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Research Overview of the Lab. of Molecula Biology, Tohoku University Graduate School of Agricultural Science

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Abstract

In our laboratory, mainly three different projects described below are now carried out. 1) "Generation and application of the cell membrane permeabilized protein", 2) "Studies on epigenetic regulation of eukaryotic genes through analyses of actin-related proteins" and 3) "Generation of the oxytocin receptor KO (*OXTR-/-*) mice, and molecular physiological and behavioral analysis of the oxytocin receptor KO (*OXTR-/-*) mice." In the first project, we generated recombinant Cre protein fused to TAT PTD (protein transduction domain), and the application of this protein into several primary cells prepared from mouse, showed significant recombinase activity. In the second project, we studied epigenetic regulation of eukaryotic genes through analyses of actin-related proteins. In the third project, we obtained several new findings in reproduction, sociosexual behaviors and in physiology of energy/temperature homeostasis of mice as the functions of oxytocin/oxytocin receptor system in vivo, using the oxytocin and its receptor genes deficient (OXT-/- and OXTR-/-) mice. Here we introduce a little about those three projects, respectively.

Key words: Protein transduction domain; TAT PTD; Cre; Actin-related protein, Epigenetics; Gene KO mice; Sociosexual behavior; Oxytocin

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Abbreviations used are: PTD, protein transduction domain; Arp. Actin-related proteins; OXT, Oxytocin; OXTR, Oxytocin Receptor

Research Project 1 ; Highly Induced DNA Recombination Mediated by Membrane Permeabilized Recombinant Cre Protein in Mouse Primary Cells

We established efficient induction of Cre mediated DNA recombination in primarily cultured cells, mouse embryonic fibroblast, keratinocyte and primary preosteoblast. We tested various recombinant Cres by modification of TAT-Cre reported by Peitz, M., fusing of protein transduction domain in HIV transactivator of transcription (TAT-PTD) to the N-and/or C-terminus. Resultant recombinant product, HTC, in which modified Cre with PTD at N-terminus, achieved the highest activity of DNA recombination for those primary cells.

Recently, several small regions of proteins, called protein transduction domains (PTD), have been developed as carriers for efficient delivery of various proteins, when by themselves cannot enter living cells. HIV-1 TAT- (48-60), Antennapedia- (43-58) (Antp) and membrane translocating sequence (MTS) peptides are known as typical PTD sequences. By genetically or chemically attaching these PTD sequences to the target protein, efficient intracellular delivery of it was often successfully achieved.

The Cre/LoxP recombination system has been widely used to induce allele dependent gene deletion, in which targeted gene was sandwiched between two LoxP elements, induced by Cre activity in a spatial and/or temporal manner. *Escherichia coli* phage P1 Cre recombinase catalyzes the site-specific recombination of DNA with two LoxP sites. Depending on the location of LoxP sites, Cre-catalyzed recombination results in DNA inversion or excision/integration. Numerous studies have demonstrated the advantage of Cre-mediated conditional mutagenesis in so-called floxed mice and cells derived from them. These mice were generated with the essential parts of the genes of interest flanked by two LoxP sites, and site specific recombination was usually performed by mating with TG mice. Those TG mice spaciotemporally expressing Cre recombinase were generated by introduction of Cre recombinase gene laid under appropriate transcriptional control element. This method is very advantageous to study the function of a gene in vivo with circumventing lethality caused by lost of essential genes, however the constitutive expression of Cre has been shown to be cytotoxic in many cell types. Moreover, it is not easy to obtain appropriate Cre TG mice, facilitating desired induction of spacio-temporal expression of Cre gene. Alternative ways to induce Cre recombination *in vivo* are to use DNA transfection or viral infection. However, DNA transfection has difficulties to achieve higher transferable efficiency *in vivo*, and viral infection has also difficulty not only with lower infection efficiency for live tissues but also with biologically potential risk for human.

Peitz et al reported that Cre fused with TAT-PTD at the N-terminus enhan-

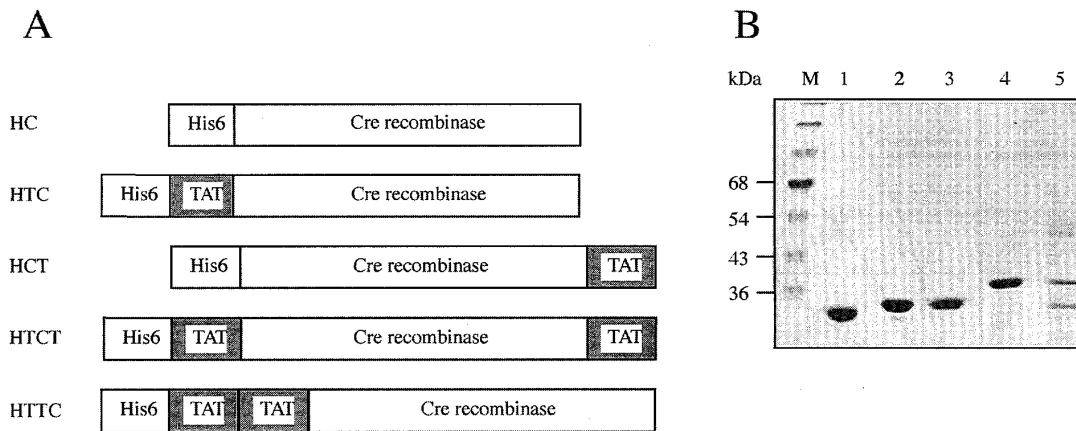

FIG. 1. Design of expression cassettes and purification of Cre fusion proteins. (A) Five expression constructs were generated. All constructs encode Cre recombinase and a His-tag as represented by white boxes. Gray boxes represent PTD-TAT (GRKKRRQRRR). Abbreviations of the constructs are given on the left. (B) Comparison of purified fusion proteins on a Coomassie stained gel. Only a faint protein band of HTTC was detected.

ced cellular uptake and subsequent recombination. We examined the properties of Cre fused with TAT-PTD located at the N- and/or C-terminus of Cre and developed highly efficient method to induce Cre-mediated gene recombination in mouse primarily cultured cells.

We generated several Cre chimeric proteins with connecting a single PTD or tandem PTDs at both terminuses of it and produced by E.coli system, expecting higher transduction efficiency of Cre with multiple PTDs. The TAT-PTD-Cre fusion proteins used in this work are shown in Fig. 1A. The purification of Cre fusion proteins was confirmed by SDS-PAGE (Fig. 1B). At first we tested recombination activity of HTC, HCT, HTCT and HC chimeric proteins *in vitro*. Consequently it suggested that *in vitro* Cre activity was not affected by location or number of TAT-PTD on the protein. In addition to *in vitro* enzyme assays, we performed recombinant Cre transduction experiments using β-galactosidase (β-gal) reporter cells from mouse line ROSA26R. We compared the transduction efficiencies with various concentrations of Cre fusion proteins (HTC, HCT and HTCT) and intact Cre (HC) protein as a control. As shown in Fig. 2A, the Cre activity in fibroblast cells with HTC (50 μg/ml) and HTCT (100 μg/ml) reached to staining of 100% cells counted in 9 mm^2 area of Petri dish surface, whereas 16% cells stained with HC, and 55% with HCT were observed at the same protein concentration (100 μg/ml). Among those Cre fusion proteins tested in this study, HTC showed most efficient recombination activity, suggesting that TAT-PTD at N-terminus can achieve higher Cre enzyme activity presumably caused by comparatively higher efficiency in protein transduction and/or refolding efficiency, although the location of TAT-PTD at the N-terminus or C-terminus

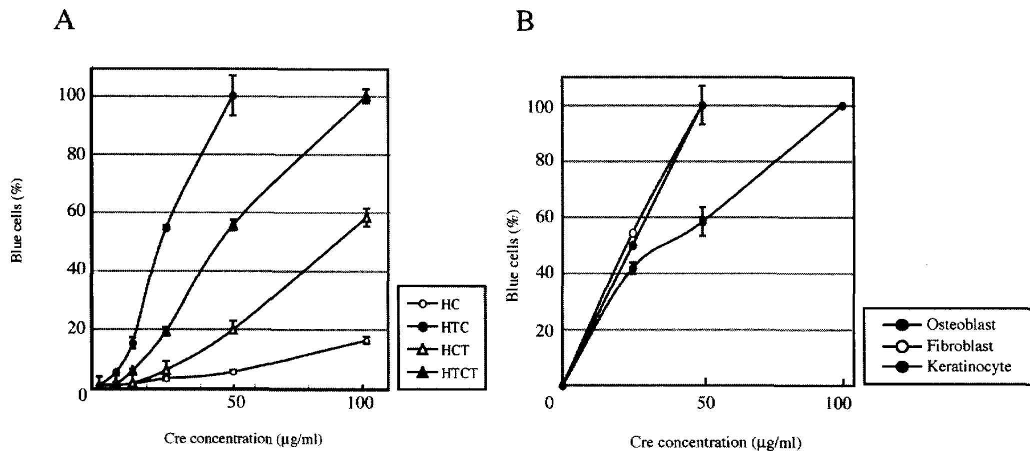

FIG. 2. Transduction of Cre fusion proteins into Cre reporter cells. Transduction of Cre fusion proteins into fibroblasts from ROSA 26R. Fibroblasts were incubated for 16 h in medium containing purified Cre fusion proteins. After incubation cells were washed, incubated with normal medium for 60 h, fixed and stained for β-galactosidase (β-gal) activity. Quantification of β-gal activities in reporter fibroblasts incubated with various concentrations of Cre fusion proteins. Percentages of blue cells are given. (A) Quantification of β-gal activities in reporter fibroblasts, osteoblasts and keratynocytes were carried out by incubation of those cells with various concentrations of Cre fusion proteins. Percentages of blue cells in each cell type are given. (B)

does not affect the Cre activities *in vitro*.

The contributive mechanism of the location and number of PTD sequence on aimed proteins for efficient transduction should be further studied. Genetic or epigenetic modification of primary cells generates transplantable self-renewing tissues suitable for therapeutic application. However it is generally difficult to be achieved because of lower efficiency in DNA transfection or viral vector infection. To overcome those difficulties, we applied some of PTD-Cre fusion protein for three typical primary cells. The transduction efficiencies of HTC into fibroblast, primary preosteoblast and keratinocyte cells, prepared from ROSA26R reporter mouse, were assessed and compared (Fig. 2B). With 50 μg/ml of purified HTC in culture medium, 100% recombination activity was achieved with fibroblast and keratinocyte cells. In contrast, fully saturated recombination efficiency was achieved with primary preosteoblast cell at $\sim$1 μg/ml of purified HTC in medium (1).

TAT-PTD mediated transduction provides several advantages over the standard DNA transfection method, to secure intracellular protein expression by introduction of exogenous genes into cells. DNA transfection or virus-mediated DNA transfection have limited availability in primary cultured cells. We can demonstrate here clearly that the TAT-PTD procedure is an alternative and effective strategy to transiently express various protein activities from exogenous genes in those cells, instead of DNA transfection. To achieve maximum protein

transduction efficiency with higher protein activity, the maximal concentration of the protein in medium and location of TAT-PTD on the fused construct should be precisely assessed in each experiment.

Research Project 2; Studies on epigenetic regulation of eukaryotic genes through analyses of actin-related proteins

Recently the total genome sequence of many organisms has been determined. However, the function of the genome can not be fully deduced only from its sequence, because the genome function is regulated by epigenetic mechanisms. In eukaryotic cells, chromatin structure and nuclear dynamics provide the molecular basis of the epigenetic regulation of the genes. On the other hand, the molecular construction of chromatin and the nucleus has not been fully disclosed yet, and it is now requited to identify and analyze functional proteins involved in the epigenetic regulation. We have approached this problem through the investigation of actin-related proteins.

Actin (Fig. 3) plays central roles in the organization and dynamics of the cytosketeton. Because of the characteristics of actin, previous researchers hypothesized that actin and/or its evolutionarily related molecules were involved in the organization and dynamics of the nucleus. However, actin filaments were observed only in the cytoplasm, and no molecule evolutionarily related to actin was identified at the time. The hypothesis was therefore regarded with skepticism for a long time. In 1992, the first molecule, other than its isoforms, that is evolutionarily related to actin was identified. This molecule was called actin-

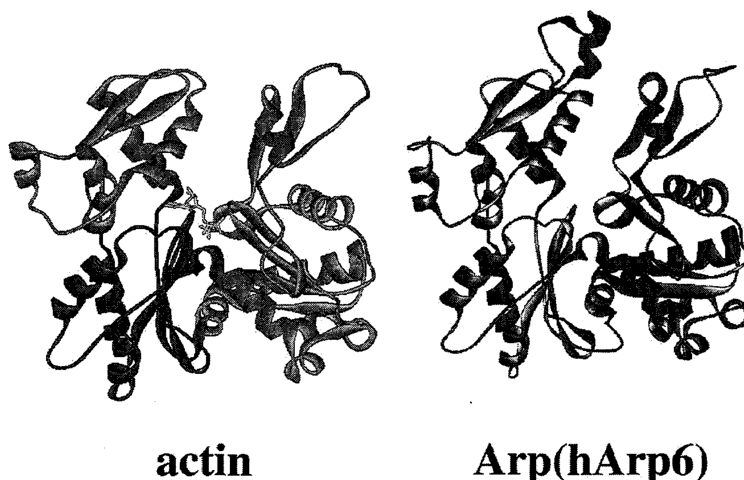

FIG. 3. The deduced three-dimensional structures of actin and Arp. The three-dimensional structures of hArp6 (right) was deduced and compared with that of rabbit skeletal muscle actin (left). The ATP molecule in the ATP-binding pocket of actin is indicated as a stick model.

related protein, abbreviated Arp. The discovery of Arp revealed that the actin family consists of actin isoforms and Arps. The first Arp identified was localized in the cytoplasm ; however, we found that some Arps were localized predominantly in the nucleus of a wide variety of organisms.

The finding of Arps localized in the nucleus shows that the actin family is an attractive candidate for the protein families involved in the epigenetic regulation of the genome. Recent biochemical approaches reveal that the nuclear Arps are components of complexes involved in chromatin modulation in both yeasts and vertebrates.

In addition we found that some Arps were included in the nuclear architecture. Advances in chromatin research, especially using the budding yeast system, have revealed that at least two classes of protein complexes in the nucleus govern the modulation of chromatin structure: one class is the ATP-dependent chromatin remodeling complexes, the other is the histone modification complexes. These complexes consist of a catalytic subunit and various specific components. The catalytic subunits, the ATPases of the remodeling complexes and the histone acetyltransferases (HATs) of the modifying complexes, are responsible for the enzymatic reactions. While many of the other components that are required for the regulation of the function of these complexes are diverse, Arps were consistently found in multiple chromatin remodeling and HAT complexes from yeast to mammals.

In budding yeast, NuA4 HAT complex contains Arp4 together with actin. In chromatin remodeling complexes, INO80 complex contains Arp4, Arp5, and Arp8 together with actin, and both SWI/SNF and the RSC complexes contain Arp7 and Arp9. SWR1 complex, which replaces histone H2A with the variant H2AZ, was shown to contain Arp4 and Arp6 (Fig. 3) together with actin. Importantly, in most cases examined, Arps are required for the activity of these complexes.

In mammals, ArpNβ/BAF53 is a component of various chromatin remodeling and related complexes, including SWI/SNF (BAF), SWI/SNF-B (PBAF), p400, cMyc-associated, and WINAC complexes. TIP60 HAT complex also contains ArpNβ/BAF53. Because of its brain-specific expression, ArpNα was not present in these isolated complexes; however, exogenously expressed ArpNα was co-immunoprecipitated with BRM, an enzymatic component of mammalian SWI/SNF (BAF) complex, as well as with ArpNβ BAF53. Recently, a SWI/SNF-like neuron-specific chromatin remodeling complex (bBAF) was identified, and its neuron-specific component was identical to ArpNα. These results, taken together with their extremely similar amino acid sequences, suggest that both ArpNα and ArpNβ/BAF53 are mutually selective components of various complexes involved in chromatin modulation (2).

Recent analyses of nuclear Arps suggest their involvement in chromatin and nuclear dynamics as components of chromatin modulation complexes and the

nuclear architecture (3). Although chromatin modulation and nuclear dynamics are realized by the collaboration of many molecules, further analyses of nuclear Arps will disclose novel molecular mechanisms in the epigenetic regulation of the genome.

Research Project 3 ; Multiple behavioral and physiological defects observed in mice lacking oxytocin and oxytocin receptor system.

The neuro peptide Oxytocin (OXT), is a neurohypophysial hormone (and recently it has been also considered to be a neurotransmitter/neuromodulator) with only 9 amino acids (Fig. 4). It was first identified as one of the prototypical hypothalamic hormones released into the bloodstream from the posterior pituitary. OXT has been considered to be essential for uterine contraction in parturition, milk ejection and sexual/ nurturing behaviors. In OXT-/- male mice, which we generated, although social amnesia was observed, OXT-/- female mice only demonstrated defect in milk ejection as a defect in reproductive abilities. On the contrary, female mice deficient in prostaglandin F2a receptor(FP) gene showed failure in delivery, and further analysis showed that no increase of the level of oxytocin receptor (OXTR) transcript in uterus immediately before term of delivery caused this disorder. Both results are implying inconsequence that OXTR but not OXT is necessary for parturition. Oxytocin receptor (OXTR) is a member of the GPCR receptors family. The cytoplasmic domain is known to functionally couple with Gq or G11 subtype of subunits of trimeric G-protein complex. Binding of oxytocin to OXTR activates sequentially G-protein subunit, phopholipase C and PKC, leading to activation of various cellular signal proteins with acceleration of the outflow of Ca^{++} from the endoplasmic reticulum. Finally the signal triggers numerous downstream cascade reactions (Fig. 5).

To define the function of OXTR *in vivo*, we generated mice deficient in *OXTR* gene (*OXTR*-/-). Firstly, we generated mice whose *OXTR* locus was

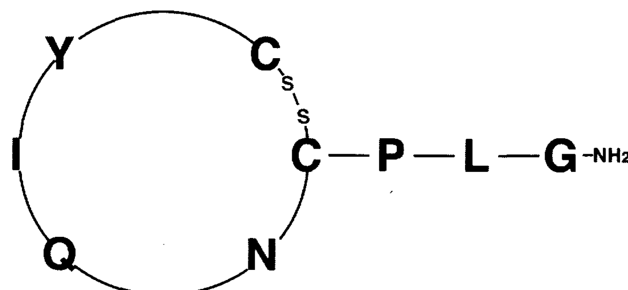

FIG. 4. Structure of Oxtocin. Oxytocin (OT) belongs to the neurogenic nonapeptide hormones family. Ring structure of OT is formed between the two Cysteine.

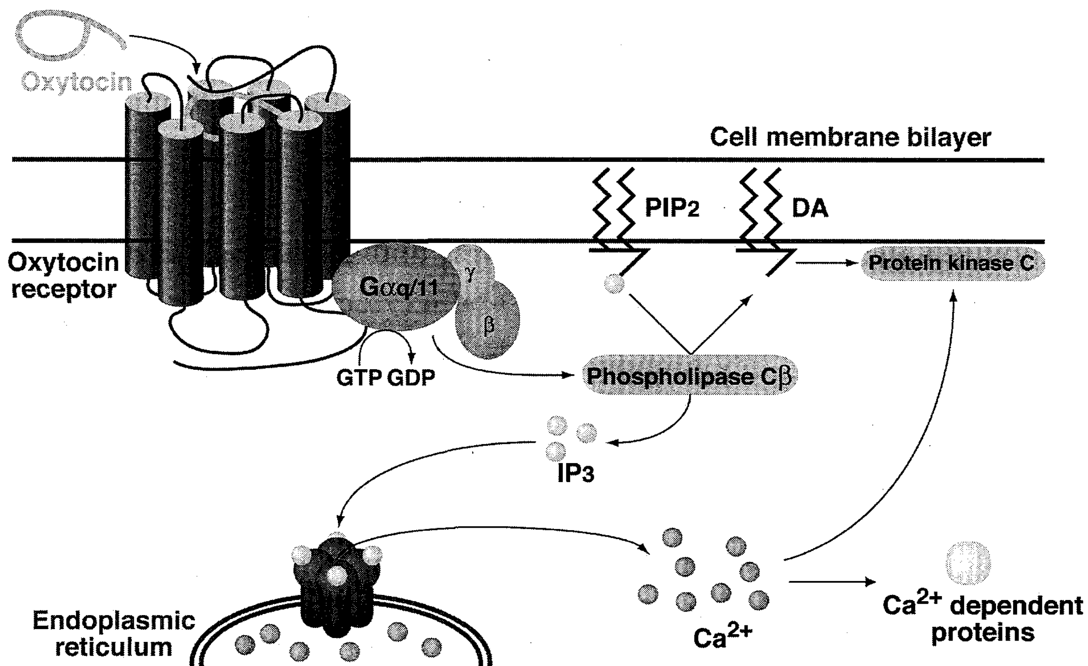

FIG. 5. The structure and signaling mechanism of OTR. Phosphatidylinositol (PI) derived cell membrane phospholipid is metabolized by kinase and it becomes phosphatidylinositol 4,5-bis phosphate (PIP2). When oxytocin binds to the OTR, inactive Gα proteins bound to GDP is activated by GTP binding. After stimulation of phospholipase Cβ (effector) with GTP-activated Gα, signal is transduced by diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP3) (second messengers) produced from PIP2 by the effector. IP3 has been thought to be a second messenger for intracellular Ca^{2+} mobilization. Both Ca^{2+} and DAG activate protein kinase C.

substituted by so-called floxed-OXTR-neo (*fx-OXTR*) knockin construct by homologous recombination with embryonic stem cell technology. For the excision of floxed cassettes, CAG-Cre transgenic mice, showing Cre-mediated recombination at early stages of development, were mated with fx-OXTR mice and we obtained conventional OXTR knockout mice. Southern blot analysis and ligand binding to brain from homozygote offspring failed to detect normal OXTR, confirming that the mutation was a null mutation.

Female OXTR-/- mice showed no obvious defect in parturition as well as OXT-/- mice. Failure in milk ejection, observed in OXTR-/- female mice, leaded to death of neonatal mice. Alveoli in mammary gland of OXTR-/- mice immediately before term of delivery were normal, but the size of alveoli in OXTR-/- immediately after term tended to be smaller than wt.

Next, we examined the abnormalities in sociosexual behaviors of the mice lacking OXTR (OXTR-/-) genes in comparison with those lacking OXT gene (OXT-/-). In results, OXTR-/- males showed elevation in their aggression, with normal plasma concentration of testosterone. In contrast, OXT-/- males showed

no elevated aggression, demonstrating discrepancy between the functions of OXT and OXTR genes. In detection of remnant receptor activity for OXT, no binding capacity to OXT in brain of OXTR-/- mice was found. In the receptor mutants, concentration of OXT and AVP in pituitary, plasma OXT, and expression level of mRNAs of OXT and AVP in hypothalamus were all not altered. We suspected that the influence of maternal OXT through placenta on the development of fetus brain to establish normal social behavior in OXT-/- male. OXT-null males from cross of OXT-null parents, showed elevated aggression, according to our expectation. On the other hand, OXTR-/- female showed abnormal maternal behavior in retrieving and crouching over the pups. Virgin OXTR-/- females also displayed a similar defect, suggesting that OXTR is required for nurturing responses to pups outside the physiological context of pregnancy and parturition. In addition, decrease in isolation-induced ultrasonic vocalizations and increase in locomotor activity of infant males, and impairment of social discrimination as well as OXT-/- was found in OXTR-/- males. Our study demonstrates that OXTR may not only play a critical role in female reproductive ability, such as milk ejection, but also roles in regulating several social behaviors, suspected to be related to developmental psychiatric disorders (4).

Other than several aberrations in their social behaviors shown in OXTR-/- and OXT-/- mice, they also showed novel physiological dysfunctions caused by deficit of OXTR or Oxt genes. We analyzed the temperature homeostasis in mice lacking oxytocin (OXT) and found that the null mice ($Oxt^{-/-}$) exhibited lower core body temperatures when they were exposed to a cold environment. $OXT^{-/-}$ mice also showed slight obese phenotype (weight gain), but there were no obvious differences in the morphology of white and brown adipose tissues between wt and ligand *OXT* KO ($OXT^{-/-}$) mice. In cold-exposed conditions, some of the OXT neurons (OXT-expressing neurons) in the paraventricular nucleus of the hypothalamus showed elevated immunoreactivity for c-Fos antibody. This is the first report suggesting that the central OXT may contribute to the thermoregulatory system to maintain the energy/body temperature homeostasis (5).

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