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Insulin-like growth factor (IGF)-like peptide and 20-hydroxyecdysone regulate the growth and development of the male genital disk through different mechanisms in the silkmoth, *Bombyx mori*

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ABSTRACT

It is well established that ecdysteroids play pivotal roles in the regulation of insect molting and metamorphosis. However, the mechanisms by which ecdysteroids regulate the growth and development of adult organs after pupation are poorly understood. Recently, we have identified insulin-like growth factor (IGF)-like peptides (IGFLPs), which are secreted after pupation under the control of 20-hydroxyecdysone (20E). In the silkmoth, *Bombyx mori*, massive amounts of *Bombyx*-IGFLP (BIGFLP) are present in the hemolymph during pupal-adult development, suggesting its importance in the regulation of adult tissue growth. Thus, we hypothesized that the growth and development of adult tissues including imaginal disks are regulated by the combined effects of BIGFLP and 20E. In this study, we investigated the growth-promoting effects of BIGFLP and 20E using the male genital disks of *B. mori* cultured *ex vivo*, and further analyzed the cell signaling pathways mediating hormone actions. We demonstrate that 20E induces the elongation of genital disks, that both hormones stimulate protein synthesis in an additive manner, and that BIGFLP and 20E exert their effects through the insulin/IGF signaling pathway and mitogen-activated protein kinase pathway, respectively. These results show that the growth and development of the genital disk are coordinately regulated by both BIGFLP and 20E.

1. Introduction

Insects grow and develop under the regulation of many hormones. In particular, ecdysteroids produced by prothoracic glands (PGs) are key hormones that control insect development, including embryonic development, molting, metamorphosis, and ovary development (Kozlova and Thummel, 2003; Yamanaka et al., 2013; Belles and Piulachs, 2015). The PGs secrete ecdysone, and this steroid is converted to 20-hydroxyecdysone (20E), an active form of ecdysteroids, in peripheral tissues including the fat body, midgut, and muscle (Petryk et al., 2003; Rewitz et al., 2006). The molecular mechanisms by which 20E regulates the larval-pupal transition have been extensively studied (Yamanaka et al., 2013; Zitnan and Adams, 2012). In general, ecdysteroids exert their effects by activating the ecdysone receptor (EcR), a member of nuclear receptor family of transcription factors, to regulate the expression of a specific set of genes (Henrich, 2012).

A vast amount of research has been conducted on the

Abbreviations: ILP, Insulin-like peptide; BIGFLP, *Bombyx* insulin-like growth factor-like peptide; PG, prothoracic gland; 20E, 20-hydroxyecdysone; IIS, insulin/IGF signaling; MAPK, mitogen-activated protein kinase; RIA, radioimmunoassay; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TR-FIA, time-resolved fluoroimmunoassay; PI3K, phosphatidylinositol 3-kinase; InR, insulin-like receptor; EcR, ecdysone receptor; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; p-AKT, phosphorylated AKT; p-ERK, phosphorylated ERK.

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mechanisms of ecdysteroid actions during larval-pupal development; however, studies on mechanism of ecdysteroid actions regulating pupal-adult development are very limited, although, based on physiological studies (Nijhout, 1994) and on their very high hemolymph titers (Mizoguchi et al., 2001), the importance of ecdysteroids in this stage has been well recognized. It is widely accepted that the role of ecdysteroids during pupal-adult development is to induce the degradation of larval tissues, such as silk glands, salivary glands, midgut, and fat body, and to promote the growth and development of adult organs. The effects of 20E on the degradation of larval tissues have been analyzed in some reports (Tsuzuki et al., 2001; Nicolson et al., 2015); however, except for its effects on wing development that starts during the final larval instar, little is known about its effects on the growth and development of adult organs (Cranna and Quinn, 2009; Moriyama et al., 2016).

Adult organs such as wings and reproductive organs in Lepidoptera develop from imaginal disks, which are kept in the larval body as small, immature tissues. The timing of their growth and development differs among disks (Koyama et al., 2013; Nijhout and Callier, 2015). For example, the wing disks in Lepidoptera grow and develop during larval-pupal development, whereas the genital disk grows after pupation. Several *ex vivo* studies have shown that 20E induces the development of imaginal disks. For example, 20E induced tracheal development in the wing disks of *Manduca sexta* (Nijhout et al., 2007) and elongation of the genital disks of *B. mori* (Shinbo and Happ, 1989).

Insulin-like peptides (ILPs) also play important roles in the regulation of insect growth, especially during larval life (Nijhout et al., 2014; Okamoto and Yamanaka, 2015; Nassel and Vanden Broeck, 2016). The first insect ILP was identified in the silkmoth, *Bombyx mori* (Nagasawa et al., 1986; Mizoguchi and Okamoto, 2013) and was named bombyxin (Mizoguchi et al., 1987). ILPs have now been identified and characterized in a wide variety of insects (Antonova et al., 2012). Insect ILPs are mainly produced by neurosecretory cells in the brain (Mizoguchi and Okamoto, 2013; Nassel and Vanden Broeck, 2016). The physiological functions of ILPs have been well studied in Lepidoptera and the fruit fly, *Drosophila melanogaster*, using physiological and genetic approaches, respectively (Okamoto and Yamanaka, 2015). Bombyxin regulates carbohydrate metabolism (Satake et al., 1997) and the growth of imaginal disks (Nijhout and Grunert, 2002; Nijhout et al., 2007). In *D. melanogaster*, ILPs (DILPs) are involved in the regulation of larval growth, metabolism, as well as longevity and stress response (Mizoguchi and Okamoto, 2013; Nassel and Vanden Broeck, 2016). Since bombyxin and DILPs are released from the brain in response to nutritional intake, insect ILPs are considered to function as key signals that couple systemic and tissue-specific growth with nutritional availability (Nijhout et al., 2014; Okamoto and Yamanaka, 2015).

Recently, we identified a new class of ILPs, the IGF-like peptide (IGFLP) in *B. mori* (Okamoto et al., 2009a). This peptide resembles bombyxin in its amino acid sequence but differs in its structural feature, production site, developmental stage of secretion, titers in hemolymph, and mode of secretion control (Okamoto et al., 2009a; Okamoto et al., 2011). *Bombyx* IGFLP (BIGFLP) retains the C-domain in its mature form (Okamoto et al., 2009a), unlike insulin and bombyxin, in both of which the C-domain in the precursor peptide is removed to produce heterodimeric mature hormones consisting of A- and B-chains (Froesch and Zapf, 1985). This peptide is mainly produced by the fat body, which is the insect tissue corresponding to the liver and adipose tissues in vertebrates, after pupal ecdysis. The titer of BIGFLP in the hemolymph during pupal-adult development is approximately 1000 times higher than that of bombyxin (Satake et al., 1999; Okamoto et al., 2009a). Gene expression and peptide secretion of BIGFLP are induced by ecdysteroid released shortly before pupal ecdysis. It has also been demonstrated that BIGFLP promotes protein synthesis and/or cell division in the primordia of adult tissues including the genital disk, sperm ducts, and flight muscles *ex vivo* (Okamoto et al., 2009a). IGFLP was also identified in *D. melanogaster* (DILP6) (Okamoto et al., 2009b; Slaidina et al., 2009). Gene expression of DILP6 is also remarkably high during metamorphosis. In addition, gene knockout of DILP6 resulted in decreases in adult size and body weight (Okamoto et al., 2009b; Slaidina et al., 2009). These results suggested that IGFLP plays critical roles in the regulation of insect growth in the post-feeding stage, when insects undergo adult development.

The titers of IGFLP and ecdysteroids are remarkably high during pupal-adult development, and both hormones have been shown to affect the growth of adult-specific tissues. Therefore, we hypothesized that the development of imaginal disks during pupal-adult development is regulated by the combined effects of IGFLP and 20E. In this study, we investigated the individual and combined effects of IGFLP and 20E on the growth and development of the genital disk and further analyzed cell signaling pathways mediating hormonal actions, using an *ex vivo* culture system. We demonstrate that the two hormones coordinately regulate growth and development of the genital disk through different mechanisms: BIGFLP promotes protein synthesis via the insulin/IGF signaling (IIS) pathway, whereas 20E induces morphogenesis via the mitogen-activated protein kinase (MAPK) pathway.

2. Materials & methods

2.1. Animals

A hybrid of different strains of the silkmoth, *B. mori*, Kinshu × Showa (Ueda Sanshu, Ueda, Japan) was used. Larvae were reared at 25 °C ± 1.5 °C under a 12-h light and 12-h dark photoperiod on an artificial diet “SilkMate” (Nihon Nosan Kogyo, Yokohama, Japan). Most larvae started wandering on days 6 (males) and 7 (females) of the fifth instar, and ecdysed to pupae on days 10 (males) and 11 (females).

2.2. Antibodies and hormones

A phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, and Grace's insect medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit monoclonal antibodies against phosphorylated extracellular signal-regulated kinase (p-ERK), total ERK, and AKT, rabbit polyclonal antibodies against phosphorylated AKT (p-AKT), mouse monoclonal antibody against alpha-tubulin, and the MAPK/ERK kinase (MEK) inhibitor U0126 were purchased from Cell Signaling Technology (Danvers, MA, USA). Ecdysone and 20E was purchased from Enzo Biochem, Inc. (Farmingdale, NY, USA). A horseradish peroxidase (HRP)-conjugated anti-mouse IgG rabbit antibody and an HRP-conjugated anti-rabbit IgG goat antibody were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Guinea pig polyclonal antibody against bombyxin-II and mouse monoclonal antibodies against BIGFLP (D7H3, D11E12) were previously produced by our group (Okamoto et al., 2009a). The D7H3 and D11E12 antibodies recognize different regions of the C-domain of BIGFLP. The anti-bombyxin-II antibody cross-reacts with BIGFLP. The anti-BIGFLP antibodies do not recognize bombyxin.

2.3. Hemolymph sampling and hormone determination

Hemolymph samples for determining the titers of ecdysone and 20E were collected twice daily from 1 day after the onset of wandering (W1) to 5 days after pupation (P5) at the same time of day

(1 h after both lights on and lights off). Collected samples were diluted 10-fold with methanol and centrifuged at 14,000 × g for 15 min. Aliquots of the supernatants were evaporated and dissolved with 250 μL of methanol, followed by centrifugation at 3000 × g for 10 min. Aliquots of the supernatant were used for the determination of ecdysone and 20E by liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to the method of Hikiba et al. (2013). Hemolymph samples for BIGFLP titer determination were collected from animals daily at 6 h after lights on. Collected samples were diluted 5-fold with Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and heated at 70 °C for 5 min, followed by centrifugation at 14,000 × g for 15 min. Aliquots of the supernatant were used for BIGFLP determination by time-resolved fluoroimmunoassay (TR-FIA), which was performed as described previously (Okamoto et al., 2009a).

2.4. BIGFLP purification

Hemolymph from day-5 or day-6 female pupae was collected on ice. Sodium diethyldithiocarbamate and *p*-amidinophenylmethanesulfonyl fluoride were added to the hemolymph to give final concentrations of 5 mM and 10 μM, respectively. After centrifugation at 1500 × g for 15 min, the hemolymph was diluted with two volumes of TBS and heated at 70 °C for 5 min. After centrifugation at 17,800 × g for 15 min, the supernatant was filtered with filter paper. The filtered hemolymph was then subjected to affinity chromatography. An affinity column was prepared by immobilizing D7H3 antibody onto Hi-Trap NHS activated HP columns (GE Healthcare, Tokyo, Japan), in accordance with the manufacturer's instructions. For desalting, the eluate was applied to a Sep-Pak Vac C8 cartridge (Waters, Associates, Milford, MA, USA). After washing the cartridge with 0.1% trifluoroacetic acid (TFA), the adsorbed materials were eluted with 60% acetonitrile in 0.1% TFA. The eluate was evaporated under reduced pressure to remove acetonitrile and then freeze-dried. The dried sample was dissolved in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and affinity-purified BIGFLP was quantified by TR-FIA.

2.5. Ex vivo culture

Male genital disks from day-8 fifth-instar larvae (2 days after the onset of wandering) were aseptically dissected and cultivated in 500 μL of basal medium (Grace's insect medium containing 0.8% BSA, 100 units/mL penicillin, and 100 μg/mL streptomycin) or hormone/inhibitor-containing medium using a 24-well culture plate. Culture medium was syringe-filtered through a disk filter with 0.22-μm pore size (Merck Millipore Ltd., Darmstadt, Germany). BIGFLP, 20E, and/or cell signaling inhibitors were added to the basal medium at selected concentrations. All cultures were maintained at 25 °C under a 12-h light and 12-h dark photoperiod, and half of the medium was replaced by fresh medium 2 and 4 days after the beginning of the culture.

2.6. Measurement of disk length and protein content

Images of genital disks were obtained using a digital camera, Nikon D5000 (Nikon, Tokyo, Japan). The length of the long axis of the disk was measured on the images. After obtaining images, the disks were homogenized by sonication in TE buffer (10 mM Tris, 1 mM EDTA, pH8.0). After centrifugation of the homogenates at 12,000 × g for 15 min, the supernatants were used for protein assays using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

2.7. Western blotting

SDS-PAGE and Western blotting for detecting AKT and ERK were performed in accordance with the method described by Rybczynski et al. (2001). Briefly, the cultivated genital disks (three disks for one sample) were homogenized in SDS sample buffer (for ERK detection) or alkaline phosphatase buffer (Takara Bio Inc., Shiga, Japan) (for AKT detection). Homogenates for detecting AKT were incubated with alkaline phosphatase from calf intestine (final concentration 1 unit/μL, Takara Bio) overnight at 37 °C. After incubation, the homogenates were mixed with SDS sample buffer. All of the homogenates mixed with SDS sample buffer were boiled for 4 min and centrifuged at 15,800 × g for 3 min. Aliquots of the supernatants were loaded onto 8% (for AKT) or 10% (for ERK) SDS-PAGE gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Immobilon®-P; Millipore, Darmstadt, Germany), and then blocked with BlockAce (MEGMILK SNOW BRAND Co., Ltd., Tokyo, Japan) in TBS at room temperature for 1 h. Blots were incubated with the primary antibody diluted 1:2500 with 2% BSA in TBS containing 0.1% Tween-20 (TBS-T) for 4 h at room temperature. After washing, blots were incubated with HRP-conjugated secondary antibody diluted 1:10,000 with 1% BSA in TBS-T for 1 h at room temperature. Following additional washing, immunoreactivity was detected with Immunostar® LD (Wako Pure Chemical Industries Ltd., Osaka, Japan) and scanned using Chem-iDoc™ XRS+ (Bio-Rad).

2.8. Immunohistochemistry

Genital disks were fixed with 4% paraformaldehyde in PBS for 2 h at room temperature. After washing with 0.5% Triton X-100 in PBS (PBS-Tx), the disks were blocked with 10% normal goat serum (Wako Pure Chemical Industries Ltd.) in PBS-Tx overnight at 4 °C. The disks were incubated with the primary antibody in PBS-Tx (1:200 dilution for anti-p-AKT antibody, 1:500 dilution for anti-p-ERK antibody) for 2 days at 4 °C. After washing, the disks were incubated with Alexa 488-conjugated anti-rabbit IgG antibody (Thermo Fisher Scientific, MA, USA) in PBS-Tx (1:1000) overnight at 4 °C. Following another wash, the disks were permeated with glycerol and mounted on slides. Fluorescence was detected with an OLYMPUS IX70 inverted microscope (Olympus, Tokyo, Japan) and scanned with an Olympus DP73 digital camera (Olympus). The gain was set at a selected value and the same settings were used to capture all images in each of two sets of experiments (Fig. 5A–C/Fig. S1 and Fig. 5D–F/Fig. S2).

2.9. Statistical analysis

Effects of BIGFLP and 20E on genital disk growth and interaction between the effects of both hormones were analyzed by two-way analysis of variance (ANOVA) with Dunnett's test. Significance in the effects of signaling inhibitors on hormone-induced growth of the disks was analyzed by Dunnett's test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Changes in 20E and BIGFLP titers in the hemolymph

It is important to use the physiological concentrations of the hormones to investigate the effects of hormones on the tissue cultured *ex vivo*. Therefore, we first measured the hemolymph titers of ecdysone, 20E, and BIGFLP (Fig. 1). Ecdysteroid titers in

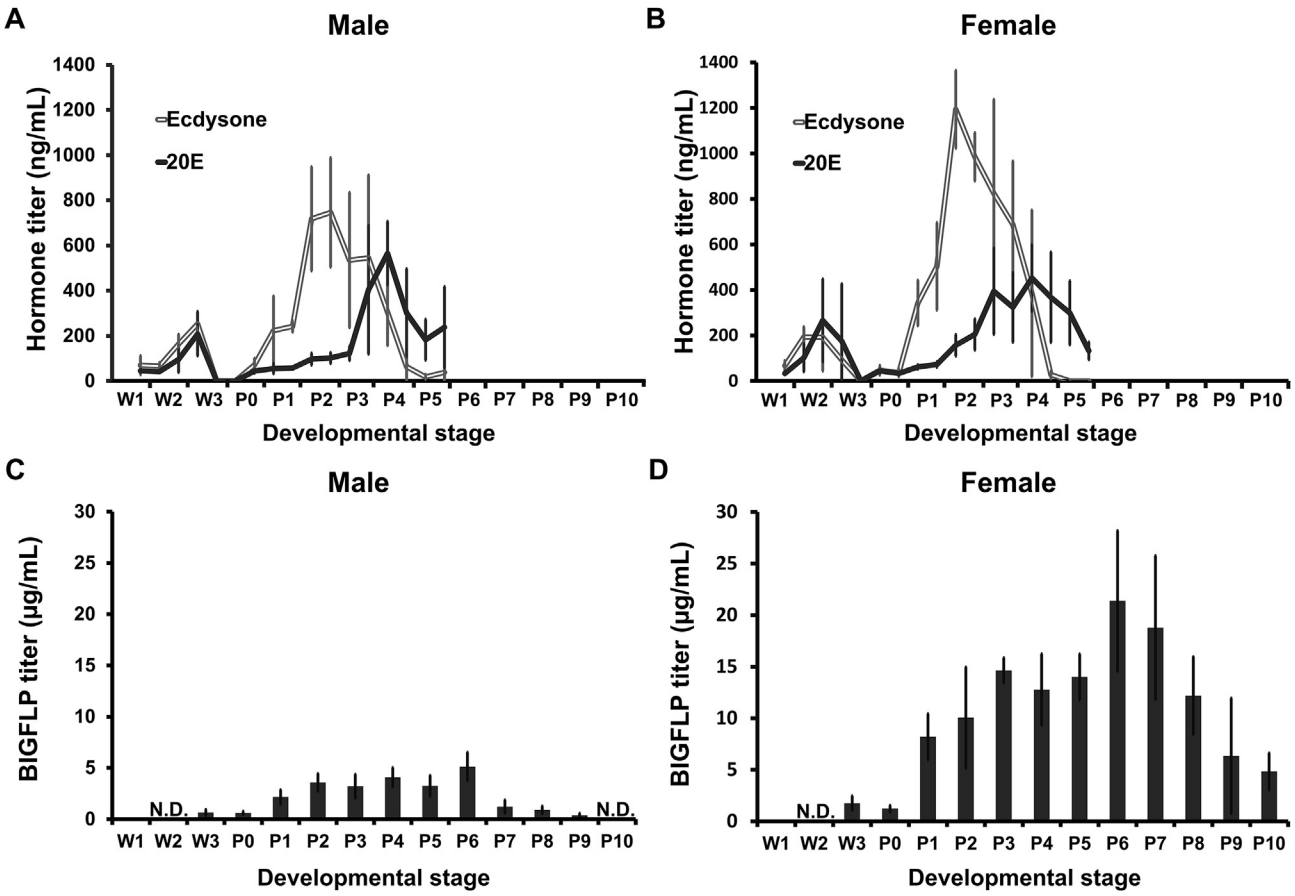

Fig. 1. Changes in ecdysone, 20E, and BIGFLP titers in the hemolymph after the wandering stage. (A, B) The concentrations of ecdysone and 20E in the hemolymph of males (A) and females (B) were determined by the LC-MS/MS method. (C, D) BIGFLP concentrations in the hemolymph of males (C) and females (D) were determined by TR-FIA. Note that ecdysteroid titers for days 6–10 pupae and BIGFLP titers for day 1 wandering larvae were not determined. The values represent the means ± SD (n = 5). Wn, n days after the onset of the wandering stage; Pn, n days after pupation.

B. mori hemolymph were previously determined by radioimmunoassay (RIA) (Calvez et al., 1976; Mizoguchi et al., 2001). However, those titers represented the titers of total ecdysteroids including ecdysone, 20E, and some other ecdysteroids, because the antibody used in these immunoassays could not distinguish between 20E and other ecdysteroids. Recently, our group established a method to quantify each ecdysteroid by LC-MS/MS analysis, and reported the changes in the titers of ecdysone and 20E during the final larval and pre-pupal stages (Hikiba et al., 2013). Using this method, we determined the concentrations of ecdysone and 20E in the hemolymph during the period from the wandering stage through several days after pupal ecdysis in both males and females (Fig. 1A and B). During the wandering stage, the profiles of changes in ecdysone and 20E titers were similar, with a peak on day 3 (males) or day 2 (females). The peak titers were also similar between the two ecdysteroids. After pupation, the ecdysone titer was highest on day 2, whereas the 20E titer was highest on day 4 in both sexes. In males, the 20E titer was approximately 0 ng/mL from 1 day before pupation (W3) to immediately after pupation (P0), approximately 50 ng/mL from P0 to P1, and approximately 100 ng/mL from P2 to P3. BIGFLP titers were determined by TR-FIA. BIGFLP was first detected in the hemolymph one day before pupation and then gradually increased, reaching the peak concentration 6 days after pupation (Fig. 1C and D). The titers were much higher in females than in males throughout pupal-adult development. The titers in males for several days after pupation were in the range of 0.5–3.5 μg/mL (70–450 nM).

3.2. Growth-promoting effects of 20E and BIGFLP on male genital disks

The effects of BIGFLP and 20E on genital disk growth was evaluated using male disks, because they were bigger in size and therefore easier to collect without damage than female disks. The male genital disk develops into inner and outer sexual organs: The upper region in Fig. 2A develops into the external genitalia (penis), while the lower region into the internal genitalia (ejaculatory duct, seminal vesicle, and accessory gland) (Suzuki et al., 2005) (Fig. 2B). During pupal-adult development, the ejaculatory duct and accessory gland elongate, and this elongation can be induced *ex vivo* by 20E (Shinbo and Happ, 1989). We therefore decided to use the length of the disk as an index of disk development. In this study, we also measured protein content in the disks as an index of cellular growth.

We set the 20E concentration in the culture medium to match the changes in this concentration in the hemolymph during the early stages of pupal-adult development as follows: 0 nM for 0–24 h, 100 nM for 24–72 h, and 200 nM for 72–120 h of culture. The concentration of BIGFLP was set at 0, 10, or 100 nM to assess the dose-dependent effects of this hormone on the disk.

The size of the genital disks was significantly increased by the addition of BIGFLP to the culture, while 20E induced a drastic change in the shape of the disks (Fig. 2C–F). Although BIGFLP did not induce a change in the shape of the disks (Fig. 2G), it promoted protein synthesis in the disks in a dose-dependent manner

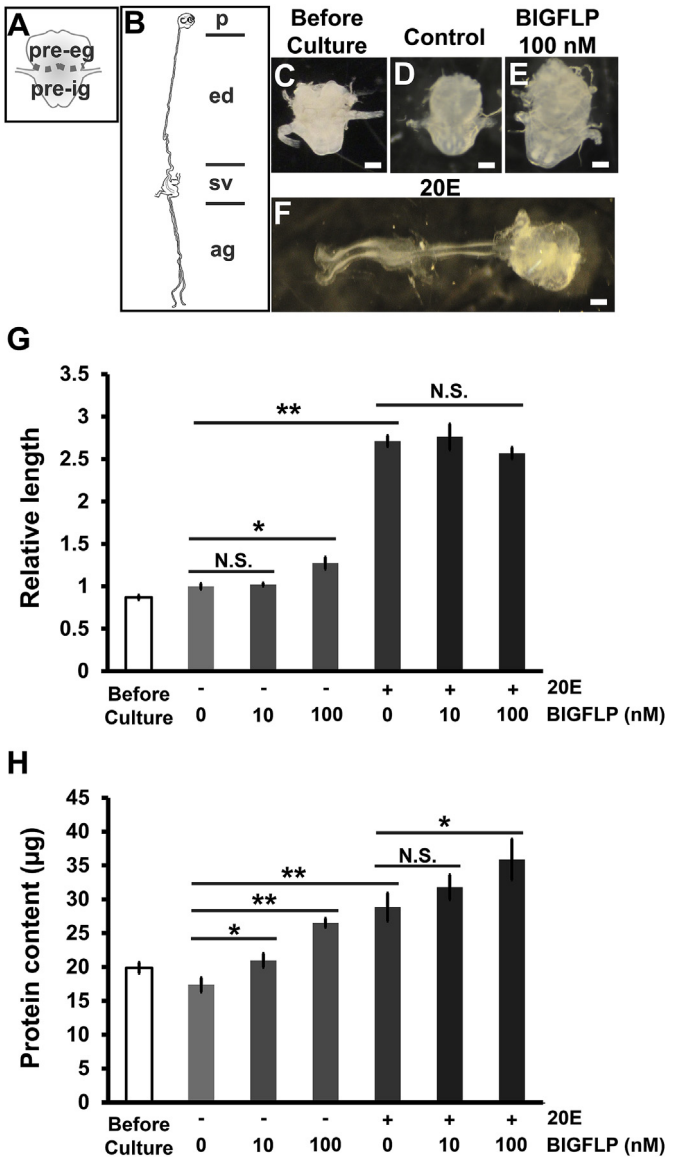

Fig. 2. Growth-promoting effects of BIGFLP and 20E on the male genital disk. (A, B) Schematic diagram of the male genital disk of day-8 fifth-instar (2 days after the onset of wandering: W2) larva (A) and adult (B). pre-eg, presumptive external genitalia; pre-ig, presumptive internal genitalia; p, penis; ed, ejaculatory duct; sv, seminal vesicle; ag, accessory gland. (C–F) Male genital disks were dissected from day-8 fifth-instar larvae, and their images were taken before (C) and after culture for 5 days in basal medium (D), 100 nM BIGFLP (E), or graded concentrations of 20E (F). The 20E concentration was 0 nM for 0–24 h, 100 nM for 24–72 h, and 200 nM for 72–120 h of culture. Half of the medium was replaced by fresh medium containing the same concentration of hormone 2 and 4 days after the beginning of culture. (G, H) The disks before and after culture were measured for the length of their long axis (G) (ANOVA, BIGFLP:20E interaction, $p = 0.016$) and protein content (H) (ANOVA, BIGFLP:20E interaction, $p = 0.84$). The disk length was expressed as the length relative to that of disks cultivated without hormone. The values represent the means ± SEM (n = 6). Note that the data in Fig. 2G and H are subsets of data in Fig. 3A, C and 3B, D, respectively. Statistics: two-way ANOVA (BIGFLP × 20E) followed by Dunnett's test (G, H). $^{*}p < 0.05$, $^{**}p < 0.01$. N.S., not significant. Scale bars: 200 μm.

(Fig. 2H). In contrast, 20E induced remarkable elongation of the disks, especially at the presumptive ejaculatory duct and accessory gland (Fig. 2G). Interestingly, 20E also promoted protein synthesis (Fig. 2H). Although both hormones significantly increased the protein content, two-way ANOVA showed no interaction between these effects [$F(2, 30) = 0.18$, $p = 0.84$], indicating that 20E and BIGFLP promote protein synthesis in an additive manner.

3.3. Prevention of BIGFLP- and 20E-stimulated growth by signaling inhibitors

To identify the cell signaling pathways involved in the effects of the hormones, signaling inhibitors were added to the culture medium. In general, insulin family peptides including insulin, IGF and insect ILPs activate the IIS pathway (Taguchi and White, 2008). Because IGFLP was expected to interact with insulin-like receptor (InR) to activate the IIS pathway, which includes PI3K as a key component (Taguchi and White, 2008), we used a PI3K inhibitor, LY294002 (Vlahos et al., 1994). LY294002 blocked the BIGFLP-induced size increase and protein synthesis in the genital disks, suggesting that BIGFLP promotes protein synthesis through the IIS pathway (Fig. 3A and B). This inhibitor did not affect the elongation and protein synthesis of the disks after treatment with 20E (Fig. 3A and B), indicating that the 20E-induced protein synthesis is not mediated by this pathway.

In general, the actions of 20E are mediated by EcR, a member of the nuclear receptors that regulate gene expression as transcription factors (Henrich, 2012). However, little is known about the cellular mechanisms by which elongation of the genital disk is regulated. To explore the signaling pathway involved in the induction of disk elongation, we hypothesized that the MAPK pathway plays a role, because this pathway is widely used in the regulation of cell proliferation and differentiation (Zhang and Dong, 2007). Thus, we next examined the effects of an inhibitor of MEK, a component of the MAPK pathway (Favata et al., 1998), on the hormone-induced growth of the disks. As expected, a MEK inhibitor, U0126, inhibited both 20E-induced elongation and protein synthesis of the genital disks in a dose-dependent manner (Fig. 3C and D), suggesting that 20E induces the development of the genital disk through the MAPK pathway. In contrast, this inhibitor did not affect the BIGFLP-inducible protein synthesis at all (Fig. 3C and D).

These findings showed that the growth-promoting effects of BIGFLP and 20E were mediated by different cell signaling pathways.

3.4. IIS pathway and MAPK pathway are activated by BIGFLP and 20E, respectively

The analyses with cell signaling inhibitors suggested that the effects of BIGFLP and 20E are mediated by activation of the IIS pathway and MAPK pathway, respectively. To confirm the involvement of these pathways in the actions of these two hormones, we investigated the effects of these hormones on the phosphorylation of AKT and ERK, components of the IIS and MAPK pathways, respectively, by Western blotting (Fig. 4).

BIGFLP was expected to activate the IIS pathway in a short time, as demonstrated in many insulin/IGF studies (Alessi et al., 1996). In fact, AKT was phosphorylated within 5 min after BIGFLP administration, with a peak of phosphorylation observed at 20 min (Fig. 4A). This phosphorylation was reduced by the treatment with the PI3K inhibitor LY294002 (Fig. 4B). In contrast, 20E did not induce AKT phosphorylation (Fig. 4B).

On the other hand, 20E promoted ERK phosphorylation (Fig. 4C and D). ERK was slightly phosphorylated in a short time (< 15 min) and further phosphorylated thereafter (Fig. 4C). The level of ERK phosphorylation fluctuated during the first 24 h after 20E administration (Fig. 4D) and subsequently became stabilized. The effect of the MEK inhibitor U0126 on ERK phosphorylation was examined after 24 h of culture, when the ERK was stably phosphorylated (Fig. 4E). U0126 not only inhibited the 20E-dependent phosphorylation of ERK, but also reduced the hormone-independent phosphorylation of ERK. BIGFLP did not enhance ERK phosphorylation (Fig. 4E), consistent with the observation that U0126 did not inhibit the BIGFLP-inducible growth of the genital disk (Fig. 3C and D).

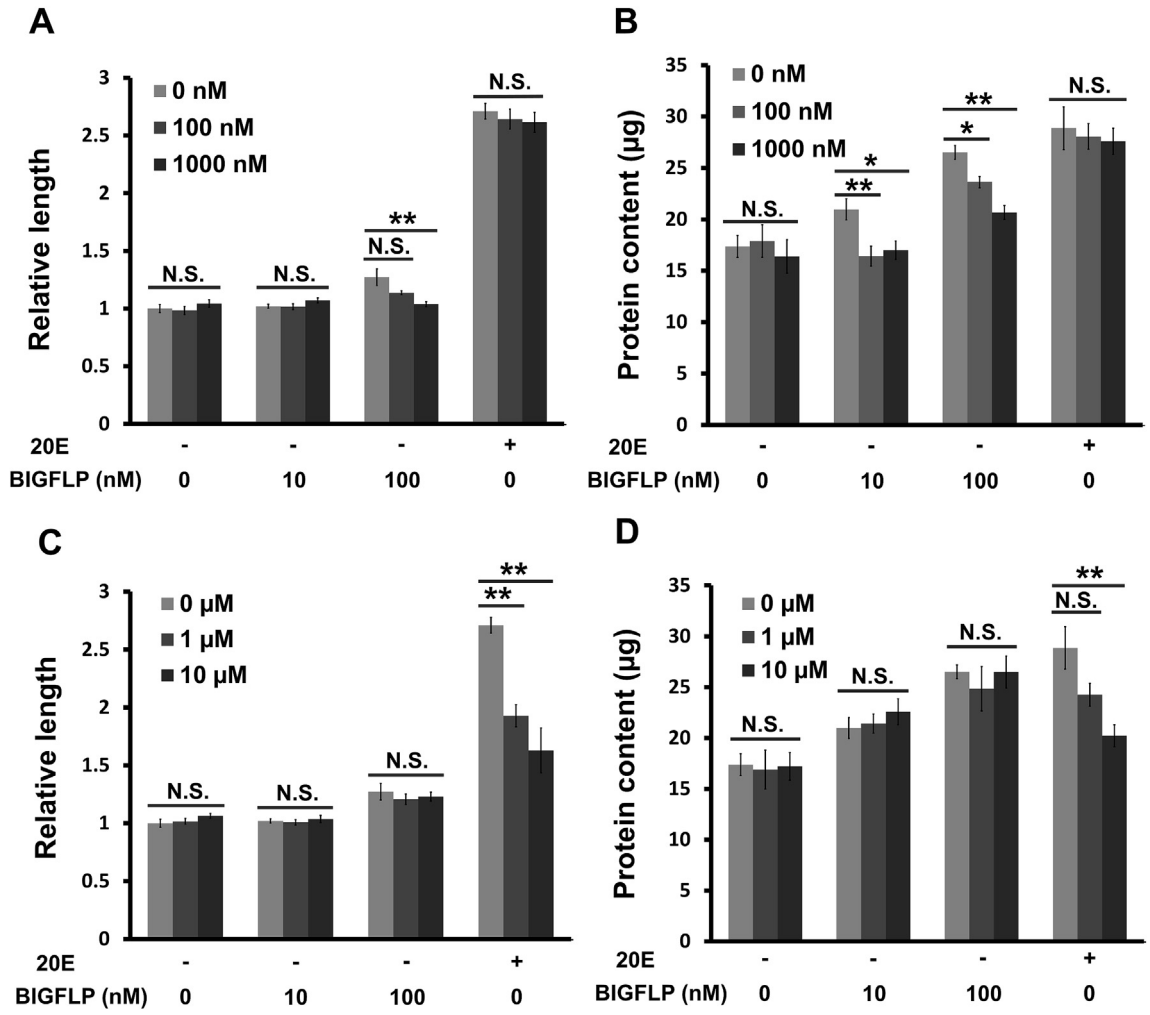

Fig. 3. Inhibition of hormone-stimulated growth of male genital disks by signaling inhibitors. Male genital disks from day-8 fifth-instar (W2) larvae were cultured for 5 days. PI3K inhibitor LY294002 (A, B) or MEK inhibitor U0126 (C, D) was added to the culture medium together with hormone. The disks were measured for the length of their long axis (A, C) and protein content (B, D). The values represent the means ± SEM (n = 6). Statistics: Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$. N.S., not significant. The concentration of 20E was changed stepwisely as described in Fig. 2.

3.5. Localization of p-AKT and p-ERK in the genital disk after hormonal stimulation

Next, we observed the localization of p-AKT and p-ERK in the hormone-stimulated genital disks by immunohistochemistry (Fig. 5). The fluorescence intensities were semiquantified by heat map images (Figs. S1 and S2). In the control and 20E-stimulated disks, the fluorescence signal for p-AKT was weak all over the tissue (Fig. 5A and C; Figs. S1A and C). After BIGFLP stimulation, p-AKT-immunoreactivity was increased in the whole disk (Fig. 5B; Fig. S1B). On the other hand, p-ERK-immunoreactivity was localized in the presumptive seminal vesicle at a low level even in the control (Fig. 5D; Fig. S2A). When stimulated by BIGFLP, the localization of p-ERK-immunoreactivity was not changed, but the signal intensity was slightly increased (Fig. 5E; Fig. S2B). After 20E stimulation, increased p-ERK-immunoreactivity was observed in the presumptive penis and seminal vesicle (Fig. 5F; Fig. S2C). Essentially the same results were obtained in at least there independent experiments (n = 6).

4. Discussion

The present study showed that the growth and development of the genital disk during pupal-adult development are regulated by both BIGFLP and 20E, but through different mechanisms: BIGFLP promotes protein synthesis via the IIS pathway, while 20E induces morphogenesis via the MAPK pathway. It is likely that both hormones act on the genital disk in concert to enable the disk to grow and develop concurrently and coordinately during metamorphosis.

Since BIGFLP production is induced by 20E (Okamoto et al., 2009a), 20E is considered as a master regulator of disk development, because this hormone directly induces morphogenesis of imaginal disks and indirectly promotes cell growth through the function of BIGFLP. It is important to note that IIS is upregulated by 20E that induces adult development because during adult development, the primordia of adult organs need IIS for their growth. During larval growth, the main insulin-like peptide in *B. mori* is bombyxin, which is secreted by the brain. Bombyxin is released in response to feeding (Masumura et al., 2000), enabling larval tissues to grow in a nutrient-dependent manner. However, because no feeding signal is available in the metamorphic stages, another source of IIS may be necessary for the adult tissues to grow. BIGFLP is likely to serve as another insulin-like peptide to support the growth of adult-specific tissues.

Considering the growth of the disk, it should be noted that the hemolymph titer of BIGFLP is extremely high (> 100 nM in males)

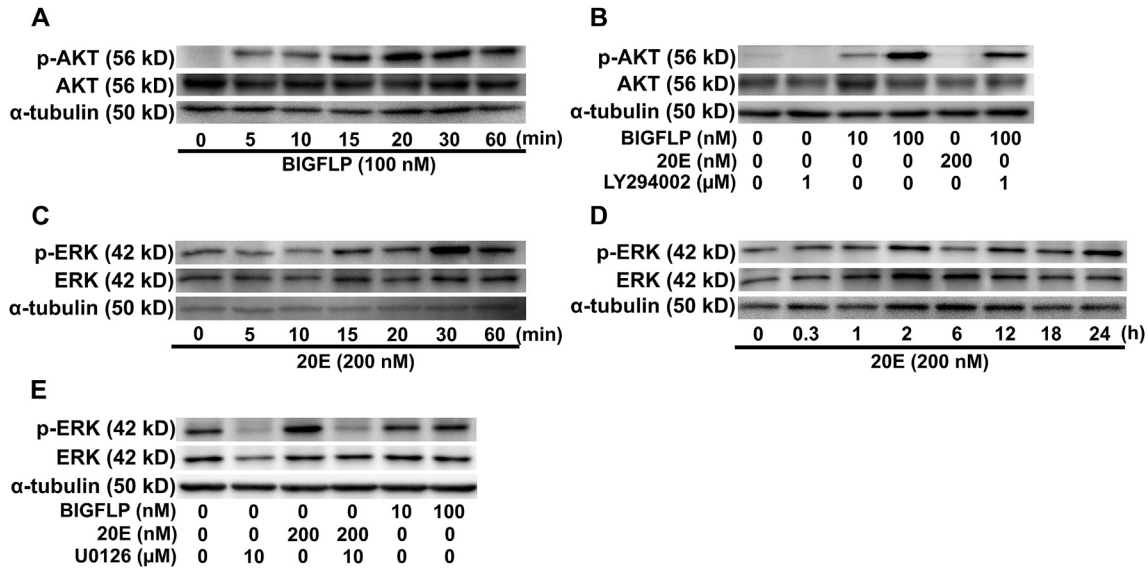

Fig. 4. Activation of IIS and MAPK pathways by BIGFLP and 20E, respectively. Male genital disks from day-8 fifth-instar (W2) larvae were incubated in the presence or absence of signaling inhibitor for 48 (A, B, E) or 72 h (C, D), and then stimulated by selected concentrations of BIGFLP or 200 nM 20E. Incubation was maintained for 0–60 min (A, C), 20 min (B), 0–24 h (D), or 24 h (E). After incubation for selected times, the genital disks were homogenized by sonication (three disks per sample), and phosphorylated AKT (p-AKT), AKT, and α-tubulin (A, B), and phosphorylated ERK (p-ERK) and ERK (C–E) in the samples were detected by Western blotting. AKT was detected after incubation with alkaline phosphatase.

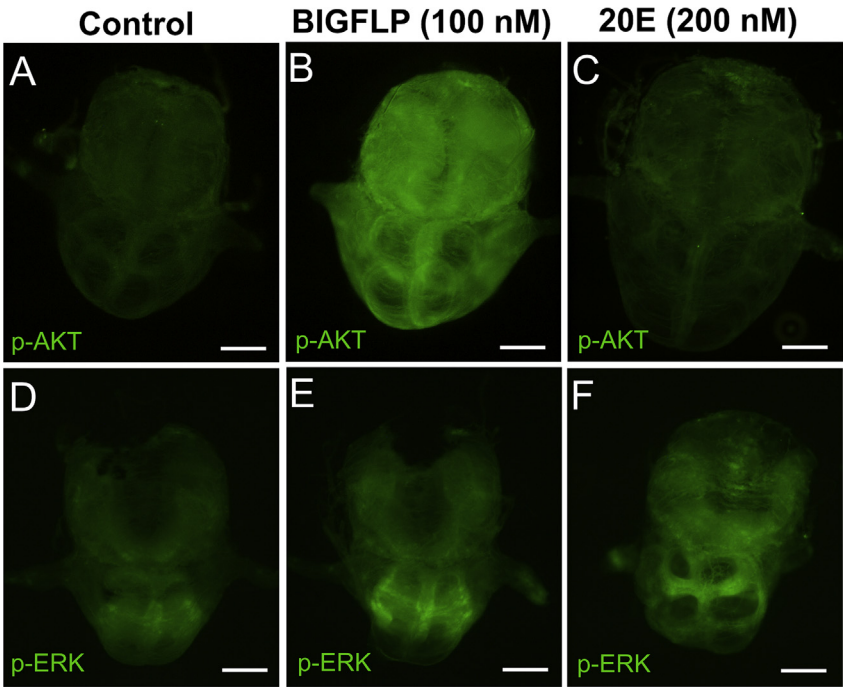

Fig. 5. Localization of phosphorylated AKT and phosphorylated ERK in hormone-stimulated male genital disks. Male genital disks from day-8 fifth-instar (W2) larvae were incubated in basal medium for 48 h, and then stimulated by 100 nM BIGFLP (B, E) or 200 nM 20E (C, F). The incubation with each hormone was carried out for 20 min (B, C) or 24 h (E, F). Control disks were incubated without hormones (A, D). Phosphorylated AKT (p-AKT) (A–C) and phosphorylated ERK (p-ERK) (D–F) were detected by whole-mount immunohistochemistry. Scale bars: 200 μm.

as compared to that of bombyxin because recent studies in *D. melanogaster* and *Trypoxylus dischotomus* demonstrated that the genital disk has low sensitivity to insulin/IGF (Tang et al., 2011; Emlen et al., 2012). Consistent with these observations, the genital disk of *B. mori* grows very little during the final larval instar, when the hemolymph bombyxin titers are well below 1 nM (unpublished result). Although larval tissues and wing disks can grow at this concentration of bombyxin, the genital disk is not likely to respond to bombyxin because of its low sensitivity to IIS. However, the input of IIS to the disk is dramatically increased after pupation because of the secretion of BIGFLP. We suggest that this is why the genital disk and some other adult-specific tissues grow only after pupation.

To our knowledge, this is the first report to demonstrate the involvement of the MAPK pathway in the regulation of imaginal disk development by 20E. The treatment of the genital disk by a

MEK inhibitor, U0126, inhibited 20E-induced elongation of the disk (Fig. 3C and D), indicative of the participation of the MAPK pathway. In fact, the phosphorylation of ERK in the genital disk was observed after stimulation by 20E, as demonstrated by Western blotting and immunohistochemistry (Fig. 4C–E and 5F). Interestingly, ERK was phosphorylated as early as 15 min after 20E administration (Fig. 4C). In general, the actions of 20E are mediated by a nuclear receptor, EcR (Henrich, 2012). Therefore, the quick activation of the MAPK pathway after 20E stimulation was unexpected. If 20E-induced elongation of the disk is mediated by EcR, phosphorylation of ERK should occur at a much later time, because cellular responses involving gene expression usually take as long as hours to occur. In contrast to the nuclear receptor-mediated response to a hormone, membrane receptor-mediated responses occur in a short time. For instance, prothoracicotropic hormone stimulation of the PG induces ERK phosphorylation in 30 min through the activation of a membrane receptor, Torso, in *M. sexta* (Rybczynski et al., 2001; Smith and Rybczynski, 2012). Therefore, our result suggests that 20E induces activation of the MAPK pathway through the function of a membrane receptor, but not through EcR.

The level of ERK phosphorylation fluctuated until 24 h after 20E stimulation (Fig. 4D), when ERK was stably phosphorylated. The genital disk contains several kinds of tissues including presumptive seminal vesicle and penis (Suzuki et al., 2005). Therefore, the timing and intensity of ERK phosphorylation may vary between parts of the disk. Another possible reason for the fluctuation in the phosphorylation level is the existence of negative feedback regulation. In mammary epithelial cells of vertebrates, ERK was found to be phosphorylated as early as 10 min after the administration of epidermal growth factor (EGF) (Fukuda et al., 2016), but was then dephosphorylated by a negative feedback mechanism. In this case, continuous EGF administration resulted in long-lasting ERK rephosphorylation at high levels (Fukuda et al., 2016). Also in genital disks, the level of ERK phosphorylation became stable at 24 h after 20E administration (Fig. 4D). Thus, we consider that both the early (15 min) and the late (24 h) responses of ERK phosphorylation to 20E are mediated by the same membrane receptor.

The involvement of a membrane receptor in some actions of ecdysteroid has recently been reported. Dopamine/ecdysone receptor (DopEcR), a kind of G protein-coupled receptor identified in *D. melanogaster*, is known to activate the MAPK pathway upon stimulation by ecdysteroid or dopamine, leading to the enhancement of memory formation (Srivastava et al., 2005; Ishimoto et al., 2013). However, the homolog of DopEcR in *B. mori* was not expressed in the genital disk, and dopamine did not activate the MAPK pathway in the disk (data not shown). Therefore, it is unlikely that DopEcR serves as a receptor mediating the regulation of genital disk development by 20E. Recently, ecdysone-responsible GPCRs (ErGPCRs) were identified in the cotton ball worm, *Helicoverpa armiera* (Cai et al., 2014). ErGPCR is reported to mediate some non-genomic actions of 20E such as an increase in intracellular calcium (Cai et al., 2014) and phosphorylation of ultraspiracle 1 (USP1), a heterodimeric partner of EcR. ErGPCR was shown to activate the expression of ecdysone response genes via USP phosphorylation (Wang et al., 2015). However, in our preliminary experiments, U0126 did not inhibit 20E-induced expression of an ecdysone response gene, *E75A*, suggesting that ErGPCR is not involved in a 20E-inducible activation of the MAPK pathway in the genital disk. In *B. mori*, it was reported that 20E induces apoptosis of anterior silk glands through an unidentified membrane receptor (Elmogy et al., 2004). In this action of 20E, cyclic AMP and Ca^{2+} were shown to serve as second messengers (Elmogy et al., 2006; Manaboon et al., 2009), but possible involvement of the MAPK pathway was not examined. Thus, we now have no promising candidate for the membrane receptor involved in the actions of 20E on the genital disk. However, it is obvious that the identification of the receptor involved is a critical need for better understanding of the 20E actions.

Even before stimulation by hormones, ERK was weakly phosphorylated in the presumptive seminal vesicle (Fig. 5D). BIGFLP slightly enhanced ERK phosphorylation in the same area (Fig. 5E). After 20E stimulation, enhanced phosphorylation of ERK was observed in the presumptive penis and seminal vesicle (Fig. 5F), suggesting that activation of the MAPK pathway is necessary for both elongation of the inner sexual organs and morphogenesis of the outer sexual organ.

BIGFLP induced the phosphorylation of AKT in the genital disk in 5 min and eventually increased the protein content of the disk (Figs. 2H and 4A). Phosphorylated AKT is known to promote protein synthesis and cell growth through activation of the target of rapamycin (TOR) pathway and/or inactivation of the forkhead-related FOXO family of transcription factors (Taguchi and White, 2008). Since the phosphorylation of AKT after BIGFLP stimulation was observed all over the genital disk, BIGFLP is likely to promote protein synthesis in the whole disk via the TOR pathway.

Immunohistochemistry demonstrated that BIGFLP slightly increased the phosphorylation level of ERK in the presumptive seminal vesicle (Fig. 5E), although Western blot analysis could not detect a significant increase in the level of ERK phosphorylation after BIGFLP stimulation of the genital disk (Fig. 4E), due probably to the localization of phosphorylated ERK to a specific small area in the disk. Okamoto et al. reported that BIGFLP induces cell proliferation of the presumptive seminal vesicle (Okamoto et al., 2009a). BIGFLP may stimulate cell proliferation in the presumptive seminal vesicle by increasing the phosphorylation level of ERK in this area.

20E also promoted protein synthesis (Fig. 2H). Simultaneous administration of both hormones stimulated protein synthesis in an additive manner, indicating that 20E and BIGFLP promote protein synthesis by different mechanisms. This conclusion was supported by the observations that 20E did not activate the IIS pathway and that PI3K inhibitor did not affect the 20E-stimulated increase in the protein content (Figs. 3B and 4B). Since the promotion of protein synthesis by 20E was inhibited by MEK inhibitor (Fig. 3D), the MAPK pathway is thought to be involved in this action of 20E as well. Cross-talk of the MAPK pathway with the TOR pathway has been suggested in some reports (Roux et al., 2004). Moreover, in *D. melanogaster* and cockroaches, it was shown that ecdysteroid regulates imaginal disk growth via 4E-BP, a downstream regulator of IIS/TOR pathway (Herboso et al., 2015). It is possible that 20E activates the TOR pathway via downstream components of the MAPK pathway.

It was reported that 20E and bombyxin promoted the growth of wing disks of *M. sexta* in a synergistic manner, where bombyxin alone exerted little effect on the disk growth but remarkably enhanced the growth-promoting effect of 20E (Nijhout et al., 2007), in contrast to our present result (Fig. 2G and H). Since *B. mori* genome has only a single insulin-like receptor gene (Antonova et al., 2012), bombyxin and BIGFLP are expected to act through the same receptor and signaling pathway. It is unknown at present why the mode of interaction between 20E and ILP (bombyxin)/IGFLP in promoting imaginal disk growth differs between the wing and genital disks.

In this study, we only used male genital disks to investigate the effects of BIGFLP and 20E on the growth and development of adult organs during metamorphosis. Currently, it is not known whether both hormones act on other adult tissues via the same mechanisms. Okamoto et al. reported that BIGFLP promoted bromodeoxyuridine incorporation into male and female genital disks, sperm ducts and flight muscle (Okamoto et al., 2009a). Therefore, it is likely that the growth-promoting action of BIGFLP is the general effect of this

hormone on adult tissues. However, the action of 20E on each adult tissue may vary because the developmental process differs among tissues, even in the female genital disk (Suzuki et al., 2005). Therefore, the effects of 20E on these tissues must be investigated individually in the future.

We determined the hormone titers of ecdysone, 20E, and BIGFLP in the hemolymph to establish culture conditions (Fig. 1). It has previously been reported that low levels of 20E induce cell division, whereas high levels of it led to cell polyploidization in the larval wing disks of *B. mori* (Moriyama et al., 2016). Therefore, in *ex vivo* studies, 20E should be administered to the culture at concentrations as similar as possible to those *in vivo*. To determine 20E titers accurately, we used the LC-MS/MS method instead of RIA (Fig. 1A and B). During the wandering stage, the peak titers of ecdysone and 20E were similar. After pupation, ecdysone titer was highest on day 2, whereas 20E titer was highest on day 4. The observed changes in ecdysone and 20E titers in the hemolymph were consistent with the mode of ecdysteroid metabolism by which secreted ecdysone is converted to 20E in peripheral tissues (Petryk et al., 2003). However, the conversion rate seems to change in a stage-specific manner. Our results suggest that ecdysone is quickly converted to 20E in the wandering stage, while the conversion rate is low during adult development. The difference in the conversion rate may be attributable to the difference in the level of expression of the *shade* gene encoding ecdysone 20-hydroxylase, in the peripheral tissues. It has been reported that, in *M. sexta*, *shade* expression is high during the last larval instar, but is low after pupation (Rewitz et al., 2006). The developmental change in *shade* expression may be the same between *M. sexta* and *B. mori*.

The sum of ecdysone and 20E titers was much lower than the titers of ecdysteroids determined by RIA (Fig. 1A and B). It is known that there are numerous metabolites and conjugates of ecdysteroids in the pupal hemolymph (Warren and Gilbert, 1986). Since antibodies used in RIA would cross-react with some of these substances, the difference in the assay results may be attributable to the presence of such substances. The titer of 20E during pupal-adult development determined in this study was about 600 ng/mL at its highest, only one-tenth or less compared with the ecdysteroid titer determined by RIA (Mizoguchi et al., 2001). The large discrepancy of the assay results between the present and previous studies indicates that we should pay more attention to the exact titer of 20E in the hemolymph, in physiological studies concerning the function of 20E.

20E induces the production of BIGFLP in the fat body and some other tissues (Okamoto et al., 2009a, 2011). BIGFLP was first detected in the hemolymph 1 day before pupation (Fig. 1C and D). The titer in males gradually increased, reaching the peak concentration of 0.5 μM several days after pupation. In the culture of genital disks, however, the BIGFLP concentration was set at 10 or 100 nM, because in the preliminary experiments, we found that the effects of BIGFLP was saturated at a concentration of about 100 nM. It is unknown why this peptide is present in the hemolymph at very high titers. One possible reason for this is the presence of binding proteins. In vertebrates, it is known that IGF-binding proteins bind to IGF-I to protect it from degradation by proteases, contributing to the maintenance of high titers of the hormone in the blood for a long time (Holly and Perks, 2006). Insects also have several ILP-binding proteins, including neuroparsins (Badisco et al., 2007) and Imp-L2 (Honegger et al., 2008), both of which are structurally related to mammalian IGFBP7, as well as a secreted decoy of InR (Okamoto et al., 2013). Thus, the titer of the free form of BIGFLP in the hemolymph might be much lower than that of total BIGFLP determined in this study.

The changes in BIGFLP titers in the hemolymph were surveyed throughout the pupal-adult development by means of TR-FIA (Fig. 1C and D). The profile of the changes was almost the same as that previously assessed by Western blot analysis (Okamoto et al., 2009a). BIGFLP was present at high concentrations on the order of 0.1–1 μM throughout adult development, with much higher concentrations in females than in males. The main reason for the higher titers in females may be that *bigflp* expression in the fat body is 5–300 times higher in females than in males (data not shown). In females, the ovaries grow drastically during adult development to fill the abdomen. Therefore, the very high titers of BIGFLP in female hemolymph might contribute to a rapid growth and development of ovaries during adult development.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2017.06.003.

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