Lentiviral vector-mediated knockdown of *Lrb* in the arcuate nucleus promotes diet-induced obesity in rats

J Bian, X M Bai, Y L Zhao, L Zhang and Z J Liu

Department of Pediatrics, The Second Affiliated Hospital, Dalian Medical University, 467 Zhongshan Road, Shahekou District, Dalian 116027, China

Correspondence should be addressed to Z J Liu
Email liuzj_1963@yahoo.com.cn

Abstract

Obesity is currently a worldwide pandemic. Leptin resistance is a main mechanism of obese human and rodents. The downregulation of the long form of the leptin receptor (*Lrb*) was involved in leptin resistance in diet-induced obese rats. In the studies, we investigated whether arcuate nucleus (ARC) silencing of *Lrb* would promote diet-induced obesity in rats. Lentiviral vectors expressing *Lrb*-shRNA were administered to 5-week-old male rats by ARC injection. Following viral delivery, the rats were provided with a high-fat diet (HFD) or a chow diet (CD). After 8 weeks of the diet, serum leptin, and insulin concentrations were measured by RIA, gene expression of *Lrb* in the ARC was detected by a real-time RT-PCR, and leptin signaling was examined by western blot. The *Lrb*-shRNA knocked down the expression of *Lrb* mRNA in infected regions by 54% for the HFD rats and 47% for the CD rats respectively. The *Lrb* knockdown reduced Stats3 activation and increased expression of *Npy* mRNA. The rats with reduced *Lrb* in the ARC showed a significant increase in energy intake and body weight (BW) again when fed with a HFD. By contrast, there were no effects of *Lrb* reduction on energy intake or BW when rats maintained on a low-fat chow. Our results provide evidence that *Lrb* knockdown selectively in the ARC promotes diet-induced obesity and associated metabolic complications in rats.

Key Words

- obesity
- metabolism
- leptin
- receptors

Introduction

Obesity has become one of the most important health problems in the world. Except for some rare types of monogenic defects (Farooqi & O’Rahilly 2006), obesity occurs due to the complex combination of multiple environmental and genetic factors (Galgani & Ravussin 2008). The hyperleptinemia in obese patients and rodents supports that leptin resistance or insensitivity to the action of leptin is a common mechanism of obesity (Fleisch et al. 2007, Augustine & Grattan 2008).

The responsiveness to leptin may vary according to the metabolic conditions (Widdowson et al. 1997) and leptin resistance is relevant to leptin receptors (*Lrb*) or their downstream signaling pathway (Martin et al. 2000). Our previous studies showed that the leptin concentrations were high in obese children (Nakanishi et al. 2001) and that gene expression of *Lrb* was reduced in diet-induced obese rats (Liu et al. 2007), which implied that downregulation of *Lrb* was involved in leptin resistance.
The suppressor of cytokine signaling 3 (SOCS3) is a negative-feedback regulator of leptin and insulin signaling. In diet-induced obese rodents, the expression of Sox3 is significantly stimulated in the hypothalamus, providing a negative control for leptin and insulin signaling (Dunn et al. 2005, Picardi et al. 2008). It is well known that genetic mutations in the Lrb (Zucker fatty rats) lead to obesity and diabetes. However, it is not known whether incomplete knockdown of Lrb in arcuate nucleus (ARC) in normally developed wild-type animals is sufficient to alter body weight (BW) on a regular chow or a high-fat diet (HFD). RNA interference (RNAi) has been proven to be ideal for long-term, stable knockdown of gene expression (Makinen et al. 2006, Kourtidis et al. 2007), which is critical for understanding and studying how reducing expression of disease-relevant genes cause disease phenotypes over extended period. In the studies, we used RNAi to investigate the possible interaction between gene and diet in the development of obesity phenotypes.

Materials and methods

Design and construction of shRNA

Lentivirus vector construction and packaging were performed by Shanghai Kangchen Biological Engineering & Technology Co. Ltd. (Shanghai, China). According to Lrb mRNA sequence (AF287268), the target oligonucleotides were designed at the position in gene sequence from 854 gene site, two cDNA template chains were designed and BamHI, Xhol enzyme cleavage sites were introduced at the gene site, two cDNA template chains were designed and annealed into one strand. For annealing, a sense, antisense, and the antisense loop was used: sense, 5'-GATCCCGAGAGATGTATCCGAGACGAGG-3', antisense, 5'-TTGATATCCGACGTGACACGTTCGGAGAACG-3', and the sequences of scrambled shRNA were sense, 5'-GATCCCC-TTCGCCGAACGTGTACGTTTCAAGAGAACGTCACCG-3', and antisense, 5'-GATCCCC-TTCGCCGAACGTGTACGTTTCAAGAGAACGTCACCG-3'. The double-stranded oligonucleotides were cloned into the linear vector containing enhanced green fluorescent protein (EGFP) gene. Escherichia coli were transformed with the recombinant linear vector and selected to obtain positive clones by PCR. The Lrb-shRNA recombinant plasmid and the scrambled shRNA control plasmid were isolated from the positive clones using plasmid preparation kit. The cloned sequence was confirmed by electrophoresis and sequencing. The Lrb-shRNA recombinant plasmids were transfected into the rat C6 glioma cells (American Type Culture Collection, Manassas, VA, USA) to determine the inhibiting efficiency by immunofluorescence microscopy and a real-time RT-PCR. The RNAi protocol specifically reduced the expression of Lrb mRNA by ~80% in transfected rat glioma cells (Liu et al. 2009).

Animal and RNAi protocols

Five-week-old male Sprague Dawley rats (n=20/group) weighing 100–120 g were housed in pairs at a constant room temperature of 20–22 °C with a 12 h light:12 h darkness schedule. The procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Stereotaxic surgery was performed under anesthesia by i.p. injection of sodium pentobarbital (60 mg/kg BW). For ARC viral delivery, Hamilton syringe needles were placed on a stereotaxic frame into the following coordinates from bregma: anterior–posterior 2.8 mm, lateral 0.4 mm, and vertical −9.5 mm. One microliter of purified virus (2.0×10⁹ infectious particles/ml) was delivered per side over a 5-min period. The injector was kept in the place for an additional 5 min before it was withdrawn in order to diffuse and to prevent backflow through the needle track. After the microinjection, the rats were fed one of two diets: the chow diet (CD; 2.6 kcal/g with 6% kcal from fat) or HFD (4.7 kcal/g with 45% kcal from fat) (Trophi Animal Feed High-Tech Co. Ltd., Nantong China) for 8 weeks. The food intake was measured daily. The BW was recorded daily for 4 weeks, after which it was measured weekly.

After 8 weeks of the diet, glucose tolerance tests were performed. The animals were fasted overnight and glucose was administered intraperitoneally (1 g/kg BW) between 0900 and 1000 h, and blood samples were taken by tail vein droplets at 0, 15, 30, 60, 90, and 120 min. Plasma glucose was measured using the glucose oxidase method (Invitrogen). Two days later, the animals were fasted overnight again and were anesthetized by i.p. injection of pentobarbital between 0900 and 1100 h. Thirteen minutes later, blood was taken by heart puncture, and serum was immediately separated through centrifugation and kept under −20 °C.

Isolation of hypothalamic tissues

The brains were harvested and the hypothalami were immediately dissected with the landmarks. The hypothalami were bordered anteriorly by the rostral border of the optic chiasma, posteriorly by the region just rostral to the

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-12-0212
© 2013 Society for Endocrinology
Published by Bioscientifica Ltd.
Printed in Great Britain

Downloaded from Bioscientifica.com at 09/12/2019 02:13:06PM via free access
mammillary bodies, and laterally 1 mm medial to the hypothalamic sulci. The depth of the excised tissue was 2 mm. The tissue samples were frozen in liquid nitrogen and stored at −80 °C until further use. For immunohistochemistry, the rats were perfused with cold PBS followed by 4% paraformaldehyde. The brains were removed, post-fixed in paraformaldehyde, and embedded with paraffin wax.

The GFP was used as a visual marker of effective transduction of the lentivirus vector and detection of the injection site, which was confirmed by visualizing GFP fluorescence using a fluorescent microscopy (Fig. 1B). The rats that did not express GFP in the ARC were excluded from any further analysis. Except for brain death after injection, anesthesia and other causes of death, and incorrect injection location, the final success rate of the animal model was 54%.

**Figure 1**

Lentivirus vector-mediated knockdown of Lrb in the hypothalamus. Schematic of shRNA expression vector (A). The vectors contain a BamHI site, a 19-nucleotide sense sequence, a loop sequence, a 19-nucleotide antisense sequence, a RNA polymerase III site, and an XhoI site. Fluorescent microscopic image showing EGFP expression (B). The lentiviral vectors expressing Lrb-shRNA or scrambled shRNA were stereotaxically injected into the arcuate nucleus (ARC). The rats were killed at 8 weeks after viral delivery. Fluorescent microscopic image showing EGFP expression in the ARC but not in the ventromedial hypothalamic nucleus (VMN). Scale bar, 100 μm. Immunohistochemistry showing Lrb protein expression in ARC (C) and VMN (E). Scale bar, 100 μm. Quantitative analysis of Lrb mRNA expression by a real-time RT-PCR in ARC (D) and VMN (F). The level of Lrb mRNA was normalized to the GAPDH values. The white bar represents scrambled control group, and the black bar represents Lrb-shRNA group. n=8–10 per group. The results are shown as mean ± S.E.M. *P<0.01 vs controls. Con, control.
Leptin signaling studies

To examine the role of Lrb knockdown on leptin signaling, the rats treated with Lrb-shRNA or scrambled shRNA (described earlier) were placed on a CD or a HFD for 8 weeks. The rats (n=5–6 for each group) were fasted overnight and then injected intraperitoneally with 15 mg/kg leptin or saline. One hour later, the rats were killed and the hypothalami were dissected. Two tissue pieces (one from each hemisphere) from individual animal were separately frozen at −80°C until further use. One piece was isolated to assess Socs3 mRNA expression via real-time PCR and the other was for quantification of phosphorylated Stat3 (pStat3) levels by western blot analysis.

Biochemical and hormonal assays

The blood samples were collected by terminal cardiac puncture and centrifuged at 1006.2 g for 10 min. The concentrations of serum leptin and insulin were determined with RIA kits provided by Linco Research (St Charles, MO, USA), and the concentrations of serum glucose, cholesterol, and triglyceride were determined with an automatic measuring analyzer (Olympus AU 1000, Tokyo, Japan).

Immunohistochemistry

Paraffin-embedded tissues including ARC were cut to a thickness of 5 µm on a microtome. The sections were deparaffinized in xylene and rehydrated using a graded series of ethanol. A method of streptavidin–biotin–HRP was used after heat-induced epitope retrieval. Briefly, endogenous peroxidase activity was blocked for 15 min in 3% hydrogen peroxide and then the slides were incubated in blocking solution for 15 min at room temperature. Thereafter, the slides were incubated with the first primary antibody for Lrb (Abcam Ltd., Hongkong, China) at a dilution of 1:100 overnight at 4°C. Detection of the immunoreaction was achieved using the secondary biotinylated antibody and the streptavidin/HRP complex (incubating for 20 min respectively). Diaminobenzidine/H2O2 was used as the chromogen and hematoxylin as the counterstain.

RNA isolation and real-time RT-PCR

In order to determine the inhibiting efficiencies of RNAi, the expression levels of Lrb mRNA in ARC were measured by a real-time RT-PCR technique. To further confirm the specificity of Lrb-shRNA in ARC, the adjacent brain sections were used to evaluate the expression levels of Lrb mRNA in ventromedial hypothalamic nucleus (VMN). The frozen samples were cut serially on a cryostat at −12°C into 300 µm sections centered on both the paraventricular nucleus of the hypothalamus and the central portion of the ARC at the level of the compact zone of the dorsomedial hypothalamic nucleus. These latter sections also contained the midpoint of the VMN and the lateral hypothalamus. The sections were placed in RNA Later (Ambion) until micropunched. The micropunctures of the ARC and VMN were performed by the method of Palkovits (1973). The tissue slices were placed on the base of a stereotaxic frame and visualized under an operating microscope. The syringe to which the punches were attached was mounted on the stereotaxic arm, and the punches were made under microscopic guidance.

Total RNA was extracted from ARC tissue using the TRizol method and purified with RNaseH according to the manufacturer’s instruction (Invitrogen). The punched samples were kept in RNA for <2 weeks before being reversed transcribed to cDNA. A real-time RT-PCR technique was used to determine relative expression levels of Lrb, Socs3, and Npy mRNA. PCR was performed using the following primers, Lrb forward: 5′-TGTTCCTTGGGCA-CAAGGACTTA-3′, reverse: 5′-ACCATAAGCTGCTGTACC-ATCTCA-3′; Socs3 forward: 5′-CTGGACCCATTCGGGA-GTTC-3′, reverse: 5′-AATCGGGAGCTACCAGCAATTG-3′; and NPY forward: 5′-ACCGAGCATGATAGGGCAAGA-3′, reverse: 5′-GGACATTCTGTGTCTTCTCATT-3′. The reactions were performed using a Light Cycler Real-Time PCR analysis (Roche Diagnostics Co.), and all amplifications were carried out in a total reaction volume of 20 µl containing 40 pmol forward and reverse primers and 2 µl cDNA by a Two-Step RT-PCR kit (TaKaRa Biotechnology Co. Dalian, China). Lrb and Socs3 were amplified using 40 cycles and NPY using 35 cycles. GAPDH (forward: 5′-GGCCAGTCAAGGCTGAGATG-3′ and reverse: 5′-ATGGTGTTGGAAGACGCAGTA-3′) was used for internal normalization. The expression levels were calculated by the standard curve method.

Protein extracts and western blot analysis

The samples from ARC (described earlier) were homogenized in ice-cold lysis buffer containing 20 mmol/l MOPS, 2 mmol/l EGTA, 5 mmol/l EDTA, 40 mmol/l β-glycerophosphate, 30 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 2 mmol/l orthovanadate, 0.5% NP-40, and complete protease inhibitors cocktail (Roche). The protein concentrations were
measured with the BCA protein quantification kit (Pierce, Rockford, IL, USA). Equal amounts (50 μg) of protein were loaded and fractionated in each lane of 10% polyacrylamide–SDS gels. After the protein was transferred to PVDF membranes, the membranes were blocked with 5% defatted milk for 60 min at room temperature and incubated with the phosphotyrosine-specific antibody against Stat3 Tyr705 (Santa Cruz Biotechnologies) at a dilution of 1:500 for overnight at 4 °C. Then the membranes were probed with the HRP-conjugated secondary antibody at a dilution of 1:5000. Blots were detected using the ECL detection kit (Amersham Life Sciences). The same blots were stripped and reblotted with an antibody specific to Stat3 to normalize the pStat3 levels. For quantitative analysis, the density of blots for pStat3 and total Stat3 was measured and expressed as ratios of pStat3 to total Stat3.

**Statistical analysis**

Statistical analysis was performed using the SPSS Software (version 11; SPSS, Inc., Chicago IL, USA). Two-way ANOVA was used to analyze energy intake and BW data (time and diet were used as the variables). One-way ANOVA with independent samples t-test was performed to analyze RT-PCR, western blot, and biochemical data. Differences were considered significant if P<0.05. Data are expressed as the means ± S.E.M.

**Results**

**Lrb knockdown in the ARC**

The rats were killed at 8 weeks after viral delivery to the ARC. Immunostaining showed that Lrb-shRNA inhibited Lrb protein expression in the ARC in both the CD group and the HFD group (Fig. 1C), but there was no affect in the VMN (Fig. 1E). The expression of Lrb mRNA was measured by real-time fluorescence PCR. The RNAi protocol specifically reduced the expression of Lrb mRNA by 47% for the CD group and 54% for the HFD group respectively (Fig. 1D). Similarly, there was no effect on Lrb mRNA expression in the VMN in both the CD group and the HFD group (Fig. 1F).

**Reduction of Lrb in the ARC promotes diet-induced obesity and associated metabolic complications**

After viral delivery, the rats were placed on a HFD or a CD for 8 weeks. Food intake was minimal immediately following surgery; however, all groups recovered to normal 2–3 days later. The Lrb-shRNA-treated rats displayed a significant increase in caloric consumption when maintained on the HFD, an effect that became apparent after 16 days of viral delivery and remained throughout the study. There were no effects of Lrb reduction on energy intake on the CD (Fig. 2).

**Figure 2**

Effect of Lrb knockdown in the ARC on energy intake in rats. The changes in caloric consumption of the HFD rats with scrambled shRNA (the white squares), the HFD rats with Lrb-shRNA (the black squares), the CD rats with scrambled shRNA (the white circles), and the CD rats with Lrb-shRNA (the black circles). n=10–12 per group. The results are shown as mean ± S.E.M. *P<0.01 vs controls.

**Figure 3**

Effect of Lrb knockdown in the ARC on body weight in rats. The lentiviral vector was stereotaxically delivered into the ARC. After the microinjection, rats were fed a high-fat diet (HFD) or a regular chow diet (CD) for 8 weeks. The white squares represent scrambled control rats on the HFD, the black squares represent Lrb-treated rats on the HFD, the white circles represent scrambled rats on the CD, and the black circles represent Lrb-treated rats on the CD. n=10–12 per group. The results are shown as mean ± S.E.M. *P<0.05 vs controls and **P<0.01 vs controls.
At the end of the study, the glucose tolerance test showed glucose intolerance in the Lrb-treated rats on the HFD. There was no difference in glucose tolerance between the scrambled control rats and the Lrb-treated rats on the CD (Fig. 4). The leptin concentrations in Lrb-shRNA rats were significantly high compared with scrambled shRNA control rats. The concentrations of insulin, fasting glucose, and cholesterol were also significantly increased in Lrb-shRNA rats on the HFD. By contrast, the biochemical parameters did not change for the CD rats treated with Lrb-shRNA (Table 1).

**Effect of Lrb knockdown on leptin signaling and Socs3 mRNA in the ARC**

To examine the role of Lrb knockdown on leptin signaling, the rats treated with Lrb-shRNA or scrambled shRNA were placed on an HFD or CD for 8 weeks. Then the exogenous leptin was injected into rats intraperitoneally and the ARC was examined with western blot analysis. One hour later, Stats3 activation was markedly reduced in Lrb-shRNA rats on an HFD or CD in infected regions (Fig. 5A). In addition, the rat treated with Lrb-shRNA revealed that leptin-induced Socs3 mRNA levels were increased in the ARC (Fig. 5B).

**Effect of Lrb knockdown on gene expression of Npy in the ARC**

The gene expression of Npy was measured by a real-time fluorescence PCR, and the levels of Npy mRNA were significantly increased in Lrb-shRNA rats on HFD or CD in infected regions (Fig. 6).

**Discussion**

Leptin is an adipocyte-derived hormone that plays a key role in the regulation of food intake, energy expenditure,
and whole-body energy balance in rodents and humans (Baile et al. 2000). Leptin acts through the Lrb, of which Lrb is the signaling-competent receptor isoform and is expressed most abundantly in paraventricular, arcuate, and ventromedial nuclei of the hypothalamus (Funahashi et al. 2003, Bjorbaek & Kahn 2004). Though there are some animal models used for obesity research, such as ob/ob mice with leptin deficiency due to mutated leptin gene, and db/db mice lacking leptin action due to mutated Lrb gene, most human obesities are not associated with deficiency of leptin secretion or Lrb bioactivity. Acquired leptin resistance or insensitivity may be a main cause of human obesity. Our previous studies showed that the obese rats induced by a HFD had hyperleptinemia and reduced expression of Lrb in hypothalamus and liver (Liu et al. 2007), suggesting that downregulated Lrb expression is one of the leptin-resistant mechanisms for maintaining obesity. In order to further study the possible interaction between gene and diet in the development of obesity phenotypes, we used a lentiviral vector-mediated RNAi technique to get long-term gene silencing of Lrb. The rats were killed at 8 weeks after viral delivery to ARC, and the EGFP-expressing cells were restricted to the deposition site. The viral delivery induced a significant reduction of Lrb mRNA expression in this region. The manipulation selectively reduced the expression of Lrb mRNA in the ARC by ~50%, which is similar to the previously reported effects of RNAi in vivo (Makimura et al. 2002) and our study (Liu et al. 2011). Thus, incomplete Lrb knockdown may have identified a metabolic role for Lrb without producing unacceptable side effects. The rats with reduced Lrb in ARC showed a significant increase in energy intake and BW in response to the HFD. However, the Lrb-shRNA-treated rats did not develop obesity when fed with a low-fat chow. A recent study (Davis et al. 2011) showed that reduction of Lrb in lateral hypothalamus led to increased food intake and BW gain, an effect that was present only when animals were maintained on a HFD, which is similar to our results. These results raise the possibility that leptin regulates BW homeostasis in a macronutrient-dependent fashion and further stresses the importance of diet in the interplay between gene and diet in the development of obesity.

Figure 5
Effect of Lrb knockdown on leptin signaling and Socs3 mRNA in the ARC. Stat3 phosphorylation in the ARC was measured by western blot (A) and Socs3 mRNA levels (B) were detected via real-time PCR. The white bars represent scrambled control rats, and the black bars represent Lrb-shRNA rats. n = 5–6 per group. The results are shown as mean ± S.E.M. *P < 0.05 vs controls. Con, control.

Figure 6
Effect of Lrb knockdown on gene expression of Npy in the ARC. The gene expression of Npy was measured by a real-time fluorescence PCR. The white bars represent scrambled control rats, and the black bars represent Lrb-shRNA rats. n = 8–10 per group. The results are shown as mean ± S.E.M. *P < 0.01 vs controls.
phenotypes. Moreover, the obese rats had hyperleptinemia, which was consistent with our previous study (Liu et al. 2007). These data proved that knockdown of Lrb in the ARC promotes diet-induced obesity and leptin resistance in rats.

Leptin activates cytokine-like signal transduction by stimulation of the JAK–STAT pathway via Lrb (Mori et al. 2004, Ueki et al. 2004). Socs3 is a member of a family of inhibitory molecules that modulate leptin action through inhibition of pathways downstream of the leptin-activated JAK–STAT pathway (Krebs & Hilton 2001). Our study showed that reduction of Lrb in ARC resulted in hyperleptinemia in both the HFD group and the CD group. The Lrb knockdown decreased Stats3 activation and increased expression of Socs3 and Npy in infected regions. These results proved that the Lrb knockdown impaired leptin signaling, supported that Socs3 was involved the etiology of leptin resistance (Myers et al. 2008).

Insulin is the second most important adipostatic signal provider to the hypothalamus and acts in the same hypothalamic areas as leptin to suppress feeding (Pliquet et al. 2006). There is an interaction between insulin and leptin action in the hypothalamus. In fact, one of the most important functions of insulin in the hypothalamus is to enhance leptin’s signal. In the hypothalamus, insulin activates signal transduction through insulin receptor substrate (Schwartz et al. 2000, Ueki et al. 2004). In addition, insulin induces a potent cross talk that with the leptin signaling pathway through the activation of JAK2 (Carvalheira et al. 2001, Myers et al. 2008). In childhood obesity, the serum level of insulin is elevated (Tamura et al. 2000). In obesity-prone juvenile rats, Lrb mRNA in hypothalamus was reduced by HFD followed by increased leptin and insulin levels (Levin et al. 2003). Hyperinsulinemia may be one of the factors for leptin resistance (Seufert 2004). Our previous study showed that HFD induced obesity and hyperinsulinemia (Liu et al. 2007). This study showed that the rats treated with Lrb-shRNA had high levels of fasting glucose and insulin after exposure to HFD, although the glucose tolerance had no pronounced change, the mild glucose intolerance took into account age and short time of diet-induced obesity. These data proved that diet-induced insulin resistance was further aggravated in rats with Lrb reduction. The mechanisms may be that Lrb knockdown in the ARC combined with a HFD results in hyperleptinemia, in turn, leads to the activation of intracellular signaling pathways that promote a negative cross talk with the leptin and insulin signaling systems, which impairs their physiological anorexogenic activities (Carvalheira et al. 2001). More importantly, the Lrb knockdown did not cause the changes in the concentrations of insulin, fasting glucose, and cholesterol of rats on the CD, emphasizing the importance of diet in the interplay between gene and diet in the development of diet-induced obesity and associated metabolic complications. In summary, these results demonstrate that Lrb knockdown in the ARC promotes diet-induced obesity and associated insulin resistance in rats, which is helpful to understand the mechanism of leptin resistance in human obesity.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by China Natural Scientific Foundation (nos 30771798 and 81273093).

References
Reduced Lrb expression by RNAi in rat


Received in final form 26 March 2013
Accepted 2 April 2013
Accepted Preprint published online 2 April 2013