male and female GM complexes at stage 56. Seven-micrometer sections of paraffin-embedded GM complexes were incubated with specific primary rabbit antibody, anti-PwCYP26 or anti-PwRALDH at the dilution 1:1,000 and then exposed to Alexa fluor 555 goat anti-rabbit IgG antibody diluted to 1:1,000 (Invitrogen Corp., Carlsbad, CA). DNA was stained with Hoechst 33342 to visualize the nuclei. Scale bars = 100 μm. GC, germ cell; MD, mesonephric duct; MuD, Müllerian duct; OC, ovarian cavity; Oo, oocyte; SC, somatic cell, WoD, Wolffian duct; asterisks indicate stained cells in testis.

Fig. 7. Molecular and histological phenotype of sex-reversed animals after metamorphosis (stage 56). **A:** Expression of the meiotic marker PwDmc1 was assessed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) on isolated gonads sampled from E_2-treated males or DHT-treated females. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) amplification served as a control for relative RNA amounts. **B:** The global morphology of the gonads and the differentiation status of germ cells were observed after hematoxylin–eosin staining of gonads from animals of the same batch at the same stage. ED, efferent duct; FB, fat body; pGC, primordial germ cell; MGC, meiotic germ cell; OC, ovarian cavity; WD, wolffian duct. Scale bars = 20 μm. **C:** Transcription levels of enzymes synthesizing (PwRaldh2) or degrading (PwCyp26b1) retinoic acid were also measured in nontreated or treated animals by semiquantitative RT-PCR. One microgram total RNA was reverse transcribed, and amplifications of PwRaldh2 and PwCyp26b1 were performed as described in the Experimental Procedures section.

Developmental Dynamics

Committee approved the experimental protocols, and guidelines for laboratory procedures were followed at all times.

Chemicals and Solutions

Leibovitz medium (L15) was diluted in sterile water (2:1) to respect the low osmotic pressure required for amphibian tissue culture and supplemented with 1% L-glutamine (Invitrogen, Cergy-Pontoise, France), 10% stripped fetal calf serum (Sigma-Aldrich, France), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen). The concentrations of compounds in culture media were as follows: all-*trans* RA 2 μM, Am580 2 μM, Citral 60 μM (all three from Sigma-Aldrich), and CYP26 inhibitor R115866 2 μM (gift from Johnson and Johnson, New Brunswick, NJ). In vivo treatments were performed by addition of hormones in rearing water: Estradiol (Sigma-Aldrich) 100 μg/L, Dihydrotestosterone (Fluka, Sigma-Aldrich) 400μg/L.

Determination of Sexual Genotype and Phenotype

For sexual genotype analysis, at stage 48, a biopsy was obtained from the tail of the animals anesthetized with 0.03% benzocaine. The tissue was homogenized in 50 mM Tris-HCl, 2.5 mM $MgCl_2$, 25 mM NaCl buffer. Samples were electrophoresed in a 0.7% agarose stacking – 10.5% starch running horizontal gel, in 25 mM Tris-Citrate pH 8.0 buffer. The patterns of peptidase-1 were revealed by specific hydrolysis of valyl-leucine substrate coupled with peroxidase colored reaction as previously described (Dournon et al., 1988).

RT-PCR

The detailed protocol for reverse transcription has been previously described (Kuntz et al., 2003b). Total RNA was extracted from pools of three GM complexes at stages 53, 54, or 54 + 60 days or from 20–50 mg of adult tissues using 200 μl of TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and quantified. Total RNA (1 μg) was reverse transcribed using hexamer primers and 100 U Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a total volume of 25 μl. A 2-μl aliquot of resultant cDNA was used for PCR.

The amplification was performed with 0.5 U of *Taq* DNA Polymerase (Invitrogen) in PCR buffer containing 25 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, and 10 pmol of each primer in a total volume of 25 μl. Specific primers and conditions of amplification for PwGapdh, PwVasa, and PwDmc1 cDNAs have been described previously (Dumond et al., 2008b). Primers for PwCyp26b1 (dir 5′-GAC-AAG-AGC-TGC-AAG-CTG-CC-3′; rev 5′-GCT-GGA-TCT-TGG-GCA-**GGT-A-AC**-3′), PwRaldh2 (dir 5′-CCC-ATT-GGA-GTG-TGT-GGA-CAG-ATC-3′; rev 5′-CTT –CTT-CCA-GCT-GCT-TCT-TG-3′), and PwSox9 (GenBank accession no. EU872027) (dir 5′-GAG-GGC-TCC-GAG-CAA-ACG-CAC-3′; rev 5′-GCT-CTG-CTC-GCT-GCC-CAG-TGT-3′) were used for a 35 cycles PCR (1 min 95°C; 1 min 55°C, 52°C, and 66°C, respectively; 1 min 72°C).

PCR products (12 μl) were run in a 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Quantitative real-time PCR analysis was performed on an Opticon 2 System (BIO-RAD laboratories) by using SYBR-green labeling. Each cDNA sample was analyzed in triplicate with specific PwVasa or PwDmc1 primers (Dumond et al., 2008b). PwDmc1 expression was normalized to PwVasa transcript expression as a germ cell control gene.

Western Blotting

Pools of 3 gonad-mesonephros complexes from larvae were sonicated on ice in PY lysis buffer (Etique et al., 2006) for 5 sec (Branson sonifier 150, Branson Ultrasonics, Danbury, MA). After 10 min at 13,000 rpm centrifugation, supernatants were sampled and boiled for 5 min in Laemmli buffer. Total proteins (30–50 μg) were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to an Amersham Hybond enhanced chemiluminescence membrane (Amersham Bioscience Corp, Piscataway, NJ). The membrane was blocked for 1 hr at room temperature in Tris buffered saline containing 0.1% Tween and 5% bovine serum albumin. After blocking of nonspecific binding, membrane was incubated overnight at 4°C with a specific polyclonal anti-PwCYP26 (amino acids 2–80) or anti-PwRALDH (amino acids 6–98) rabbit serum, produced in the laboratory and diluted to 1:1,000 in the blocking buffer. The secondary anti-rabbit antibody was used following manufacturer's instructions (Amersham Bioscience Corp). Immunoreactive proteins were detected using enhanced chemiluminescence (ECL kit, Amersham Bioscience Corp).

Organ Culture

GM complexes were removed aseptically from the larvae anesthetized with 0.03% benzocaine (stage 48 to 54) under a binocular microscope. Whole explants were transferred to the in vitro culture system: the complexes were placed on a single Millipore filter (HA, pores size: 0.45 μm; Bedford, MA). The filters bearing the explants were floating on 1 ml of culture medium in tissue culture dishes and incubated at room temperature (20 ± 2°C) under noncontrolled gas composition. The complexes were maintained for 2–5 days with the medium being replaced once a day. Cultured explants were harvested for RNA extraction and RT-PCR analyses, or incubated for 48 hr in Bouin's fixative for histological analysis. No necrosis was ever observed in any part of the tissues.

Histology

GM complexes were fixed in Bouin's solution, embedded in paraffin, and sectioned at 7 μm. Sections were stained with hematoxylin–eosin–light green as described previously (Dumond et al., 2008b).

Immunohistochemistry Analysis

GM complexes were fixed overnight in 0.05 M Tris-HCl buffer pH 7.4 containing 4% paraformaldehyde, embedded in paraffin, and sectioned at 7 μm. PwCYP26 and PwRALDH immunolabeling were performed on sections deparaffinized in Histolemon (Carlo-Erba Corp., Val de Reuil, France) and gradually rehydrated. Slices were incubated with BSA 4% for 10 min at

room temperature to block nonspecific binding. Then these sections were incubated for 30 min with specific primary rabbit antibody, anti-PwCYP26 or anti-PwRALDH at the dilution 1:1,000. After two washes with Tris-HCl buffer, they were exposed for 30 min to Alexa fluor 555 goat anti-rabbit IgG antibody diluted to 1:1,000 (Invitrogen). Finally, the sections were rinsed with Tris-HCl buffer for 10 min and DNA was stained with Hoechst 33342 to visualize the nuclei. Fluorescence labeling was observed under a Nikon microscope.

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Estrogens promote proliferation of the seminoma-like TCam-2 cell line through a GPER-dependent ERα36 induction

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ABSTRACT

Seminoma, originated from carcinoma in situ cells (CIS), is one of the main causes of cancer in young men. Postpubertal development of these testicular germ cell tumors suggests a hormone-sensitive way of CIS cell proliferation induction.

Using the unique seminoma TCam-2 cell line, we demonstrate that both estradiol and testosterone can stimulate TCam-2 cell proliferation in the absence of the estradiol receptor ERα. We establish that estradiol can activate GPER-cAMP/PKA signalling pathway. TCam-2 cells express ERα36, a truncated isoform of the canonical ERα receptor, the expression of which is rapidly induced after estrogen treatment in a GPER-dependent manner. ERα36 knockdown indicates that ERα36 is (i) a downstream target of E_2-activated GPER/PKA/CREB pathway, (ii) required for estradiol-dependent EGFR expression, (iii) necessary for cell proliferation. Colocalization of ERα36 with cytoskeleton microfilaments suggests a role of estrogens in cell motility.

Our results highlight the functional role of ERα36 in context of seminoma cell proliferation and the importance of testing ERα36 in vivo as a possible future prognostic marker.

1. Introduction

Among malignant tumors of the testis, 95% are type II germ cell tumors (GCT), which incidence has worryingly risen over the last 50 years to become the most common tumors and the leading cause of cancer among young men aged 15–40 years. Based upon histologic traits, type II GCTs are classified into two main categories: seminoma and nonseminoma, both derived from a common precursor cell type called carcinoma in situ (CIS) (Skakkebaek, 1972).

CIS are believed to originate from developmentally arrested primordial germ cells or gonocytes (Almstrup et al., 2005; Rajpert-de Meyts and Hoei-Hansen, 2007; Sonne et al., 2009) that enter a period of dormancy until after puberty when type II GCT emerge. This prepubertal dormancy suggests a hormone-dependent mechanism for type II GCTs development at puberty, or tumor progression (McIntyre et al., 2007, 2008). Additionally, several pathologies related to testis cancer, such as cryptorchidism, hypospadias as well as decreased sperm counts, have been observed mainly after exposure to endocrine disruptors, i.e. environmental molecules having either estrogenic or anti-androgenic effects (Martin et al., 2008; Main et al., 2010).

The androgen/estrogen balance is essential for reproduction in mammals, especially for gonad development. In the testis, this balance is tightly regulated by endocrine and paracrine factors and the enzyme aromatase that converts androgens to estrogens. *Cyp19* transcripts were detected in Leydig and Sertoli cells as well as differentiated germ cells extending from spermatocyte to spermatozoan stage (Carreau and Hess, 2010; Carreau et al., 2010). The role of estrogens in male gonads and reproductive tract was highlighted by the discovery of patients genetically deficient in aromatase who display variable degrees of fertility disorders up to impaired spermatogenesis (Jones et al., 2006). Depletion of endogenous estrogens in the aromatase deficient mice (ArKO) resulted in an impairment of sexual behaviour and an age-dependent disruption of spermatogenesis. This disruption occurred during early spermiogenesis, possibly due to an increased number of apoptotic round spermatids (Murata et al., 2002).

Once produced, estrogens can act through an interaction with estrogen nuclear receptors (ERs, i.e. ERα and ERβ) which in turn exert their biological effects via both genomic and nongenomic signalling and transcriptional stimulation of estrogen responsive genes. The genomic estrogen signalling is mediated directly by binding to nuclear ERα and ERβ responsive elements. Through nongenomic ways, estrogen-like molecules can target kinase cascades, and tyrosine kinases or membrane-associated molecules such as

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ion channels and G protein-coupled receptors (Simoncini et al., 2002, 2004). ERβ is expressed in either somatic (Leydig, peritubular, Sertoli) or germ cells (gonocytes to spermatozoans) of human testis whereas ERα is absent from gonocytes, peritubular and Sertoli cells (Pentikäinen et al., 2000; O'Donnell et al., 2001). Studies in rodents, namely the phenotype of single ERαKO or ERβKO or of double ERα/ERβ KO mice have demonstrated that ERβ triggers a negative signal toward germ cell proliferation (Delbès et al., 2006).

Ten years ago, Filardo (Filardo et al., 2000; Filardo, 2002) described the transmembrane receptor GPR30, an orphan member of the G protein-coupled receptor superfamily that can mediate estrogen responsiveness of ER-negative breast cancer cells. Now renamed GPER, this receptor mediates a wide range of responses to estrogen in a large variety of cell types through cAMP, MAPK and PI3K pathways, some of which being redundant with those of nuclear estrogen receptors (Pandey et al., 2009). Furthermore, several environmental contaminants (bisphenol A, zearalenone, nonylphenol) which were shown to exert estrogenic actions through binding to ERs also bind to GPER with a sufficient affinity to allow the activation of alternative estrogen signalling pathways in ER-negative GPER-positive cell lines (Thomas et al., 2005; Thomas and Dong, 2006; Maggiolini and Picard, 2010; Bouskine et al., 2010).

In 2005, Wang and colleagues described a novel 36 kDa ERα isoform called ERα36, a truncated form of the canonical human estrogen receptor ERα66, retaining the DNA-binding, partial dimerisation and ligand–binding domains. Owing to the lack of both transcriptional activation domains (AF-1 and AF-2), this protein was thought to function as a dominant-negative of ERs. The involvement of ERα36 in nongenomic estrogen signalling, concurrently to GPER, was further demonstrated in various breast cancer cell lines (Wang et al., 2005, 2006; Lee et al., 2008; Lin et al., 2009, 2010). More recently, Zhang et al. (2010) demonstrated that ERα36 mediates E_2 dependent signalling in ER-negative breast cancer cells suggesting that a subset of ER-negative tumors thus retains responsiveness to mitogenic estrogen signalling.

Although the transcriptional responses mediated by the nuclear ERs have been extensively characterized, the changes in gene expression elicited by signalling through GPER or ERα36 have not been studied in the context of either germ cell neoplasia or testicular germ cell cancer. Our aim was to decipher the estrogen-dependent transduction pathways in TCam-2 cells, a unique seminoma cell line isolated by Mizuno from a pure seminoma (Mizuno et al., 1993). First, we show that either estradiol or testosterone can increase the TCam-2 proliferation rate by triggering the stimulation of ERα36 expression. Second, we indicate that those effects depend on GPER-PKA pathway activation. Finally, we demonstrate that estrogen signalling pathway can trigger ERα36-dependent EGFR expression.

2. Materials and methods

2.1. Reagents

Steroids, BSA-conjugated steroids, H89 (cAMP/PKA inhibitor) and dimethylsulfoxyde (DMSO) were purchased from Sigma–Aldrich (France). All compounds were solubilized in DMSO for 1×10^{-2} M stock solution and further diluted in RPMI medium without phenol red. Working concentrations were 1 nM for 17β-estradiol (E_2) and BSA-conjugated 17β-estradiol (E_2BSA), 1 μM for testosterone (T) and BSA-conjugated testosterone (TBSA). Control cells were treated with 0.01% DMSO.

2.2. Cell culture

TCam-2 cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (FCS, Sigma) and 2 mM L-glutamine (Sigma) in 5% CO_2 at 37 °C. Briefly, cells were plated in 10% FCS containing RPMI medium for 24 h and then starved for 24 h in 0.5% stripped FCS containing RPMI medium without phenol red. Treatments were performed on FCS deprived cells plated at a density of 8×10^4 cells per well in 6-well plates. In case of inhibitor use, the corresponding compound was added to the medium 30 min before steroid treatment.

2.3. Cell proliferation assay

Cells were seeded in 6-well plates at a density of 4×10^4 cells/well in 2 mL RPMI medium supplemented with 10% FCS and 2 mM L-glutamine. Cells were washed with PBS (GIBCO) once they had attached and then incubated in phenol red-free RPMI containing 1% charcoal-stripped FCS for 24 h. Cells were then treated with the indicated treatments; medium was renewed every 2 days (with treatments). At the end of the treatment, cells were washed with PBS, trypsinized and counted with the CellTiter-GloTM Luminescent Cell Viability Assay (Promega, Charbonnières, France) on a VICTOR™ *X*3 Multilabel Plate Reader (Perkin–Elmer). Each treatment was performed in triplicate.

2.4. Colony formation assay

Clonogenic assay was performed into 6-well plates coated with 0.5% Agar containing 10% FCS supplemented RPMI. Each well then received 3×10^2 TCam-2 cells dispersed into 1.2% methylcellulose (R&D systems, Lille, France), 20% FCS supplemented RPMI. Plates where then incubated at 37 °C, in a 0.5% CO_2, humid atmosphere, for 14 days before colony counting. Additionally, cells were treated with 1 nM E_2, 1 μM T or DMSO as vehicle. Colonies containing more than 100 cells, i.e. ⩾0.5 mm diameter in size, were taken into account and counted by using an inverted microscope.

2.5. Real-time PCR analysis

RNA was extracted using TRIzol reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions. Reverse transcription was performed using random hexamer primers and mouse Moloney leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions. PCR reactions were performed in the Opticon 2 detection system (Biorad, Marnes-la-Coquette, France), using 0.1 μM of each primer, IQ SYBR Green Supermix (Biorad, Marnes-la-Coquette, France) in a total volume of 15 μL. The ribosomal protein *RPLPO* encoding mRNA was used as a control to obtain normalized values. Primers are listed in Table 1. Assays were performed at least in triplicate, and the mean values were used to calculate expression levels, using the $\Delta\Delta C(t)$ method. For each gene expression tested, the value indicated on figures was calculated as the ratio gene of interest/reference gene (*RPLPO*). When steroid treatments were performed, the variation of expression was measured as steroid treated/DMSO treated cells.

2.6. Semi-quantitative RT-PCR

Total RNA (1 μg) was reverse transcribed using hexamer primers and 100 U M-MLV reverse transcriptase in a total volume of 25 μL. A 2 μL aliquot of resultant cDNA was used for PCR.

The amplification (40 cycles for *Cyp19* or 24 cycles for *RPLPO*) was performed with a 55 °C hybridization temperature with 0.5 U *Taq* DNA Polymerase (Invitrogen) in PCR buffer containing 25 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$ and 10 pmol of each primer in a total volume of 25 μL. PCR products (12 μL) were run in a 1% agarose gel containing 0.5 μg/mL ethidium bromide.

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Table 1
Primer list.

Gene	Forward primers (5′–3′)	Reverse primers (5′–3′)
RPLPO (QPCR)	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
ERα36 (QPCR)	CCAAGAATGTTCAACCACAACCT	GCACGGTTCATTAACATCTTTCTG
CYP19	CTGGAAGAATGTATGGACTT	GATCATTTCCAGCATGTTTT
RPLPO	ACAACCCAGCTCTGGAGAAA	TCGTTTGTACCCGTTGATGA
GPER	TGGTGGTGAACATCAGCTTC	AAGCTCATCCAGGTGAGGAA

2.7. RNA interference

The small-interfering RNA (siRNA) duplexes for targeting GPER and scrambled control (negative control) were purchased from Eurogentec (Belgium). The sequences of the human GPER siRNAs were 5′-GCACCUUCAUGUCGCUCUU-3′ and 5′-AAGAGCGACAUGAAGGUGC-3′ for duplex n°1, 5′-UUGAACACCUCAAUGAGGG-3′ and 5′-CCCUCAUUGAGGUGUUCAA-3′ for duplex n°2, 5′-AAGACUGCUUGCAGGGAGC-3′ and 5′-GCUCCCUGCAAGCAGUCUU-3′ for duplex n°3. The control duplex was provided by Eurogentec (SR-CL000-005, Angers, France). The day before transfection, 8×10^4 cells/well were plated into 6-well plates, in 2 mL of RPMI medium supplemented with 10% FCS and 2 mM L-glutamine. Cells were transiently transfected with either GPER (200 nM of the mix duplex 1, duplex 2 and duplex 3) or control siRNAs using the Oligofectamine™ Reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were washed with PBS and the medium was replaced with phenol red-free RPMI supplemented with 1% charcoal-stripped FCS and 2 mM L-glutamine. 24 h later, cells were treated in phenol red-free and FCS free RPMI and harvested for further analyses.

2.8. Western immunoblotting

Total proteins were separated by 12% SDS–PAGE. Proteins were then transferred onto Hybond-ECL nitrocellulose membrane (Amersham), where non specific binding sites were blocked in TNT buffer (5 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat powder milk, and incubated overnight at 4 °C with polyclonal anti-ERα36 kindly provided by Dr Wang Z.Y. (Creighton University Medical School, Omaha, USA) diluted at 1:500, anti-cyclinD1 (1:500; Cell signalling #2922, Saint Quentin, France), anti-ERα (sc73479, Santa Cruz Biotechnology Inc., Santa Cruz, USA), anti-EGFR (1:1000; Cell signalling #2232, Saint Quentin, France), anti-phospho CREB (1:1000; Epitomics #1113-5, Montrouge, France) or anti-alpha tubulin antibody (Santa-Cruz) diluted at 1:5000 as a loading control. Next, the membranes were washed 3 times with TNT and incubated for 1 h with a peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com). Western blot signals were detected by the use of ECL Western Blotting Substrate (Pierce) and exposition to Clear Blue X-ray film (CL-XPosure, Thermo Scientific).

2.9. Immunocytochemistry (ICC)

After a 30 min pre-treatment with 10 μM H89 (cAMP/PKA pathway inhibitor) and a 15 min treatment with 1 nM E_2, cells were fixed for 15 min in PBS, 4% parafolmaldehyde (Merck). Then, samples were permeabilized in 0.25% Triton X-100 (Sigma) in PBS for 10 min and a 30-min step of saturation was performed in 4% bovine serum albumin (BSA, Sigma) and 0.1% Triton X-100 containing PBS. Primary antibody incubations were carried out for 1 h with anti-EGFR (1:200; Cell signalling #2232, Saint Quentin, France), anti-phospho CREB (1:1000; Epitomics #1113-5, Montrouge, France) or anti-CREB (1:200; Upstate NL904, Euromedex, Mundolsheim, France) antibodies. Cells were washed three times in 0.1% Triton X-100 in PBS for 10 min. Secondary antibody incubations were performed during 45 min with a fluorescent-conjugated secondary antibody Alexa555-conjugated goat anti-rabbit (Invitrogen). Then, samples were washed three times in 0.1% Triton X-100 in PBS for 10 min, and nuclei were stained with Hoechst (bis-Benzimide H 33342 trihydrochloride, Sigma–Aldrich France, 0.1 μg/mL in PBS). Slides were mounted with AquaPoly/Mount (Polysciences Inc., Warrington, PA).

2.10. Transient transfection and establishment of stable cell line

TCam-2 cells transfected with the empty expression vector or the ERα36-specific shRNA expression vector kindly provided by Dr Wang Z.Y. (Creighton University Medical School, Omaha, USA) using the ExGen500 in vitro transfection reagent (Euromedex, France). Transfected cells were kept for 6 h, and then the ExGen500-DNA complex was removed. Two days after transfection, cells were incubated in medium containing 400 μg/mL of G418 Geneticin (Invitrogen) for 3 weeks; the cells proliferated continuously in the presence of G418 and formed a large number of expanding undifferentiated colonies which named as Neo-TCam-2 and sh36-TCam-2, respectively. The transfected cell lines were not subcloned. Rather, knocked down level of ERα36 expression was confirmed by western blot and RT-PCR analysis before each experiment.

2.11. Transmission electron microscopy

Cells were pelleted and fixed for 4 h in 2.5% glutaraldehyde (Euromedex, Mundolsheim, France) in 0.1 M sodium cacodylate buffer, pH 7.2 (Euromedex). Cells were then rinsed with sodium cacodylate buffer and postfixed in 1% osmium tetroxide (Euromedex) for 1 h at room temperature. Samples were washed and then dehydrated through a graded series of ethanol solutions followed by propylene oxide, and then infiltrated in 1:1 propylene oxide/poly Bed 812 (Euromedex). Samples were kept overnight embedded in Poly Bed 812, mounted in molds and left to polymerize in an oven at 56 °C for 48 h. Ultrathin sections (70–90 nm) were obtained with a Reichert-Jung Ultracut S microtome (Wien, Austria).

Sections were exposed to anti-ERα36 antiserum (1:200 dilution in TBS) for 1 h at room temperature. The specimen were subsequently incubated with goat anti-rabbit IgG antibodies conjugated with colloidal gold (particle size of 10 nm) (BioCell, Cardiff, UK) at a 1:10 dilution for 45 min. Finally, sections were stained for 20 min with 2% (w/v) uranyl acetate, viewed, and photographed with a Philips CM12 transmission electron microscope operated at 80 kV.

2.12. Statistical analysis

Realtime-PCR results were analyzed as follows: variance analysis of steroid-treated vs. vehicle-treated cells was performed using Dunnett's test for multiple comparisons. Differences in which P was less than 0.05 were statistically significant.

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3. Results

3.1. Estradiol and testosterone treatment increase TCam-2 seminoma cell proliferation rate

Since both proliferative and antiproliferative effects of estrogens have been demonstrated in various cell lines depending on the dose and the effectors involved, we started this study by measuring TCam-2 cell growth in response to steroids. After 24 h serum deprivation (see Section 2 for details), cells were treated for either 24 h or 48 h with 1 nM estradiol or 1 μM testosterone. Those doses were chosen as a result of dose–response experiments (data not shown) and were in the range of those detected in an adult human testis – 600 ng/mL i.e. about 2 μM for testosterone and 2.4 ng/mL i.e. about 8 nM for estradiol- (Roth et al., 2010). The relative percentage of viable cells after steroid treatment compared to vehicle-treated ones indicates that both estradiol and testosterone significantly stimulate cell proliferation after a 48 h treatment (Fig. 1A). This result was confirmed after 24 h treatment with the same hormone doses by using western blotting analysis of cyclin D1 expression level as a marker of cell cycle progression into S-phase (Fig. 1B). Both estradiol and testosterone induced cyclin D1 protein expression.

A colony formation assay was also performed by resuspending cells into a steroid complemented medium containing 1.2% methylcellulose. Clones containing more than 100 cells were counted after 14 days (Fig. 1C). Both estradiol and testosterone significantly increased the number of colonies once again demonstrating a proliferative effect.

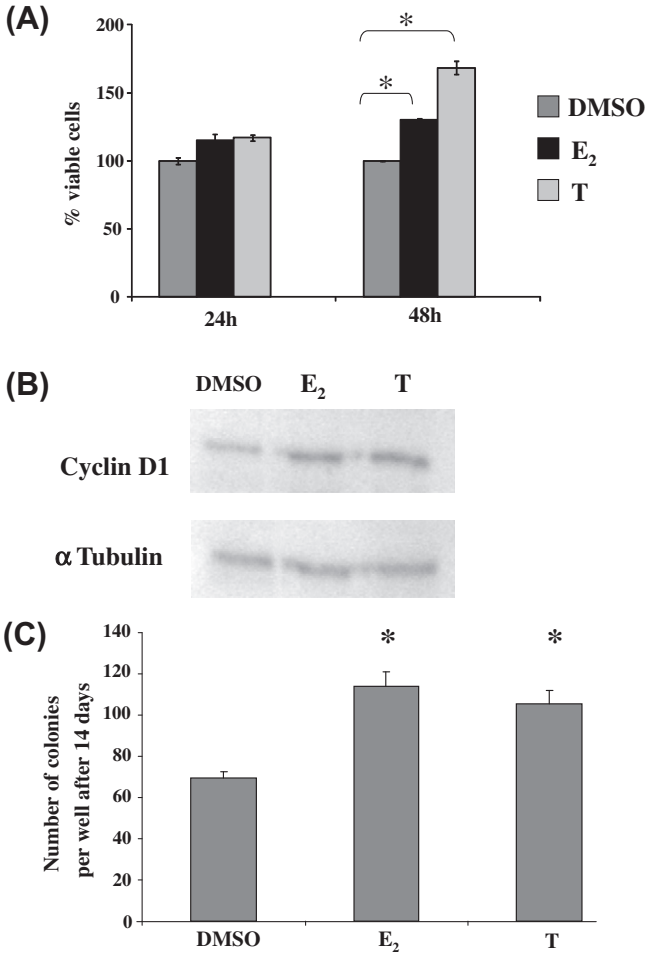

Fig. 1. Steroids can induce TCam-2 cell proliferation. (A) Proliferation test was performed by TCam-2 cell treatment with 1 nM estradiol (E_2) or 1 μM testosterone (T) in medium containing 1% charcoal-stripped FCS for 24 h or 48 h. Control cells were treated with DMSO (0.01%). Cell number was evaluated using the CellTiter-Glo™ Luminescent Cell Viability Assay. Control cell growth was considered as 100%. Results are given as mean ± s.e.m. of 3 different counts. (B) Western-blot analysis of cyclin D1 expression after 24 h of E_2 or T treatment. Cells were cultured for 24 h in phenol-red free, 0.5% FCS containing medium in the presence of 0.01% DMSO, 1 nM E_2 or 1 μM T. Total cell lysates were then subjected to western blot analysis using anti-cyclin D1 or anti-tubulin antibodies. (C) Colony formation assay. For clonogenic growth test, cells were placed into 1.2% methylcellulose 20% FCS and treated with E_2 or T as in (A). Colonies (>100 cells) were counted after 14 days. Results are given as mean ± s.e.m. of 3 different counts. *Significantly different from DMSO treated control ($P < 0.05$).

3.2. Estradiol induces the expression of a 36 kDa ERα isoform

Since ERα36 expression was recently demonstrated to portray E_2 response in some breast tumors (Kang et al., 2010), we investigated the potential role of ERα36 in the response to steroid stimulation in two cell lines originating from type II GCT: TCam-2 seminoma cells and NT2/D1 embryonal carcinoma cells. First, we probed both cell lines for ERα isoforms. Fig. 2A indicates that both TCam-2 and NT2/D1 cells namely express the truncated ERα46 and

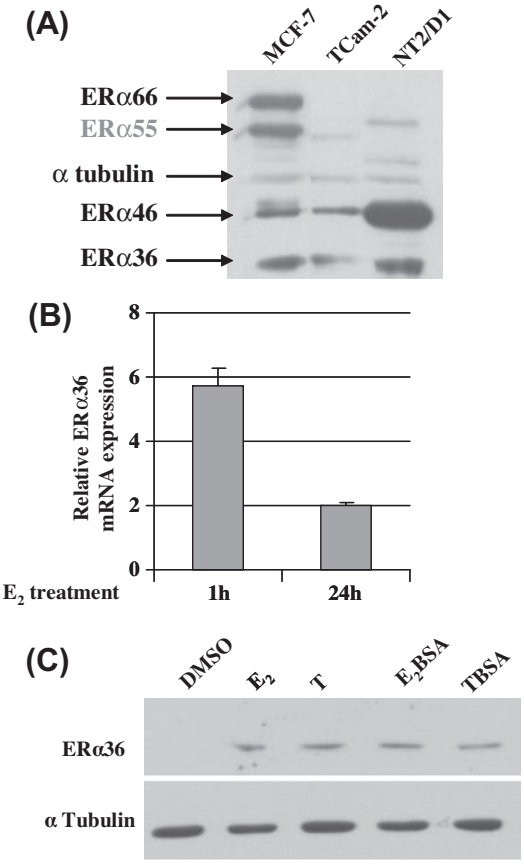

Fig. 2. Estradiol can induce the expression of ERα36 in a GPER-dependent manner. (A) Western blot analysis of ERα isoform expression in TCam-2 seminoma or NT2/D1 embryonal carcinoma cells compared to MCF-7 breast cancer cells. Cells were cultured for 24 h in 10% FCS containing RPMI medium and harvested in 2x Laemmli buffer. The amount of proteins loaded in each well represents 1×10^5 cells. Immunoblotting was performed with an anti-ERα66 antibody. MCF-7 and NTera2/D1 cell lines were used as a control. (B) Expression levels of ERα36 after 1 h or 24 h E_2 treatment in TCam-2 cells as measured by realtime-PCR. Control cells received DMSO. The values represent the means ± s.e.m. of at least 3 different experiments. (C) Western blot analyses of ERα36 protein expression level after 24 h treatment with DMSO (0.01%), E_2 (1 nM), E_2BSA (1 nM), T (1 μM), TBSA (1 μM) in TCam-2. Expression of alpha-tubulin is given as a control.

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ERα36 isoforms but not the full length ERα66 estrogen receptor alpha. MCF-7 breast carcinoma cells were used as control since those cells are known to express the 3 isoforms cited above (Wang et al., 2005).

When TCam-2 cells were treated for 1 h or 24 h by 1 nM E_2, realtime-PCR analysis showed a 6-fold and a 2-fold stimulation of ERα36 expression, respectively (Fig. 2B). This induction was also detectable by western blotting analysis after a 24 h but not a 1 h estradiol or testosterone treatment (Fig. 2C).

Since (i) ERα36 has been shown to localize into the plasma membrane in breast cancer cells (Wang et al., 2006) and (ii) TCam-2 cells express the non specific steroid receptor GPER (Franco et al., 2011), we tested the ability of E_2-coupled BSA and testosterone-coupled BSA, the non permeable form for E_2 and testosterone, to activate ERα36 expression. Fig. 2C shows that both E_2BSA and TBSA stimulate ERα36 protein expression. Similar results were obtained when NT2/D1 cells were treated in the same conditions (data not shown). These results suggest that estradiol signals mainly through membrane receptor signalling (through GPER or directly through ERα36 activation) whereas testosterone could also act through intracellular dependent mechanisms (through androgen receptor activation or aromatization into estradiol).

3.3. ERα36 is not located into the plasma membrane in TCam-2 cells

ERα36 polypeptide chain contains three myristoylation sites which could address the receptor to the plasma membrane in breast cancer cells (Wang et al., 2005, 2006). ICC experiment indicated that all the TCam-2 cells observed were labelled with anti-ERα36 antibody (Fig. 3A). Staining was observed into the nuclei and in cytoplasmic filamentous structure localized beneath the plasma membrane (Fig. 3A). Therefore, we used transmission electron microscopy to precisely localize ERα36 in TCam-2 cells (Fig. 3B–D). The results did not differ between vehicle- or E_2-treated cells (data not shown) since in both cases, ERα36 gold-labelling

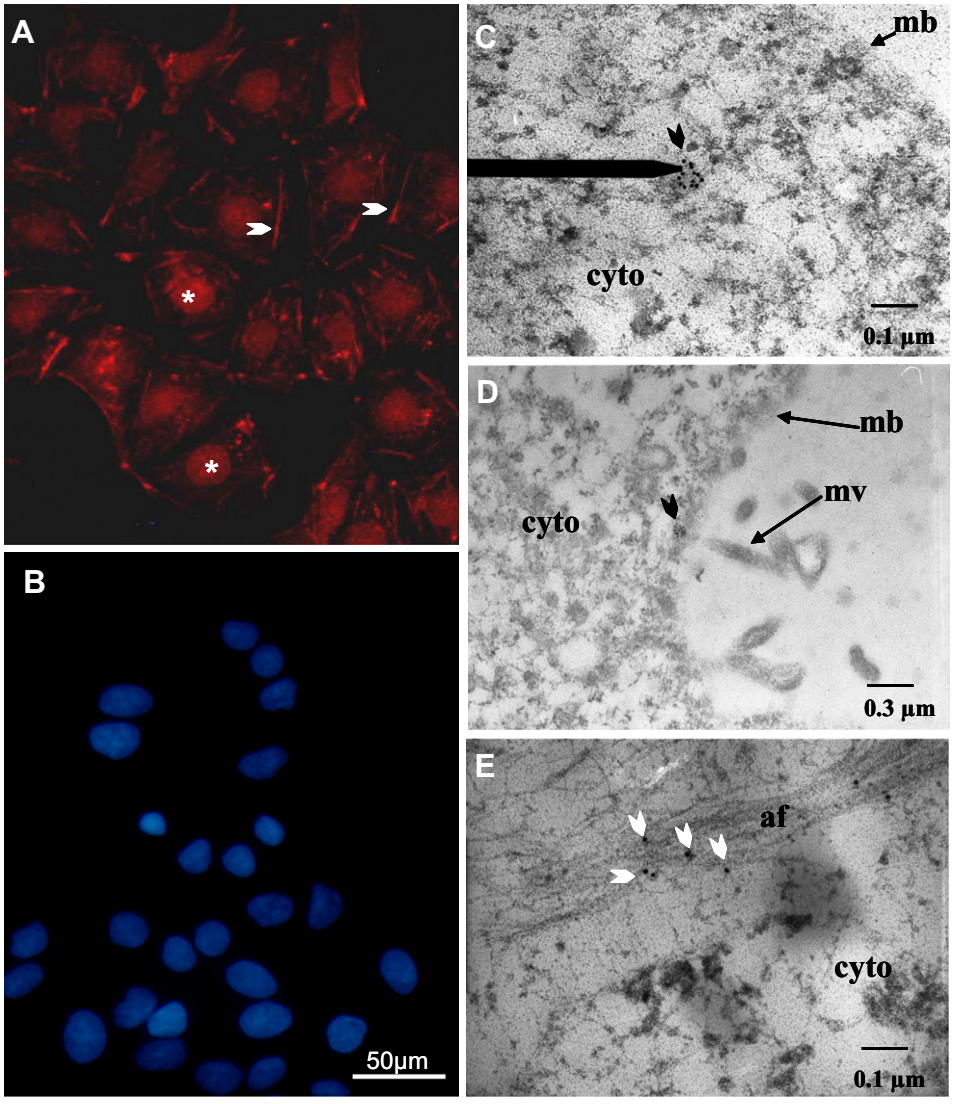

Fig. 3. Localization of ERα36 in TCam-2 cells by immunofluorescence (A and B) and transmission electron microscopy (C–E). (A) ICC was performed with specific anti-ERα36 antibody provided by Pr Z.Y. Wang. Staining is observed in all cells, into the nucleus (*) and in filamentous structures below the plasma membrane (arrowhead). (B) ICC performed with secondary antibody alone is shown as a control. Nuclei are stained with Hoechst. (C–E) Gold-labelled ERα36 is observed (arrowheads) at ecto-endoplasm border (C), at the basis of microvillosity (D) and colocalized with actin cytoskeleton (E). mb: plasma membrane, cyto: cytoplasm, mv: microvillosity, af: actin filament.

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was found underneath the membrane (Fig. 3B and C), at the endoplasm/ectoplasm border, and colocalized with actin microfilaments (Fig. 3D). This observation could rule out the possibility that BSA-coupled steroid can trigger an intracellular signal through direct binding or activation of a membrane bound ERα36.

3.4. Testosterone can trigger estrogenic signal after aromatization in TCam-2 cells

After demonstrating the stimulating effect of steroid treatment on TCam-2 cell proliferation, we aimed to decipher the involved signalling pathway. Since testosterone is an aromatizable androgen, we first addressed the question of whether it acts via an estrogen- or androgen-like pathway. First, we detected *Cyp19* gene expression by semi-quantitative RT-PCR in TCam-2 (Fig. 4A). *Tfap2c* gene encodes AP2γ protein, known to be involved in maintenance of the undifferentiated state in carcinoma in situ and embryonic stem cells and to control hormone response in breast cancer cells through multiple estrogen signalling pathways (Bouskine et al., 2010; Weber et al., 2010; Woodfield et al., 2007). Cells were treated for 24 h with E_2, T, E_2BSA or TBSA in the presence or absence of an aromatase inhibitor (AI1-10 nM). Expression of *Tfap2c* was measured by realtime-PCR. Indeed, E_2 triggered a 2.5-fold increase in *Tfap2c* expression in TCam-2 cells (Fig. 4B). This stimulation was even higher in the case of co-treatment with AI1 (3.3-fold). Testosterone induced a 2-fold increase of *Tfap2c* mRNA expression, which is not significantly different from that observed in the case of estradiol treatment (Fig. 4B). This up-regulation was partially impaired in the presence of AI1 (a 1.6 fold induction level was observed when comparing testosterone + AI vs. AI treatments). These results suggest that testosterone could act, in part, through an estrogen signalling pathway after aromatization. In contrast, AI1 did block neither TBSA nor E_2BSA-dependent stimulation of *Tfap2c* expression (Fig. 4B), suggesting that testosterone could also display an estrogenic-like signalling through a plasma membrane receptor.

3.5. Establishment of a stable TCam-2 cell line knocked down for ERα36

Thereafter, we focused on estradiol signalling in TCam-2 cells and established a TCam-2 cell line with stable ERα36 knockdown, as described by Kang et al. (2010). Protein extracts from TCam-2 cells transfected by either the empty vector (Neo-TCam-2 strain) or the ERα36 shRNA vector (sh36-TCam-2 strain) were subjected to western blot analysis. As shown in Fig. 5A, no expression of ERα36 was found in the sh36-TCam-2 cell line. To further understand the role of ERα36 in TCam-2 cell physiology and proliferation, we performed a 5 day long proliferation assay in 2.5% and 10% FCS containing medium with Neo-TCam-2 and sh36-TCam-2 cell lines. We observed that the basal proliferation rate of sh36-TCam-2 strain was significantly reduced compared to Neo-TCam-2, indicating that ERα36 is necessary for normal cell growth even in rich culture medium (Fig. 5B). We were not able to repeat the same experiment in 1% stripped FCS containing medium because sh36-TCam-2 cells did not survive starvation. This suggests that TCam-2 cells proliferation is hormone-sensitive.

3.6. ERα36 induction depends on GPER activation

Taken together, the above results pointed out the potential role of GPER in estrogen-dependent stimulation of ERα36 expression. Therefore, we tested the ability of E_2 to induce ERα36 protein expression in TCam-2 cells transfected by GPER targeted siRNA. In control siRNA transfected cells, a 24 h E_2 treatment stimulated ERα36 expression whereas this induction was blocked in cells transfected by GPER targeted siRNA (Fig. 6A). This confirmed that GPER is necessary for E_2 to trigger ERα36 stimulation of expression.

In order to study short to mid-term E_2 effects on ERα36 expression, we performed a kinetic of GPER blockage after E_2 treatment.

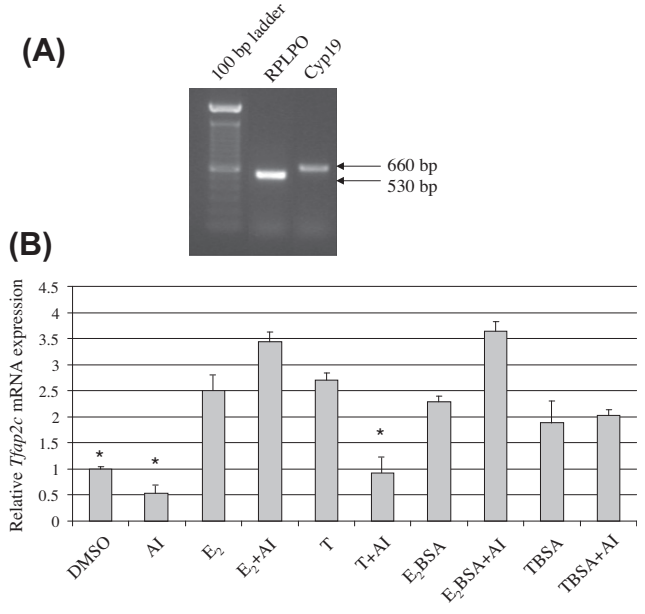

Fig. 4. Testosterone acts via an estrogenic pathway, partly through aromatization. (A) Semi-quantitative RT-PCR detection of *Cyp19* transcripts (660 bp) in TCam-2 cells. *RPLPO* amplicon (530 bp) is shown as a control. (B) TCam-2 cells were treated with DMSO (0.01%), E_2 (1 nM), E_2BSA (1 nM), T (1 μM), TBSA (1 μM) for 24 h in the presence or absence of 10 nM P450 aromatase inhibitor (AI1). *Tfap2c* mRNA expression was measured by realtime-PCR. Control cells received DMSO. The values represent the means ± s.e.m. of 3 different experiments. *Significantly different from T treated cells ($P < 0.05$).

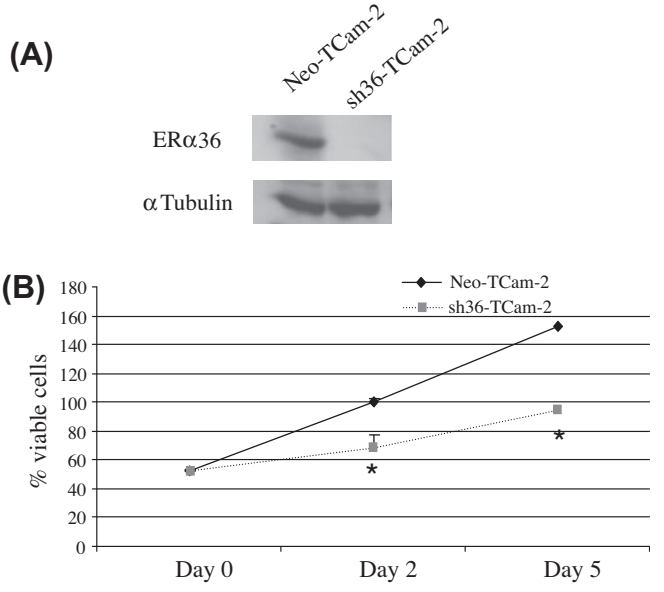

Fig. 5. Establishment and growth phenotype of the sh36-TCam-2 cell line. (A) Western blot analysis of ERα36 expression in Neo-TCam-2 and sh36-TCam-2 cells. (B) Cell proliferation rate analysis of Neo- and sh36-TCam-2 cells was performed in 2.5% charcoal-stripped FCS containing medium for 2 or 5 days. Cell number was determined by using the CellTiter-Glo™ luminescent cell viability assay. Results are given as mean ± s.e.m. of 3 different counts. *Significantly different from Neo-TCam-2 cells ($P < 0.05$).

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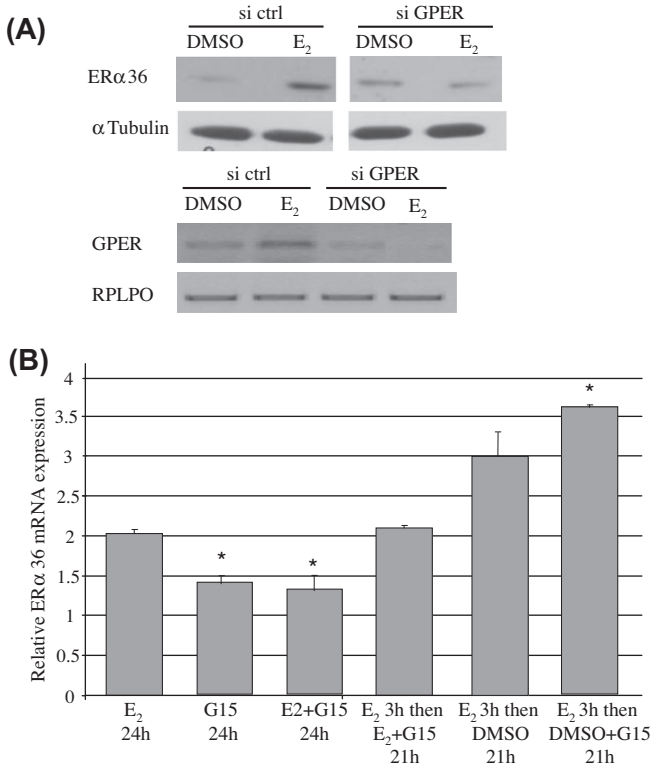

Fig. 6. Estradiol can induce the expression of ERα36 in a GPER-dependent manner. (A) Western blot analysis of ERα36 expression levels, after 24 h E_2 treatment, in TCam-2 cells knocked-down for GPER by small interfering RNA (siGPER) in comparison with a non-targeting control siRNA (siCtrl). Control cells were treated with DMSO (0.01%). Semi-quantitative RT-PCR of GPER mRNA expression is shown as a control for knock-down efficiency. (B) ERα36 expression analysis as measured by realtime-PCR in TCam-2 cells after 24 h 1 nM E_2 treatment and 1 μM G15 treatment added at the same time or 3 h after E_2 addition. Results are given as mean ± s.e.m. of 3 different experiments. *Significantly different from E_2 treated cells ($P < 0.05$).

G15, the specific antagonist of GPER (Dennis et al., 2009) was added either alone, or at the same time as E_2, or 3 h after E_2 addition. Then, ERα36 expression was measured by realtime-PCR. As previously described in other cell lines, G15 treatment alone triggered a moderate stimulating effect on ERα36 expression. Nevertheless, the antagonist did block ERα36 induction when added concomitantly with E_2 but not when added after 3 h (Fig. 6B). This suggests that GPER is necessary for the early and rapid (3 h) stimulation of ERα36 but not for the maintenance of its expression at a high level.

3.7. Estradiol signals through a GPER-cAMP/PKA pathway

Since many signalling pathways were described downstream GPER in various cell types, we then tested the ability for E_2 to activate the cAMP/PKA, the PI3K or the MAPK pathway in TCam-2 cells. Since ERK phosphorylation seems to be constitutive in TCam-2 cells (De Jong et al. (2008), no more stimulation of either ERK1/2 or AKT phosphorylation was detected following E_2 treatment (data not shown). The activation of cAMP/PKA pathway was studied by measuring CREB phosphorylation by western blotting. E_2 triggered a moderate stimulation CREB phosphorylation level and this effect was impaired in the presence of the PKA inhibitor H89 (10 μM) (Fig. 7A).

This result was confirmed by immunofluorescent microscopy analysis using both anti-CREB and anti-phospho-CREB antibodies. CREB protein was observed mainly into the nuclei, but also slightly into the cytoplasm of both DMSO and E_2 treated cells. In contrast, the phosphorylated and transcriptionally active form was absent in DMSO treated cell nuclei. Following 15 min exposure to 1 nM E_2, phospho-CREB protein was detected in more than 80% of TCam-2 cells nuclei (Fig. 7). A 30 min incubation in the presence of H89 before E_2 treatment impaired the induction of CREB phosphorylation. Finally, the stimulation of CREB phosphorylation was also observed in sh36-TCam-2 cells, suggesting that ERα36 could be a downstream target rather than an upstream component of this phosphorylation pathway. Taken together, these results indicate that E_2 signals mainly through GPER-cAMP/PKA-CREB dependent pathway in TCam-2 cells and further suggest that ERα36 could be the end point target of this rapid signalling pathway.

3.8. ERα36 is necessary for E_2-mediated EGFR regulation

Since an estrogen-dependent positive feedback loop involving ERα36 and EGFR was recently described in breast cancer cells (Zhang et al., 2010), we investigated the relationship between both receptors in TCam-2 cells. Western blot analysis shows that E_2 can stimulate EGFR protein expression (Fig. 8A) in TCam-2. Moreover, the basal level of EGFR is greatly diminished in sh36-TCam-2 cells and the stimulation is no more detectable after E_2 treatment (Fig. 8B). This result indicates that ERα36 could be one of the factors necessary for EGFR expression and for E_2-mediated EGFR stimulation.

4. Discussion

4.1. TCam-2 seminoma cells are hormone-sensitive

Several studies demonstrated that CIS, and their invasive type II GCT counterparts are diagnosed with increased frequency in DSD patients, namely those with partial androgen insensitivity, OCT3/4 and TSPY positive germ cells (Pleskacova et al., 2010; Cools et al., 2011). CIS cells derive from arrested gonocytes due to maldifferentiation of the somatic niche during fetal life and genetic adaptation during infancy (Sonne et al., 2008; Kristensen et al., 2008). Dormant CIS remain in the seminiferous tubules and tumors often become detectable in young adults. It has been hypothesized that exposure to androgens during and after puberty stimulates the development of type II GCTs in male DSD patients (Cools et al., 2009; Looijenga et al., 2011). However, the underlying mechanism remains unclear since germ cells do not express the androgen receptor. For the first time, we provide evidences for hormone sensitivity of TCam-2 seminoma cells and demonstrate that proliferation rate is stimulated by either estradiol or testosterone through an estrogen-related ERα36 dependent pathway. This phenotype as well as underlying signalling mechanisms described in this paper support the hypothesis of an increased risk in type II GCT tumor progression after exposure to endogen estrogen or aromatizable androgen under ERα36 testicular expression. It remains to be determined if CIS cells and/or normal gonocytes do express ERα36, and could escape dormancy in response to estrogen related compounds. Additionally, our results would be of interest in order to understand the endocrine disrupting compound (EDC) effects on germ cell pathology.

4.2. Estrogens act through a GPER-dependent signalling pathway

The mechanisms underlying EDC exposure-response trends are still not known. In our study, we evaluated the impact of estrogen treatment and deciphered the signalling pathways involved in the absence of ERα. Thus, we showed that estradiol and BSA-coupled estradiol act via a GPER-cAMP/PKA-CREB dependent pathway. These results extend previous data obtained by Fenichel's group which first demonstrated an opposite effect of E_2 and E_2BSA on

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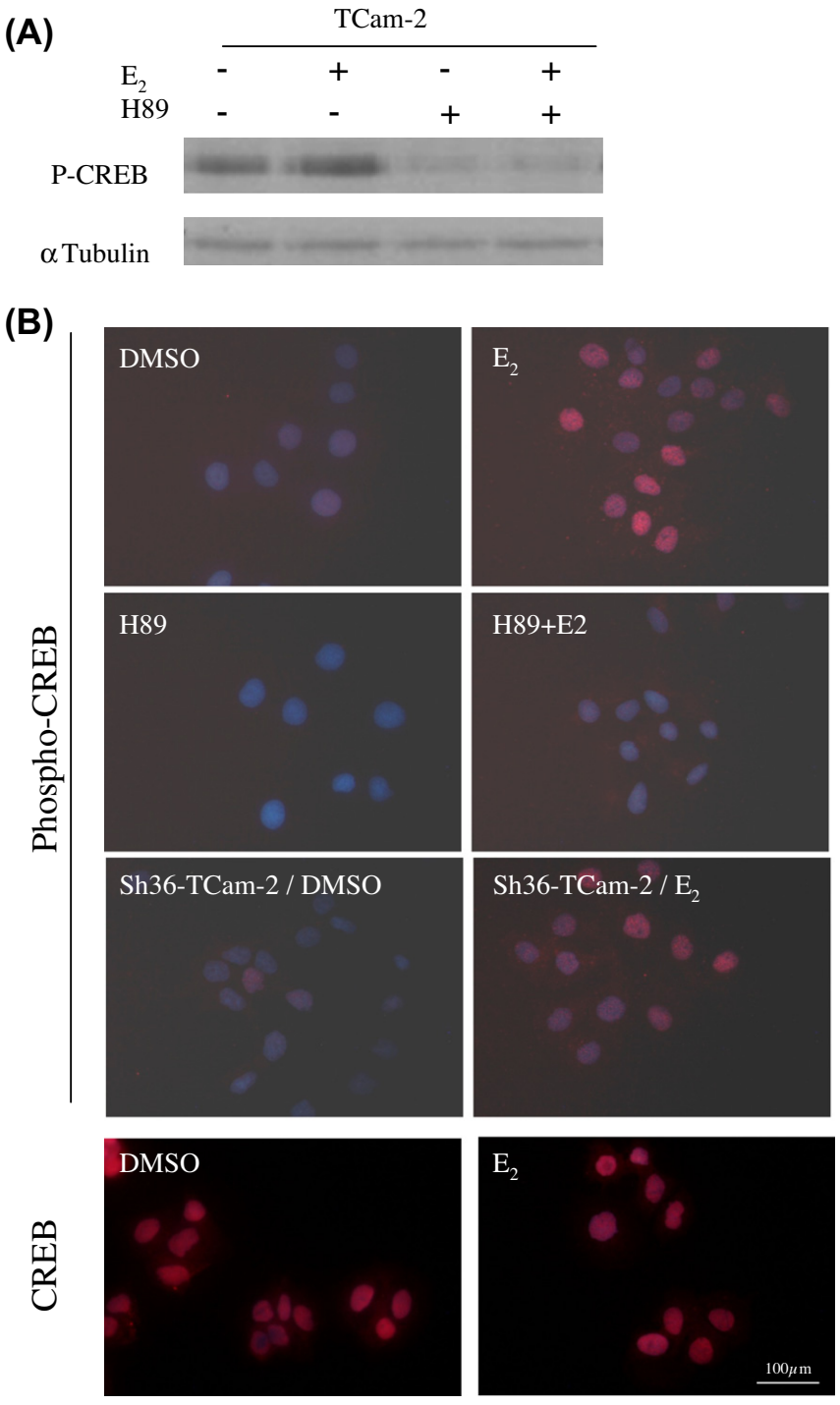

Fig. 7. E_2 can act through a cAMP/PKA dependent pathway. (A) Phospho-CREB (P-CREB) protein expression analysis in TCam-2 cells. Cells were treated with 1 nM E_2 for 15 min in the presence or absence of 10 μM H89 PKA inhibitor. Expression of alpha-tubulin is given as a control. (B) TCam-2 cell ICC was performed using anti-P-CREB antibody. TCam-2 as well as sh36-TCam-2 cells treated for 15 min with E_2 show an intense staining of CREB phosphorylation into the nucleus. This staining is impaired by pre-treatment with 10 μM H89. TCam-2 stained with an anti-CREB antibody are shown as a control for the presence of CREB in DMSO or E_2 treated cells. The scale bar represents 100 μm.

JKT-1 cell proliferation (Roger et al., 2005; Bouskine et al., 2008; Bouskine et al., 2009). Recently, they addressed the role of bisphenol A in promoting cell growth and showed the involvement of GPER and PKA/PKG pathway (Bouskine et al., 2009; Chevalier et al., 2011a). Therefore, we can hypothesize that JKT-1 and TCam-2 cells, both isolated from type II GCTs and lacking ERα66, do respond to estrogenic signal with similar molecular components. Different expression levels of GPER and ERα or the presence/absence of ER cofactors in both cell lines could explain the discrepancy between E_2 mediated proliferation in TCam-2 and E_2 growth inhibition in JKT-1. In fact, Eckert et al. (2008) and De Jong et al. (2008) previously demonstrated that JKT-1 lacks expression for most of the genes detectable in type II GCTs, thus providing evidences for great differences between TCam-2 and JKT-1.

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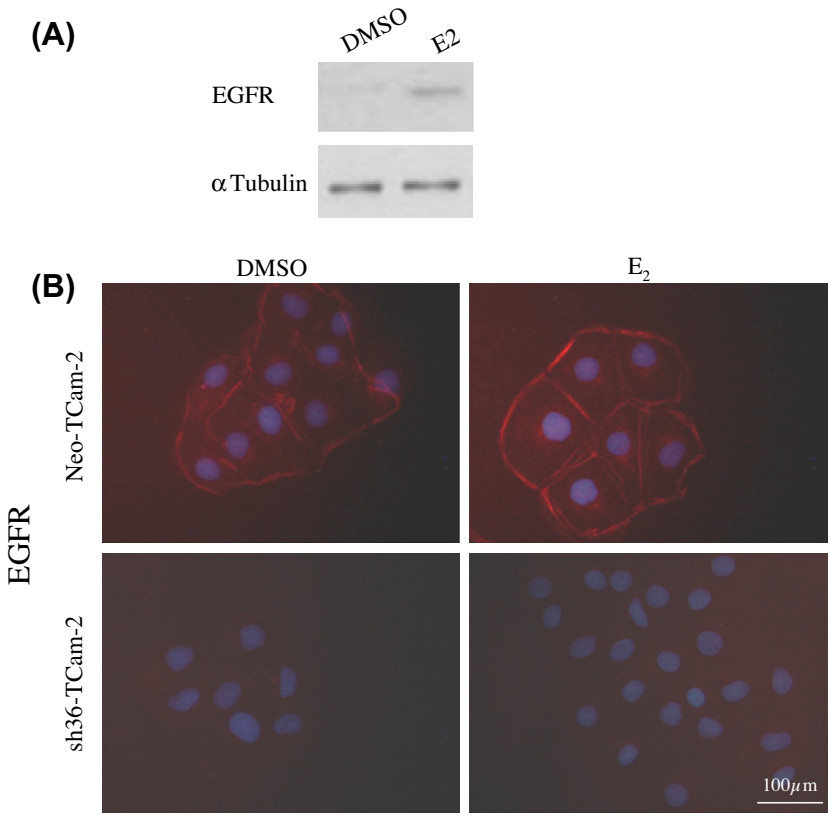

Fig. 8. E_2 stimulates EGFR expression in an ERα36-dependent way. (A) Western blot analysis of EGFR expression was performed in TCam-2 cells treated by E_2 (1 nM) for 24 h. (B) ICC analysis of EGFR expression and localization in Neo-TCam-2 and sh36-TCam-2 cells treated by E_2 (1 nM) for 15 min. In Neo-TCam-2 cells, EGFR is localized at the cell surface. In sh36-TCam-2 cells, EGFR expression was detected neither in DMSO nor in E_2 treated cells. The scale bar represents 100 μm.

GPER was also detected at a high level in intratubular germ cell tumors, seminoma and embryonal carcinoma (Franco et al., 2011; Rago et al., 2011). Since we demonstrated that GPER is necessary to induce ERα36 expression in response to estradiol, leading to an elevated proliferation rate, GPER seems to be a promising therapeutic target in type II GCTs treatment as recently proposed by Chevalier et al. (2011b).

4.3. ERα36 expression portrays seminoma cell E_2 exposure

Based on the work of Wang and colleagues who analyzed the upstream signals leading to ERα36 activation in several breast cancer cell lines (Zhang et al., 2010; Wang et al., 2010), we showed that the GPER-dependent E_2 signalling ends to ERα36 expression induction. Moreover, the growth phenotype of our stably transfected sh36-TCam-2 cell line suggests that a threshold of ERα36 protein should be reached to respond to estradiol otherwise cell activity is compromised. Therefore, a high level of ERα36 expression seems to be sufficient to portray estradiol exposure in TCam-2 and might be tested as a marker for EDC exposure.

More recently, Zhang et al. (2010) further demonstrated that ERα36 mediates nongenomic estrogen signalling through the EGFR/Src/ERK signalling pathway in ER-negative breast cancer cells suggesting that these tumors can retain an estrogen mitogenic signalling in the absence of estrogens. This process is also suspected to be involved during acquired tamoxifen resistance of hormone-treated breast tumors (Lin et al., 2010). Indeed, we show that a minimal ERα36 expression level is necessary for both EGFR membrane localization and E_2-mediated stimulation of EGFR expression in TCam-2 cells. Similar data were obtained in 22Rv1 prostate cancer cell line which expresses unreported AR-truncated forms in the course of hormone resistance acquisition (Marcias et al., 2010). They also demonstrate that the expression of these AR mutants is an essential molecular event leading to androgen independency in prostate tumors that escape androgen deprivation therapy. Therefore, high ERα36 protein level may also reveal hormone independency in type II GCTs. One can even hypothesize that a transient exposure to EDCs during early life or puberty could trigger a positive feedback loop of ERα36/EGFR activation which could, in turn, lead to estrogen-independent long term promotion of seminoma cell proliferation and tumor progression in adulthood.

4.4. Is ERα36 a modulator of cytoskeleton remodeling in seminoma cells?

In most animal cells, dynamic changes of the cytoskeleton are related to the ability of the cell to respond to external signals from the surrounding environment and result in the recruitment of specific intracellular signalling cascades. In these complex processes, a critical role is played by the dynamic remodelling of the actin cytoskeleton. The role of sex steroids in the regulation of cell movement and actin-dependent signalling has been demonstrated in numerous physiological processes and diseases, namely cancer cell migration (Giretti et al., 2008; Flamini et al., 2011). Indeed, three papers from Simoncini's laboratory (Sanchez et al., 2010a,b) recently reported that E_2 can trigger cytoskeleton remodelling and cell motility in several cell lines including breast cancer cells through ERα-dependent recruitment and phosphorylation of Focal Adhesion Kinases (FAK) and N-WASP. This process seems to be necessary for cancer cell migration and invasion. In our study, ERα36 was found to colocalize with actin microfilaments, suggest-

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ing a potential regulatory role for the truncated form of ERα in actin cytoskeleton remodelling and cell migration. Since ERα36 expression is enhanced by an E_2 treatment, one can hypothesize that a protein critical threshold must be reached to trigger cell motility. However, the precise domain of ERα36 protein that could be involved in actin regulation remains to be determined. The expression of ERα36 was not tested in the cell lines used by Sanchez and Flamini so that the involvement of ERα36 in the processes they describe remains to be elucidated.

5. Conclusion

Taken together, our results indicate a positive role of E_2 on TCam-2 seminoma cell line proliferation. The described pathway involves a GPER-dependent signalling that triggers ERα36 upregulation. Our study also suggests a potential role of the ERα36 isoform in actin cytoskeleton remodeling. Case control study would be interesting in order to validate ERα36 expression as a marker of endocrine disruptor exposure and/or as a prognostic marker in type II GCTs.

Disclosure statement

The authors have nothing to disclose.

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RESUME

Au cours de mon doctorat puis de mes différents stages post-doctoraux, j'ai développé des approches de physiologie et génomique comparatives pour identifier et caractériser des médiateurs moléculaires impliqués dans le contrôle de la prolifération des cellules eucaryotes en réponse aux variations de l'environnement. Je me suis également attachée, dans des modèles biologiques divers, à replacer les mécanismes moléculaires et cellulaires étudiés dans un contexte plus général de physiologie de l'organisme.

Depuis ma nomination en tant que Maître de Conférences à l'Université Nancy 1, je me suis focalisée sur l'influence des hormones stéroïdes sur la prolifération et la différenciation normale ou pathologique des cellules germinales.

Le projet de recherche que je souhaite développer s'articule autour de 3 mots-clefs : tumeurs hormono-sensibles, perturbateurs endocriniens, voie non conventionnelle de signalisation œstrogénique. Le premier axe renferme un projet concernant les effets d'un mélange d'alkylphénols sur l'initiation et la progression tumorale dans un modèle de cancer testiculaire d'origine germinale puis de cancer mammaire, *in vitro* et *in vivo*. Le but est de caractériser le rôle éventuel de ERα36 dans ces processus afin de valider cette forme du récepteur des oestrogènes comme marqueur d'exposition aux perturbateurs endocriniens dans les cellules hormono-. Le second axe s'inscrit dans un objectif à plus long terme qui sera de valider ERα36 comme marqueur prédictif de réponse aux traitements anti-tumoraux dans les cancers hormono-sensibles et en particulier dans le cancer du sein.