Generation and Primary Phenotypes of Imidazoline Receptor Antisera-Selected (IRAS) Knockout Mice

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The ontology of imidazoline receptors was first proposed by Bousquet P in 1980s [1] and was classified as I₁, I₂, and I₃(non-I₁/I₂) subtypes. However, deficiency in high selective ligand restrains the illumination of I₁ imidazoline receptor functions. In 2000, Piletz JE screened human hippocampal expression library and found a strong candidate protein for I₁ imidazoline receptor, named “imidazoline receptor antisera-selected” (IRAS)[2]. Concurrently, Alahari SK discovered a protein with identical sequence as IRAS and named it as “nischarin” [3]. Afterward, this protein was proved to be similar with I₁ imidazoline receptor in tissue distribution, ligand-binding property, and intracellular signalings and mediate many processes such as hypotensive effect of rilmenidine, inhibition of opioid addiction, inhibition of cell migration, antiapoptosis, and so on [4]. Herein, we originally generated IRAS conditional knockout mice (IRAS^{flxed/flxed}) and IRAS null mice (IRAS^{-/-}), which might be valuable tools for functional exploration of IRAS/nischarin and I₁ imidazoline receptors.

Mouse IRAS gene is located on chromosome 14 and scatters into 21 exons, and we generated IRAS^{flxed/flxed} mice by flanking exon 4 with loxP sites and then obtained IRAS^{-/-} mice by crossing EIIa-Cre mice (Figure 1A and Data S1 for more details). As for IRAS^{-/-} mice, genotyping with the primer pair 5loxP-f and 3loxP-r could distinguish wild-type, heterozygote, and knockout mice

(Figure 1B). Sequencing result of the PCR product from 5loxP-f and 3flank primer pair revealed that only a loxP site remained between upstream and downstream homologous arms. In RT-qPCR, the amplification curve of IRAS^{-/-} was normal when the primer pair was originated from exon 3 and exon 5; however, the product is smaller than the wild type (Figure 1C). Moreover, the amplification curve of IRAS^{-/-} was null when the primer pair was originated from exon 2 and exon 4 (Figure 1D). All the results above illustrated the normal transcription and the absence of exon 4 counterpart in the transcription product of IRAS^{-/-} mice, which resulted in a new stop code in the following exon 5. In the Western blot assay, three bands, between 95 and 130 kD, were absent in cerebellum tissue of IRAS^{-/-} mice (Figure 1E). As several bands that represent functional IRAS have been reported, including 85 and 33 kD in human and 67 kD in bovine, these characteristic bands were proposed to be the splicing products in mouse cerebellum. All the above declared the functional deletion of IRAS gene.

The IRAS^{-/-} mice were born small compared with wild-type littermates (Figure 2A, B). Furthermore, weights of knockout embryos at day 12.5 (0.121 g, 0.133 g, and 0.143 g) were smaller than those of wild-type littermates (0.173 and 0.173 g) (Figure 2C). These results suggested the participation of IRAS in

Figure 1 Construction of Imidazoline Receptor Antisera-Selected (IRAS) knockout mice. **(A)** Targeting strategy and primers used in screening and verification. **(B)** Genotyping of progenies from inter-heterozygote crossing by P4 primer pair, the wild-type allele is 1.3 kb (the upper band), and the IRAS is 400 bp (the lower band). **(C)** Products of real-time PCR with the primer pair originated from exon 3 and exon 5, the KO band was smaller than the WT band. **(D)** Amplification curves (left) and melting curves (right) of real-time PCR with the primer pair originated from exon 2 and exon 4. The curves were abnormal for knockout mice. **(E)** Western blot with C terminal antibody against IRAS. 1. HEK-293T cell lysate, 2. cell lysate from HEK-293T cell transfected with pCMV-IRAS expressing vector, 3. cerebellum lysate from IRAS null mouse, 4. cerebellum lysate from wild-type mouse. A band >170 kD was obvious in 1 and 2, and three discrepant bands between 130 and 95 kD were revealed in 3 and 4.

prenatal growth and the postnatal growth retardation might be an after effect. The developmental retardation of IRAS^{-/-} mice is similar to Silver–Russell syndrome (SRS, OMIM: 180860), a

genetic disease with disturbances of genes in insulin-like growth factor pathway, such as GRB10, IGF1, IGF2, and so on [5]. On the other hand, Sano H et al. reported the interaction between

Figure 2 Phenotypes of Imidazoline Receptor Antisense-Selected knockout (IRAS^{-/-}) mice. **(A)** Representative IRAS^{-/-} mouse (left) and wild-type mouse (right) of 8 weeks. **(B)** Postnatal body weights, that is, at weaning (3 weeks, wild type $n = 18$, knockout $n = 33$), 7 weeks (wild-type $n = 9$, knockout $n = 8$) and 11 weeks (wild type $n = 16$, knockout $n = 14$). Mean $\pm$ SEM, *** $P < 0.001$, knockout versus wild-type, two-way ANOVA. The difference between body weights of knockout and wild-type (the lowest curve) reduced a little after 7 weeks, which was a reflection of catch-up growth. **(C)** Representative embryos at E12.5. The knockout embryo was smaller than wild-type. **(D and E)** Basal pain threshold in hot-plate test and tail-flick test (wild-type $n = 16$, knockout $n = 14$). Mean $\pm$ SEM, ** $P < 0.01$ and *** $P < 0.001$, knockout versus wild-type, two-way ANOVA with repeated measurements followed by Bonferroni *post-hoc* test. **(F)** Cumulative dose-response curves of methadone analgesia before (Day 1) and after chronic methadone (10 mg/kg, sc) treatment (Days 3, 6 and 9). $n = 8$ of each genotype, mean $\pm$ SEM. **(G)** Analgesia of methadone at the dosage of 10 mg/kg (Data were normalized by the corresponding MPE values of pre-tolerance induction). $n = 8$ of each genotype, mean $\pm$ SEM. ** $P < 0.01$ and *** $P < 0.001$, knockout versus wild-type, two-way ANOVA with repeated measurements followed by Bonferroni *post-hoc* test.

IRAS and insulin receptor substrate, which is the intermediate molecule transferring the signal from IGF receptor to the downstream second messages [6]. Therefore, we proposed that IRAS knockout might lead to dysfunction of IGF receptor signaling and, as a consequence, restrict the fetal growth.

There was no significant difference between wild-type and IRAS^{-/-} mice in spontaneous locomotion, grip strength, motor coordination (rotarod test), spatial learning and memory (Morris water maze test), and sensorimotor gating function (prepulse inhibition of acoustic startle response test). Moreover, IRAS knockout did not alter the systolic blood pressure and heart rate of conscious mice (14.4 ± 0.7 kPa and 565 ± 45 beats/min for wild-type mice, $n = 6$; 14.0 ± 0.6 kPa and 553 ± 64 beats/min for knockout mice, $n = 9$, measured by Softron BP-98A system), which challenged the idea that IRAS/I₁ receptor mediated the central modulation of blood pressure, and the underlying mechanism still needs further investigation.

Besides, pain threshold of IRAS^{-/-} was lower than that of wild type in both hot-plate test and tail-flick test, indicating the noci-

ceptive perception was potentiated in IRAS^{-/-} mice (Figure 2D, E). Additionally, compared with wild-type mice, the analgesia of methadone in IRAS^{-/-} mice was attenuated in 55°C hot-plate test (genotype: $P < 0.001$, dose: $P < 0.001$, genotype $\times$ dose: $P = 0.186$, two-way ANOVA with repeated measurements for WT-day1 vs. KO-day1) (Figure 2F). After chronic methadone (10 mg/kg, sc) treatments, the dose-response curves of methadone analgesia shifted to right and forward in both wild-type and IRAS^{-/-} mice, suggesting the analgesia tolerance developed (Figure 2F). Because of the difference of methadone analgesia before tolerance induction between wild-type and knockout mice, we normalized the MPE values of Days 3, 6, and 9 (after chronic methadone treatment) by that of Day 1 (before chronic methadone treatment). The normalized data revealed that IRAS knockout exacerbated chronic methadone-exposure-induced analgesia tolerance (genotype: $P < 0.01$, treatment: $P < 0.01$, genotype $\times$ treatment: $P = 0.471$, two-way ANOVA with repeated measurements) (Figure 2G). Moreover, after chronic methadone (10 mg/kg, sc) treatment for 8 days, naloxone-precipitation-induced withdrawal

jumping was different between the two genotype mice, in which the incidences of jumping were 4 of 8 and 1 of 8 for IRAS^{-/-} and wild-type mice, respectively ($P < 0.01$, χ^2 -test). This result suggested that IRAS knockout exacerbated the development of physical dependence as well. In 1994, Li G discovered agmatine as the endogenous ligand for imidazoline receptors [7]. Our previous works revealed that exogenous agmatine could enhance morphine analgesia and alleviate morphine tolerance and dependence, which may be mediated by IRAS or I₁ imidazoline receptor [8–10]. Meanwhile, increasing the level of endogenous agmatine resulted in the same effects as exogenous agmatine; decreasing endogenous agmatine or blocking imidazoline receptors resulted in the reverse effects [8–10]. Therefore, we proposed that “agmatine and imidazoline receptors might be a novel endogenous modulating system for opioid functions.” In this study, IRAS^{-/-} mice not only showed a reduction in pain threshold, but also displayed hyporeactive in analgesia and hyperreactive in tolerance and physical dependence to exogenous opioid substances, which was in full consistent with our hypothesis. Therefore, by means of knockout mice, we not only certified that IRAS is a functional molecule in modulating the opioid functions, but also proved the

existence of the novel endogenous modulating system for opioid functions, that is, agmatine and I₁ imidazoline receptor.

In conclusion, we generated IRAS knockout mice for the first time. Primary phenotype assays indicated that this protein may play an important role in embryo development, nociceptive perception, and modulation of opioid functions.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Data S1. Methods of generation of IRAS^{-/-} mice and methadone analgesia, tolerance and physical dependence.