Role of the RIP140 Corepressor in Ovulation and Adipose Biology

Jennifer H. Steel, Roger White and Malcolm G. Parker

Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College, Du Cane Rd, London W12 1ONN, UK

Tel 44 20 7594 2177

Fax 44 20 7594 2184

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Abstract

RIP140 is a ligand-dependent corepressor for most, if not all nuclear receptors. It is expressed widely in many different tissues, but the phenotype of mice devoid of RIP140 indicates that it plays a crucial role in the ovary and in adipose biology. Ovarian expression of RIP140 is cell type-specific during follicular development and it is essential for oocyte release during ovulation, but not for luteinisation of mature ovarian follicles. In adipose tissue, RIP140 is essential for normal fat accumulation and RIP140 null mice show decreased lipid storage even on a high fat diet, with upregulation of mitochondrial uncoupling protein (UCP1) in some fat depots. Thus RIP140 plays a crucial role in female fertility and in energy homeostasis, and could be a target for infertility treatment, new contraceptive strategies, or prevention of obesity.

Introduction.

Nuclear receptors control a vast array of endocrine responses by controlling the activity of specific subsets of target genes in cells, usually for precise periods of time. Their ability to regulate gene transcription depends on the recruitment of coactivators and corepressors that remodel chromatin in the vicinity of their promoters. Numerous coactivators have been reported but the p160/SRC (steroid receptor coactivator) family of coactivators and CBP/p300 seem to play an important role in transcriptional activation by many members of the nuclear receptor family (McKenna & O'Malley, 2002). NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor) (Jepsen *et al.*, 2000, Nagy *et al.*, 1997) were the first corepressors to be identified and found to block the transcriptional activity of a subset of nuclear receptors. RIP140 is a corepressor that binds to most, if not all, nuclear receptors (Cavailles *et al.*, 1995, L'Horset *et al.*, 1996) and its importance in endocrine responses is evident from the observation that it is essential for female fertility (Leonardsson *et al.*, 2002, White *et al.*, 2000) and energy homeostasis (Leonardsson *et al.*, 2004). This review will focus on the function of RIP140 in the ovary to regulate ovulation and in adipose tissue to regulate fat accumulation.

RIP140 binds to nuclear receptors in a ligand-dependent manner by a mechanism which resembles that of the p160 coactivators. These cofactors contain helical leucine-rich LXXLL motifs that dock into a cleft formed by the activation surface in the ligand binding domain of nuclear receptors when a hormonal ligand is bound (Heery *et al.*, 1997) (Torchia *et al.*, 1997). The mechanisms that determine whether, for example a p160 coactivator or the RIP140 corepressor is recruited to a particular nuclear receptor and precisely when and for how long are not known but there are hints that several mechanisms may be involved. These include variations in their relative cellular concentrations, alterations in their subcellular location in response to other signalling pathways and regulation of their activity brought about post-translational modifications. The binding properties of NCoR and SMRT are quite distinct from these cofactors since they contain extended LXXLL-like motifs (Hu & Lazar, 1999) that can not fit into the cleft formed on activated receptors but bind to certain nuclear receptors, including retinoic acid and thyroid hormone receptors, in the absence of hormone and to steroid receptors in the presence of antagonists (Huang *et al.*, 2002, Liu *et al.*, 2002, Shang & Brown, 2002). Despite differences in the binding properties of cofactors to nuclear receptors they all seem to act as docking or scaffold proteins for the assembly of enzyme complexes at specific sites on a promoter. The function of these enzymes, which include acetylases/deacetylases, kinases/phosphatases and methyl transferases, is to catalyse the modification of histones, that results in the remodelling of chromatin and ultimately gene activation or gene repression.

A schematic diagram of the structure of RIP140 is shown in Figure 1. It consists of ten leucine-rich motifs that allow its recruitment to the ligand binding domain of nuclear receptors (Heery *et al.*, 1997) (Torchia *et al.*, 1997) and four repression domains (Christian *et al.*, 2004). Individual LXXLL motifs are assumed to allow selective binding to different receptors; for example motif 10, LXXML, is reported to bind preferentially to retinoid receptors (Lee & Wei, 1999). The repression domains exhibit autonomous activity suggesting that they function by recruiting enzymes that modify histones or DNA. Repression domain 1 has been found to bind histone deacetylases (HDACs) (Wei *et al.*, 2000) while repression domain 2 binds carboxy-terminal binding protein (CtBP) that then bind enzymes,

including histone deacetylases (Vo *et al.*, 2001). The mechanism of action of repression domains 3 and 4 are unknown (Christian *et al.*, 2004). Nevertheless it seems likely that the four repression domains allow the regulated recruitment of distinct sets of chromatin modifying enzymes each of which is capable of repressing transcription from target genes.

RIP140 is detected in most tissues but only in specific cell types where its expression is often under developmental or hormonal control. This is achieved by the presence of multiple promoters that precede a number of alternately spliced untranslated exons which are differentially regulated (Donna Nichol, unpublished data). The biological roles of RIP140 have been determined by generating mice devoid of the gene. The coding sequence of the RIP140 gene was replaced by lacZ to allow for the detection of β−galactosidase at sites at which the RIP140 promoter is active (White *et al.*, 2000). The highest levels of RIP140 expression are in the gonads and in metabolic tissues, particularly adipose tissue, where it clearly plays important roles. While RIP140 is not required for viability it is essential for female fertility since it plays a crucial role in oocyte release by the ovary during ovulation (Leonardsson *et al.*, 2002). It is important for fat accumulation in white adipose tissue so that in its absence mice are extremely lean (Leonardsson *et al.*, 2004). It also plays a role in growth and probably has many other functions in view of its widespread expression in tissues, but these are yet to be determined.

Role of RIP140 in Female Reproduction.

Female mice devoid of RIP140 (RIPKO mice) are completely infertile because they fail to ovulate. Thus RIPKO mice fail to release oocytes from mature follicles following the pre-ovulatory surge of luteinising hormone (LH), (Figure 2 A,B), although luteinisation still occurs (White *et al.*, 2000). This phenotype closely resembles that of luteinised unruptured follicle syndrome often associated with infertility in women, and suggests that suppression of nuclear receptors is essential for the normal coordinated control of ovarian function, with the process of oocyte release dependent on the activity of RIP140.

Further investigation of the ovarian phenotype demonstrated that ovulatory failure is due to a primary defect in the ovary itself. Standard hormone treatments (PMSG and hCG) designed to "superovulate" mice had no effect on oocyte release (White *et al.*, 2000), indicating that the defect is independent of gonadotrophin stimulation of follicle growth or the LH surge. In addition ovarian transplantation experiments gave direct evidence that the defect lies at the ovarian level rather than the hypothalamic-pituitary axis (Leonardsson *et al.*, 2002). Ovaries from 4-week old immature RIPKO mice were transferred into wild-type or heterozygous littermates and vice-versa. Continuous breeding experiments indicated that the transfer of ovaries from wild-type into RIPKO mice rescued their fertility whereas control mice with RIP140 null ovaries generated very few pups, all of which were dead at birth or died within 72 hours. The conclusion was that RIP140 expression in the ovary is necessary for ovulation and that expression in other tissues is not essential for this process to occur.

The precise site of RIP140 action in the ovary required for ovulation has not been determined definitively. RIP140 expression, as determined by analysing β-galactosidase activity as a marker of promoter activity (Figure 2), indicated that the highest levels are found in granulosa cells but low levels are also present in thecal and interstitial cells. No expression was detected in the granulosa cells of primary follicles or small follicles, but expression increased during follicular maturation, with the highest level detected in the outer mural cells in pre-ovulatory follicles (Figure 2C). Expression levels dramatically decreased after luteinisation but were subsequently regulated in the corpora lutea of pregnant mice (Figure 2D-G). In ovaries of heterozygous or RIPKO mice used for embryo transfer there is negligible expression from the RIP140 promoter in corpora lutea at day 6.5 post-coitus (p.c.), but expression increases at mid-gestation, is maintained until at least day 13.5 p.c., and then declines to negligible levels at day 17.5 p.c., correlating with initiation of the regression of the corpora lutea. While initial failure of oocyte release did not affect luteinization and production of progesterone in the early stages of pregnancy, the normal mid-gestation rise in progesterone levels was not as pronounced in embryo-transferred RIP140 null mice. This is despite the fact that the corpora lutea were still large and apparently healthy (Leonardsson *et al.*, 2002).

RIP140 may also play a role in uterine function given that it is in the glandular epithelium, stroma and myometrium of the non-pregnant uterus (Figure 2H). To investigate its function during pregnancy embryo transfer experiments were performed. The transfer of wild-type embryos into pseudopregnant RIPKO females resulted in a similar number of implantation sites as wild-type mice, indicating that uterine RIP140 is not required for implantation. Nevertheless RIP140 was expressed in primary decidual cells around the time of implantation and, by 9.5 days p.c., in differentiating decidual cells on the anti-mesometrial side of the uterus (Leonardsson *et al.*, 2002). At later stages of pregnancy, however, there was a progressive reduction in the number of surviving embryos in the RIP140 null mice suggesting that uterine RIP140 expression might be required to maintain the pregnancy. In addition, while the majority of pups born from control mothers survived after birth, 75% of the pups from RIP 140 null mothers were dead within 24 hours (Leonardsson *et al.*, 2002).

The high incidence of fetal loss in mid-pregnancy and the death of the majority of pups at or shortly after birth following embryo transfer suggests that some aspects of the maternal expression of RIP140 are sub-optimal for the maintenance of pregnancy and for the subsequent survival of offspring. However, the ovarian transfer experiments indicated that uterine and mammary gland RIP140 expression is not essential for pregnancy and survival. We conclude from this apparent disparity that the pregnancy failure and the death of pups may reflect a defect in the RIP 140 null ovary which fails to support fully the function of the uterus and the mammary gland.

Role of RIP140 in Adipose Tissue.

The defects in fertility are accompanied by metabolic changes in the RIP140 null mice, which are lean with a 20% reduction in body weight in both males and females compared to wild type mice. Magnetic resonance imaging (MRI) and spectroscopy (MRS) of whole body fat content revealed marked decrease in subcutaneous fat and other fat depots (Leonardsson *et al.*, 2004). Total body fat content was reduced by approximately 70% as analysed by whole body proton MRS while the weight of inguinal white adipose tissue (WAT) was reduced by 40-60%, according to the age of

the mice. The reduction in body weight and fat content was not due to increased physical activity, although RIPKO mice showed increased oxygen consumption. In addition, food intake was 4.93 ± 0.37 g/mouse/day and 4.70 ± 0.20 g/mouse/day in wild type and null mice respectively, corresponding to a small increase in the null mice relative to total body weight (Leonardsson *et al.*, 2004).

Further investigation showed that the RIP140 null mice are lean because they fail to store triglycerides and are resistant to high-fat diet induced obesity, suggesting that alternative mechanisms are involved in the dissipation of excess fuel (Leonardsson *et al.*, 2004). The most striking finding was the upregulation, by more than 100 fold, of mitochondrial uncoupling protein 1 (UCP1) mRNA, in inguinal WAT of RIPKO mice. In RIPKO mice fed on a normal diet, adipocyte volume was 2.7 fold less in inguinal WAT, compared to wild type littermates, while there were no differences in the adipocytes in the interscapular brown adipose tissue (BAT) and there was no evidence from Oil red O staining or MRI/MRS that fat was being stored in alternate tissues such as the liver. In animals fed on a high fat diet, RIPKO mice gained less weight than wild type mice and showed a smaller increase in adipocyte volume. Lipid accumulation in the liver on high fat diet was seen in wild type but not in RIPKO mice, showing that the absence of RIP140 protects against hepatic steatosis (Leonardsson *et al.*, 2004).

White and brown adipose tissue can be distinguished by the morphology of the adipocytes; unilocular white adipocytes store energy as triglycerides and release it when required, whereas multilocular brown adipocytes dissipate energy as heat. White and brown adipocytes are distributed in different proportions in distinct fat depots in mice, and the depots can be classified as subcutaneous or visceral in origin (Figure 3A). In mature animals, most of the BAT is found in the interscapular depot, but in young animals it can also be found in the posterior subcutaneous (dorsolumbar, inguinal and gluteal) and mediastinic and perirenal depots, mainly composed of WAT. Visceral WAT depots include mesenteric and gonadal, epididymal or ovarian, fat (Cinti, 2002).

The dramatic increase in UCP1 expression in the subcutaneous inguinal fat depot in RIPKO mice was accompanied by a change in the balance between unilocular and multilocular adipocytes, as shown by histology and immunohistochemistry for UCP1 (Leonardsson *et al.*, 2004). The different fat depots in RIPKO and wild type mice were compared**.** In inguinal fat, regarded as a typical subcutaneous WAT depot**,** UCP1-immunoreactive multilocular and unilocular cells were present in RIPKO mice but not in their wild type littermates (Figure 4 A,B). In unilocular adipocytes of RIPKO, the UCP1 immunoreactivity was localised mainly in the perinuclear area, similar to expression seen in mice expressing an aP2-*Ucp1* transgene (Rossmeisl *et al.*, 2002) (Figure 4B). Interscapular brown fat, regarded as a typical BAT depot, was similar in wild type and RIPKO mice (Figure 4 C,D) and the peri-renal fat was composed of similar amounts of brown and white adipocytes in both genotypes (Figure 4 E,F)**.** In contrast to the inguinal fat, visceral WAT depots such as epididymal and ovarian WAT did not appear to contain multilocular cells or UCP1-immunoreactive cells in RIPKO mice (Figure 4 G,H). This suggests that RIP140 plays a more important role in subcutaneous than in visceral depots in mice. It is now clear that the adipocyte itself may act as an endocrine cell such that altered adipocyte function cause changes in systemic energy balance (Spiegelman & Flier, 2001), therefore regulation of lipid storage and metabolism by adipocytes in different fat depots is increasingly seen as an important mechanism for controlling energy balance in humans and may be involved in other physiological processes.

There is evidence of nuclear receptor action in adipose tissue in humans, because estrogen (acting through ERα) controls fat accumulation in subcutaneous rather than visceral adipose tissue in females by upregulating α2A-adrenergic receptors (Pedersen *et al.*, 2004). Such depot-specific expression of key genes involved in fat deposition and mobilisation may also be mediated by peroxisome proliferator-activated receptor-γ (PPAR-γ), since PPAR-γ agonists, used to treat insulin resistance, exert depot-specific effects upon human fat deposition (Laplante *et al.*, 2003). In rats, leptin mRNA levels are higher in gonadal and retroperitoneal (intra-abdominal depots) than in inguinal (subcutaneous) adipose tissues (Zheng *et al.*, 1996), while glucocorticoid receptor expression is higher in epididymal than inguinal and retroperitoneal depots (Zhang *et al.*, 2002). Angiotensinogen expression changes

on high-fat feeding in visceral fat depots in mice, but not in subcutaneous depots nor in brown adipose tissue (Rahmouni *et al.*, 2004). The differential regulation of adipocyte function between subcutaneous and visceral depots in humans is an important area for investigation because increased visceral adipose tissue in human obesity is associated with pathological effects such as hypertension and metabolic syndrome, whereas subcutaneous fat deposition is less harmful.

Other Sites of RIP140 Action

RIP140 is widely expressed and so it is likely that the corepressor may play a role in other tissues, apart from the obvious phenotypes seen in reproductive system and adipose tissue. Real-time TaqMan PCR showed the highest levels of RIP140 mRNA in WAT, followed by skeletal muscle, with lower levels in BAT and liver (Figure 3B).

Expression of RIP140, as revealed by detection of β-galactosidase by histochemical staining, showed that the gene is expressed in specific cells in a variety of other tissues, mainly in hormone-responsive cell types. In the salivary gland the gene is expressed in ducts of the sublingual gland and in granular convoluted tubules (GCT) of the submandibular gland (Figure 5A,B). The differentiation and maintenance of GCT cells are under the control of the synergistic actions of androgens, thyroid hormones and adrenocortical hormones (Kim *et al.*, 2001), suggesting that nuclear receptor cofactors have a role to play in these cells. In the kidney RIP140 is expressed in a variety of epithelial cells, in proximal and distal convoluted tubules, the loop of Henle and collecting ducts (Figure 5C,D). In male mice, RIP140 is expressed in epididymal epithelial cells, in the prostate and the testis. In addition, β-galactosidase staining is present in blood vessels and neuronal ganglia in a variety of tissues.

RIP140 is present in neurones in many different brain regions, such as the cerebral cortex, cerebellum (Figure 5E,F) and hippocampus. It is also expressed in anterior and intermediate lobes of the pituitary gland but not in the posterior lobe (Figure 5G),

and in sections of RIPKO mouse pituitary, anterior pituitary cells immunoreactive for different pituitary hormones (ACTH, GH, PRL, TSHβ, LHβ, FSHβ) all expressed β-galactosidase. However, comparison of immunostained pituitary sections from wild type and RIPKO mice showed no apparent differences in the distribution of cells producing these trophic hormones**,** suggesting that although RIP140 is expressed in these cells, it's absence does not result in altered pituitary hormone production.

Conclusion

The phenotype of RIP140 null mice shows that RIP140 plays a crucial role in reproduction and in energy homeostasis and demonstrates a crucial role for the corepressor in ovarian and adipocyte function. Since RIP140 is recruited to nuclear receptors in a ligand-dependent process, it appears that it is required to prevent the expression of genes that might otherwise disrupt the normal biological function of these tissues. Thus, one important role for RIP140 in white adipose tissue may be to prevent UCP1 expression. However, the absence of RIP140 in adipose tissue not only allows increased expression of certain genes, but also causes decreased expression of other genes such as acetyl CoA carboxylase 1 (ACC-1), fatty acid synthase (FAS) and stearoyl CoA desaturase (SCD-1) (Leonardsson *et al*., 2004). This might reflect the ability of the RIP140 corepressor to block transrepression, reported in the case of glucocorticoids (Subramaniam *et al.*, 1999), so that the expression of such target genes would increase in cells lacking the corepressor. Alternatively, the expression of these genes may be subject to regulatory mechanisms, not only intrinsic to the adipocyte, but also to systemic factors that might be disrupted by the lack of RIP140 in distinct endocrine tissues.

RIP140 may therefore provide a novel therapeutic target for the treatment of obesity and related disorders. Further investigation of the role of this corepressor in ovulation may bring about new opportunities for treating infertility or the development of new contraceptive strategies.

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Figure legends

Figure 1: Schematic representation of the RIP140 protein showing repression domains (RD1-RD4) and leucine motifs (numbered 1-10).

Figure 2: Ovarian phenotype:
Sections of wild type (A) and RIP140 null (RIPKO) (B) ovary stained with haematoxylin and eosin, showing retained oocytes (arrows) in corpora lutea.
(C) β-galactosidase staining in specific cell types in RIPKO ovarian follicle;
and in ovaries from non-pregnant (D) and pregnant (E-G) RIPKO mice, showing corpora lutea at 6.5, 10.5, and 17.5 days p.c. CL, corpus luteum, TC, thecal cells; GCm, mural granulosa cells; GCc, cumulus granulosa cells; O, oocyte.
(H) β-galactosidase staining in RIPKO uterus, mainly in stroma and glandular epithelium. S, stroma; LC, luminal epithelium; GE, glandular epithelium.

Figure 3: (A) Diagram of distribution of fat depots in the mouse (after Cinti, 2002); inguinal fat was used as typical depot for white adipose tissue (WAT) and interscapular fat as a typical depot for brown adipose tissue (BAT). (B) Histogram showing relative levels of RIP140 mRNA in different tissues; WAT, white adipose tissue; BAT, brown adipose tissue; Mu, skeletal muscle; Li, liver.

Figure 4: Immunocytochemistry for UCP1 in different fat depots in wild type (A,C,E,G) and RIPKO (B,D,F,H) mice (Chemicon antibody AB3038, avidin-biotin peroxidase method). (A,B) subcutaneous inguinal WAT, (C,D) interscapular BAT, (E,F) peri-renal WAT and BAT, (G,H) epididymal WAT.

Figure 5. β-galactosidase (blue) staining in various tissues in RIPKO mice showing cells normally expressing RIP140, nuclei counterstained with Nuclear Fast Red. Labelling is found in ducts in the sublingual salivary gland (A) and in granular convoluted tubules in submandibular (B) salivary gland. Labelling was also found in proximal (PCT) and distal (DCT) convoluted tubules in the renal cortex, but not in glomeruli (C), and in collecting tubules in the renal medulla (D). Labelling was also

found in the cerebral cortex (E), cerebellum (F) and in the pituitary gland (G); a, anterior lobe; i, intermediate lobe; p, posterior lobe.