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Transgenic silkworms expressing human insulin receptors for evaluation of therapeutically active insulin receptor agonists

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A B S T R A C T

We established a transgenic silkworm strain expressing the human insulin receptor (hIR) using the GAL4/UAS system. Administration of human insulin to transgenic silkworms expressing hIR decreased hemolymph sugar levels and facilitated Akt phosphorylation in the fat body. The decrease in hemolymph sugar levels induced by injection of human insulin in the transgenic silkworms expressing hIR was blocked by co-injection of wortmannin, a phosphoinositide 3-kinase inhibitor. Administration of bovine insulin, an hIR ligand, also effectively decreased sugar levels in the transgenic silkworms. These findings indicate that functional hIRs that respond to human insulin were successfully induced in the transgenic silkworms. We propose that the humanized silkworm expressing hIR is useful for *in vivo* evaluation of the therapeutic activities of insulin receptor agonists.

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1. Introduction

Understanding the mechanisms of pathogenicity and exacerbation in human disease is important toward establishing prophylaxis and therapy. Although many animal models have been developed for preclinical studies, inconsistencies between humans and model animals are a major stumbling block. To address this issue, humanized mice, i.e., mice expressing human genes, have been constructed and used in many studies [1]. Humanized mice are categorized into three groups, in which either (i) human genes are introduced, (ii) human cells are transplanted, or (iii) human tissues or organs are transplanted [1–5]. Humanized mice expressing human receptor proteins required for the infection of pathogens such as poliovirus have been established [6,7]. Immunodeficient mice transplanted with human immune cells or cancer cells are used for immunologic and cancer therapy studies [1,8]. In recent studies, humanized mice receiving transplantation of human tissues established from human embryonic stem cells or induced pluripotent stem cells were used as animal models to study the exacerbation mechanisms of various diseases and for vaccine development [1,9–11]. The large number of mice needed for drug screening, however, is both costly and associated with ethical problems in terms of animal welfare. Invertebrates, on the other hand, are less costly to rear and house, and are associated with fewer ethical problems compared to mammals; therefore, the development of alternative invertebrate animal models would be highly advantageous.

We previously proposed using silkworms as an invertebrate animal model for screening drug candidates for therapeutic efficacy and toxicity. The silk industry has a long history with established methods for handling a large number of silkworms. Silkworms cost much less and occupy less space than mammals such as mice and rats. Silkworms move slowly and their large body size is suitable for sample injection by syringes. Moreover, drug metabolism is similar between silkworms and mammals. We compared the toxic effects of various chemical compounds between both silkworm and mammalian models, and demonstrated that the LD_{50} values of toxic compounds were similar between models [12]. Furthermore, we previously established various silkworm infection models using bacteria, fungi, and viruses, and reported that the ED_{50} values of medicines were comparable to those in mammals [13–15]. These findings suggest that the pharmacokinetics of various medicines in silkworms is similar to those in mammals, including humans.

Abbreviations: hIR, human insulin receptor; PI3, phosphoinositide 3; UAS, upstream activating sequence.

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Transgenic techniques to express exogenous genes in silkworms are established [16]. We previously generated a transgenic silkworm line expressing GFP in a tissue-specific manner by applying the GAL4/UAS system [17]. Silkworm strains expressing human genes have been established [18]. These reports provide examples of the use of silkworms as efficient machinery for recombinant protein production. To date, however, the *in vivo* functions of human gene products have not been assessed in live transgenic silkworms. Based on previous studies using silkworms as disease models, we considered the transgenic technique applicable for generating humanized silkworms for *in vivo* evaluation of drugs that target human proteins. In the present study, we constructed humanized silkworms expressing the human insulin receptor (hIR). We previously reported that silkworms fed a glucose-containing diet exhibited hyperglycemia, and the increased sugar levels in hyperglycemic silkworms were decreased by intra-hemolymph injection of human insulin [19]. Furthermore, administration of human insulin stimulated Akt phosphorylation in fat body cells, which was inhibited by wortmannin, a pharmacologic inhibitor of the upstream phosphoinositide 3 (PI3) kinase. These findings indicate that the intracellular insulin-signaling pathway, which is well elucidated in mammals, is conserved in silkworms. In our previous study, the concentration of human insulin required for therapeutic effects in silkworms was 100-fold higher than that used clinically for human patients. This could be explained by the lower affinity of the silkworm insulin receptor for human insulin. We therefore considered that transgenic expression of a human-type insulin receptor in silkworms could decrease the amount of drug required and make the silkworm model more accurately reflect the activity of human insulin. Here, we examined the utility of the transgenic hIR-expressing silkworm as a model animal to evaluate the *in vivo* therapeutic effects of insulin receptor agonists. This is the first report of the use of transgenic silkworms expressing a receptor gene of human origin to evaluate the activities of various therapeutic compounds.

2. Materials and methods

2.1. Insects

The silkworm strain w1-pnd, *actin A3*-GAL4/3×P3-DsRed2 (w1) [20], is maintained at the Transgenic Silkworm Research Unit of National Institute of Agrobiological Sciences. Silkworms were reared with an artificial diet, SilkMate 2S (Nihon Nosan Kogyo, Yokohama, Japan), at 27 °C.

2.2. Chemicals

Recombinant human insulin and recombinant bovine insulin were purchased from Wako and Sigma, respectively, and dissolved in 0.9% NaCl containing 0.1% acetic acid. Wortmannin was purchased from Wako and dissolved in DMSO for stock solution at 10 mM.

2.3. Construction of the hIR expression vector

The full-length cDNA of the hIR cloned in pCR-XL-TOPO was purchased as a Funakoshi IMAGE clone from Open Biosystems (clone ID: 40125723). The ORF region was amplified by PCR using a primer set with a cleavage site for *Xba* I (Supplementary Table 1). This PCR product was ligated into a pZErO-2 vector (Invitrogen), and transformed into a competent *Escherichia coli* strain. Plasmid pZErO-2-hIR was extracted from a drug-resistant colony after overnight incubation at 37 °C and the DNA size of the insert was verified by electrophoresis. The DNA sequence of the *hIR* gene in the plasmid was further confirmed using primers, as shown in Supplementary Table 1.

Construction of the pBacMCS[UAS-SV40, 3×P3-EGFP] vector, encoding *upstream activating sequence* (*UAS*) and *egfp* genes, was previously described [21]. The *Xba* I fragment of pZErO-2-hIR, which contains the hIR ORF, was cloned into the *Bln* I site of pBacMCS[UAS-SV40, 3×P3-EGFP]. The resulting plasmid was named pBac-UAS-hIR-3×P3-GFP. The complete hIR sequence was analyzed and confirmed by DNA sequencing using primers, as shown in Supplementary Table 1.

2.4. Generation of transgenic silkworms expressing hIR

The method for generating transgenic silkworms was previously reported [21]. The plasmid constructed above (pBac-UAS-hIR-3×P3-EGFP) was micro-injected into silkworm eggs (w1-pnd strain), thus producing a line expressing the *hIR* gene under regulation of the UAS (UAS-*hIR*). Because the vector encodes GFP downstream of the 3×P3 promoter specific to compound eyes, transgenic silkworms could be selected by observation under a fluorescence microscope. This transgenic line was crossed with another line transformed by a plasmid, pBac-A3-GAL4-3×P3-DsRed, containing both the *GAL4* gene downstream of the cytoplasmic *actin A3* promoter and the *DsRed2* gene as an eye marker [20]. Silkworms expressing both EGFP and DsRed2 in the compound eyes (UAS-*hIR*; *actin A3*-GAL4) were selected as the "hIR-induced strain" under a fluorescence microscope. Western blotting analysis

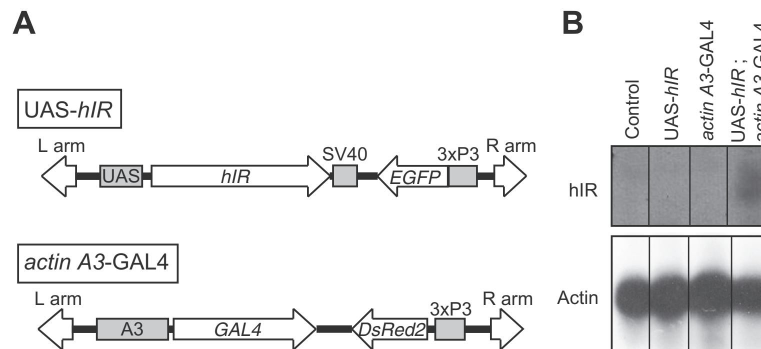

Fig. 1. Development of transgenic silkworms expressing the human insulin receptor using the GAL4/UAS system. (A) Structures of UAS-*hIR* and *actin A3*-GAL4 for expressing hIR in silkworms. "UAS-*hIR*" represents the genotype for UAS-*hIR* with the green fluorescent protein marker, and "*actin A3*-GAL4" represents the genotype for *actin A3*-GAL4 with the red fluorescent protein marker. Note that the "UAS-*hIR*; *actin A3*-GAL4" strain possesses both UAS-*hIR* and *actin A3*-GAL4, which enable the induced expression of hIR in whole body. hIR: human insulin receptor. (B) Detection of human insulin receptors in the fat body of transgenic silkworms. Fat bodies of the silkworms, (Control), (UAS-*hIR*), (*actin A3*-GAL4) and (UAS-*hIR*; *actin A3*-GAL4), were isolated. Human insulin receptor and ß-actin were determined by Western blot analysis using each antibody.

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