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Review

Ecdysone signalling and ovarian development in insects: from stem cells to ovarian follicle formation ☆

Xavier Belles *, Maria-Dolors Piulachs

Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37, 08003 Barcelona, Spain

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ABSTRACT

Although a great deal of information is available concerning the role of ecdysone in insect oogenesis, research has tended to focus on vitellogenesis and choriogenesis. As such, the study of oogenesis in a strict sense has received much less attention. This situation changed recently when a number of observations carried out in the meroistic polytrophic ovarioles of *Drosophila melanogaster* started to unravel the key roles played by ecdysone in different steps of oogenesis. Thus, in larval stages, a non-autonomous role of ecdysone, first in repression and later in activation, of stem cell niche and primordial germ cell differentiation has been reported. In the adult, ecdysone stimulates the proliferation of germline stem cells, plays a role in stem cell niche maintenance and is needed non-cell-autonomously for correct differentiation of germline stem cells. Moreover, in somatic cells ecdysone is required for 16-cell cyst formation and for ovarian follicle development. In the transition from stages 8 to 9 of oogenesis, ecdysone signalling is fundamental when deciding whether or not to go ahead with vitellogenesis depending on the nutritional status, as well as to start border cell migration. This article is part of a Special Issue entitled: Nuclear receptors in animal development.

1. Introduction

When Henry Hagedorn and co-workers discovered, in the 1970s, that vitellogenin production in the fat body of mosquitoes was regulated by ecdysteroids produced in the ovary [1,2], they opened up a new field of research on the ecdysone-dependent mechanisms of regulation of insect oogenesis and oocyte development. Before this discovery, juvenile hormone was thought to be the main if not unique gonadotrophic hormone in insects, triggering the expression of vitellogenin genes in the fat body and inducing the formation of large intercellular spaces between follicle cells thus facilitating vitellogenin uptake in the growing oocyte [3,4]. Indeed, this still holds true in practically all groups, except in most panorpids (Lepidoptera, Diptera and other less diverse orders), where ecdysteroids play an important role in many species [5,6]. Although much has been achieved in the study of the role of ecdysteroids on oocyte development and growth since the 1970s, most research has focused on oocyte maturation, from vitellogenesis to chorion formation, as proven by copious information contained in comprehensive reviews on these aspects [3,5,6]. In this sense, the most thoroughly studied models have been the fruit fly *Drosophila melanogaster* and the silkmoth *Bombyx mori*, where the important role of the ecdysone signalling pathway has been highlighted in the transition from previtellogenesis to vitellogenesis, and that from early to middle and late vitellogenesis and final choriogenesis, as summarized in recent reviews [7,8]. Conversely, the study of oogenesis itself, especially concerning the early steps, around the establishment of the stem cell niche, has received much less attention until very recently, when a handful of (key) studies conducted in *D. melanogaster*, mainly between 2000 and 2014, started to unveil the fundamental roles played by the ecdysone signalling pathway in this process.

The present review will deal specifically with these most recent contributions describing the role of ecdysone in oogenesis, from the establishment and maintenance of the stem cell niche to formation of the ovarian follicle or egg chamber. In addition, we will cover a number of important events at the interface between formation of the ovarian follicle and the incorporation of vitellogenin into the growing ovarian follicle. In *D. melanogaster*, the transition from stages 8 to 9 of oogenesis, which represents a temporal boundary framing two important decisions, is of particular importance. One of these decisions concerns whether to proceed to vitellogenesis if nutritional conditions are appropriate, and the other is whether to initiate border cell migration. The role played by ecdysone signalling in both decisions is fundamental.

For the sake of brevity, although the active ecdysteroid that interacts with the ecdysone receptor and gives the biological response generally is 20-hydroxyecdysone, we will use the common terms “ecdysone” and “ecdysone signalling” throughout the text.

2. A brief overview of the oogenesis process

The ovariole, which is the basic unit of egg production, comprises three regions from the distal to the basal part, namely the terminal

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* Corresponding author. Tel.: +34 932309636; fax: +34 932211011.
E-mail address: xavier.belles@ibe.upf-csic.es (X. Belles).

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filament, the germarium and the vitellarium [9,10]. The terminal filament is a thread-like structure at the apical part of the ovariole containing a stack of disc-shaped cells. The germarium lies beneath the terminal filament and contains the germline stem cells within the stem cell niche (accompanied by cap and escort cells) (Fig. 1A), where differentiation of germline stem cells into cystoblasts, and eventually to the cystocyte complex, occurs. The cystoblast surrounded by follicle cells gives rise to the cystocyte complex, which moves down to the vitellarium region, were the last steps of ovarian follicle maturation takes place [9,10].

Two main types of ovariole can be distinguished in insects: the panoistic and the meroistic (Fig. 1B). In panoistic ovarioles, all germline stem cells become functional oocytes, which are surrounded by a monolayer of follicular cells and thus constitute an ovarian follicle; all RNA types that accumulate in the oocyte cytoplasm are expressed in the oocyte nucleus. In meroistic ovarioles, some of the germline stem cells differentiate into oocytes whereas others become nurse cells, the main function of which is the synthesis and subsequent transport of macromolecules and organelles to the oocyte cytoplasm. Meroistic ovarioles can be further classified into telotrophic and polytrophic, depending on the anatomical distribution and relationships between the oocyte and the nurse cells. In the polytrophic type, the nurse cells and oocytes alternate along the ovariole, whereas in the telotrophic type, the nurse cells are located in the germarium and are connected to oocytes in early stages of their development by cytoplasmic processes called nutritive chords (Fig. 1B) [9,10]. Panoistic ovaries are found in insects belonging to the more basal orders, from Thysanura to Polyneoptera (Archeognatha, Zygentoma, Ephemeroptera, Odonata, Plecoptera, Phasmida, Orthoptera, and Dictyoptera). Telotrophic meroistic ovaries occur in Paraneoptera (Hemiptera) and in basal Holometabola (Coleoptera, Raphidioptera and Megaloptera), whereas Polytrophic meroistic ovaries are also present in Paraneoptera (Psocoptera, Phthiraptera) and in more distal Holometabola (Hymenoptera, Trichoptera, Lepidoptera and Diptera) [9]. Therefore, there is no exact correspondence between ovariole type and phylogenetic position, although there is a consensus considering that the panoistic ovaries correspond to the ancestral type, from which the meroistic had derived.

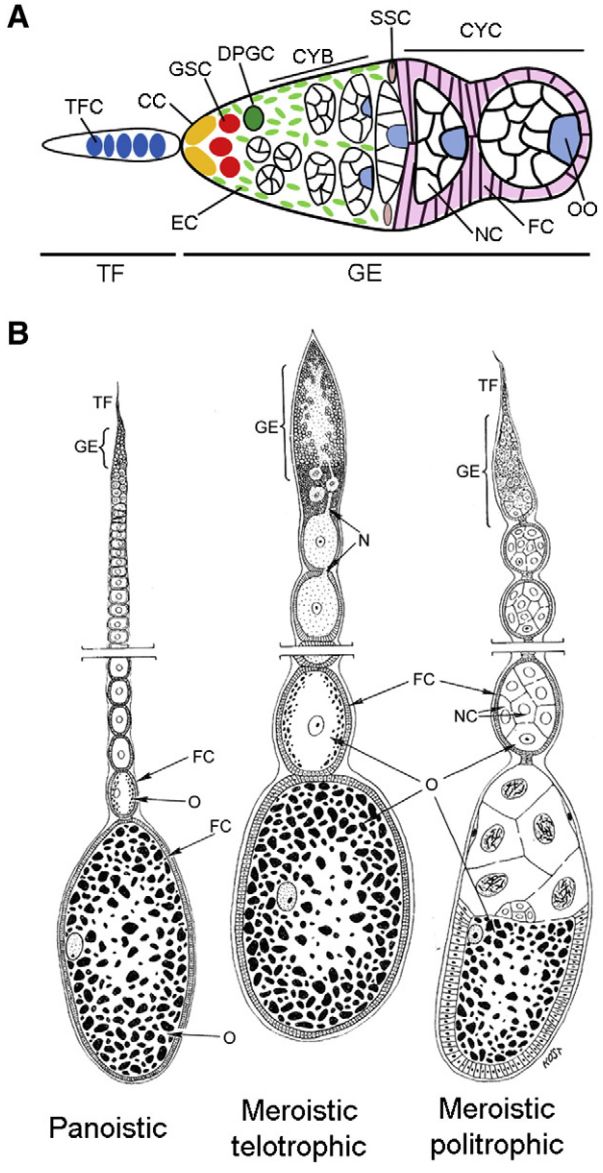

Fig. 1. A. Scheme of the terminal filament and germarium of a *Drosophila melanogaster* ovariole. And B: Scheme of the three kinds of ovariole in insects. CC: cap cells; CYB: cystoblasts; CYC: cystocytes; DPGC: differentiating precursors of germline stem cells; EC: escort cells; FC: follicle cells; GE: germarium; GSC: germline stem cells; NC: nurse cells; OO: oocyte; SSC: somatic stem cells; and TF: terminal filament. Figure B from Mahowald [42], with permission.

The meroistic polytrophic ovary of *D. melanogaster*, with 16–20 ovarioles, has been the most influential model for understanding the interactions between stem cells and their niches [11,12], and a great deal of information is available regarding the mechanisms and signals (mainly coming from the cap cells) that maintain the niche environment and regulate the behaviour of their cells [13]. The data reported herein deal specifically with ecdysone signalling and most of them have also been obtained in *D. melanogaster*. Thus, if not stated otherwise, we will refer to the meroistic polytrophic ovarioles of this fly. A basic morphological and dynamic description of the oogenesis of *D. melanogaster*, including the definition of the different stages used to divide the process, can be found in relatively recent reviews [14,15].

3. Establishment and maintenance of the stem cell niche

During embryogenesis, the germ cells of the reproductive organs originate from the pole cells, which are amongst the first to differentiate. Most of the ovarian tissues subsequently originate from the splanchnic mesoderm. The portions bearing the germ cells develop into ovarioles, whereas other, anterior cells form a suspensory ligament at the distal region, which unites all ovarioles and anchors the whole ovary to the body wall [10].

In the early larval stages of *D. melanogaster*, both gonadal somatic cells (the precursors of niche cells) and primordial germ cells (the precursors of germline stem cells) proliferate. Somatic proliferation is needed during these stages to allow correct formation of 16–20 niches, whereas primordial germ cell proliferation is needed to produce sufficient germline stem cell precursors to fill the developing niches [16]. Differentiation of the terminal filament commences towards the mid-third larval instar, and late in this instar, 16–20 terminal filament stacks have formed, with cap cells developing at the base of these stacks. Primordial germ cells can subsequently attach to them to become adult germline stem cells (Fig. 2) [17].

Using experiments involving RNAi depletion of the ecdysone receptor components, ecdysone receptor (EcR) and ultraspiracle (Usp) in somatic cells, and immunodetection of the transcription factor broad complex isoform Z1 (BR-C-Z1), Gilboa and co-workers [18] unveiled a non-autonomous role of ecdysone (i.e. ecdysone signalling in somatic cells) first in repression, and later in activation, of niche and primordial germ cell differentiation. The results showed that in the early third larval instar of *D. melanogaster*, when the first, proliferative stage of gonadogenesis takes place, EcR/Usp-transduced ecdysone signalling inhibits niche formation and primordial germ cell differentiation, and this inhibition appears to be mediated by the repression of BR-C-Z1 (Fig. 2). Inhibition of niche formation and primordial germ cell differentiation allows the germarium to generate sufficient niche and stem cell precursors, which

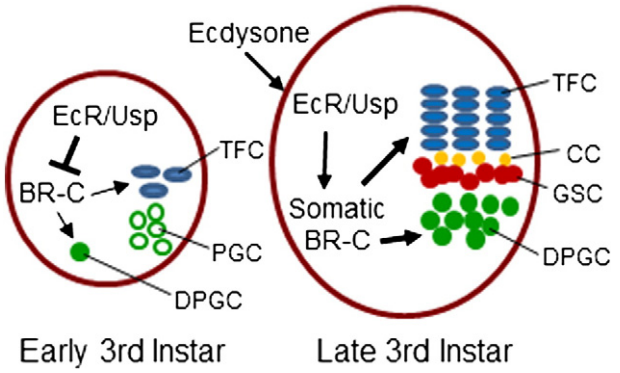

Fig. 2. Ecdysone coordination of niche formation with the establishment of germline stem cells. At early third instar, ecdysone signalling repress niche formation and primordial germ cell differentiation, which allows the gonad time to grow and generate enough precursor cells of both cell populations; repression of the target gene BR-C is a key component of this repression. At mid-third instar and later, ecdysone signalling activates BR-C-Z1 expression in the somatic cells through the components of the ecdysone receptor, EcR and Usp, and this firstly leads to formation of niches and later to primordial germ cell differentiation. CC: cap cells; DPGC: differentiating precursors of germline stem cells GSC: germline stem cells; PGC: precursors of germline stem cells; and TFC: terminal filament cells. From Ganz et al. [18], slightly modified.

will eventually end with the formation of 16–20 stem cell units. Conversely, ecdysone signalling activates BR-C-Z1 expression in the somatic cells of the ovary in the late third larval instar. This first leads to formation of niches, and later (coinciding with the wandering behaviour that precedes pupation) to primordial germ cell differentiation. Only primordial germ cells located next to niches are protected from differentiation and become adult germline stem cells (Fig. 2). As such, ecdysone signalling orchestrates the entire sequence of germline stem cell unit formation in the ovary of *D. melanogaster* in pre-adult stages in a non-autonomous manner [18]. A recent contribution by Gancz and Gilboa [19] has shown that insulin and Target of Rapamycin (TOR) pathways, which play fundamental roles in the maintenance and function of the *D. melanogaster* adult niche —germline stem cell unit [20–22], affect germ cells and their somatic support cells during ovary formation. Interestingly, the molecular mechanisms underlying the insulin and TOR pathways in this larval context appear to be independent from those operating in ecdysone signalling [19], in contrast with what occurs in the control of body size, where the principal regulators are insulin, ecdysone and their respective receptors, interact in complex networks [23].

With regard to stem cell niche maintenance in the adult stage, Ables and Drummond-Barbosa [24] have reported that EcR mutants of *D. melanogaster* exhibit reduced cell division of germline stem cells, thus suggesting that ecdysone normally stimulates proliferation of these cells, and the same authors showed that this effect is mediated by E74, an Ets transcription factor associated with the genetic cascade triggered by ecdysone. Intriguingly, other transcription factors from the ecdysone cascade, such as E75 and BR-C, appear not to be involved in inducing this germline stem cell proliferation. These authors also show that ecdysone signalling contributes to the maintenance of germline stem cells as EcR mutants exhibit a rapid loss of them; in contrast to the larva, ecdysone signalling in this case is cell-autonomous (i.e. signalling within germ cells) and does not appear to rely (at least not very strongly) on BR-C factors [24]. The above work additionally shows that ecdysone signalling regulates germline stem cells by interacting with chromatin remodelling factors such as ISWI (an intrinsic epigenetic factor that is required for germline stem cell fate and activity) and Nurf301 (the largest subunit of the ISWI-containing NURF chromatin remodelling complex). This highlights the functional link between ecdysone signalling and the intrinsic chromatin remodelling machinery as a potential mechanism for promoting general transcriptional programmes such as that required for adult stem cell self-renewal [24] Finally, Shcherbata and co-workers have shown that adult-specific dominant-negative EcR expression in escort cells (the somatic cells of the stem cell niche that contact early germline cysts) disrupts early germ cell differentiation, thereby suggesting that ecdysone signalling is required non-cell-autonomously for correct differentiation of adult daughter germline stem cells [25].

4. From germline stem cells to cystoblasts, cystocytes and ovarian follicles

When a germline stem cell divides asymmetrically, the daughter cell closest to the terminal filament and cap cells remains a stem cell whereas the one closest to the inner sheath cells differentiates into a cystoblast. The newly formed cystoblast subsequently undergoes cell division with incomplete cytokinesis, which in *D. melanogaster* ultimately gives rise to a cyst of 16 cystocytes that are connected to each other by a series of cytoplasmic bridges, or ring canals [15]. Once the cyst has been completely surrounded by follicle cells, it enters to the vitellarium, and one of the two cystocytes that contains four ring canals becomes the oocyte, whereas all others become nurse cells, with the whole ensemble constituting the ovarian follicle or egg chamber.

Ecdysone signalling suppression experiments in *D. melanogaster* have shown that ecdysone is required to maintain the germline stem cell number and that downregulation of the ecdysteroid pathway in somatic cells severely impairs new 16-cell cyst formation (whereas that of 2-, 4- and 8-cell cysts is practically unaffected). Moreover, entry into meiosis, a fundamental event that takes place just after the formation of 16-cell cysts, was severely impaired in those specimens where ecdysone signalling was reduced in somatic cells [26]. After 16-cell cyst progress through meiosis, the envelope of escort cells is subsequently replaced by follicle cells, which proliferate and cover the new ovarian follicle. The same experimental approach led Morris and Spradling [26] to show that follicle formation also requires ecdysone signalling in somatic cells. As stated above, the insulin and TOR signalling pathways also play a regulatory role in these germline stem cell divisions and cyst growth processes [20–22].

5. The checkpoint at stage 8: adjusting egg production to available nutrient reserves

In *D. melanogaster*, it has been shown that ecdysone signalling regulates the transition of follicles through a checkpoint at stage 8 that prevents the onset of vitellogenesis and egg maturation if nutritional resources are insufficient [27,28]. A significant finding that ecdysone signalling may be instrumental in deciding whether the ovarian follicles will proceed to vitellogenesis and maturation in normally fed insects, or die by apoptosis under conditions of food shortage or starvation, was obtained from measurements of ecdysone concentration in ovaries under different conditions of food supply. Specific measurements showed that the ecdysone concentration increased significantly when the insects were insufficiently fed [29]. Further results from the same laboratory indicated that the progression of egg chambers to vitellogenesis or to apoptosis is dictated by an intricate interplay between ecdysone and different isoforms of BR-C and E75 transcription factors in which BR-C isoforms regulate E75A and E75B, which should have opposite effects: E75A promoting apoptosis and E75B preventing it [30]. On the basis of the detailed results, the authors proposed a model in which, in fed *D. melanogaster*, only BR-C-Z1 expression occurs in follicle cells at stage 8, thus means that normal levels of E75B suppress E75A expression and the egg chamber progresses towards vitellogenesis and maturation. In starved flies, higher levels of ecdysone activate the expression of BR-C-Z2 and BR-C-Z3 at stage 8, which in turn induce the expression of E75A in follicle cells and repress that of E75B. As a result, E75A expression in follicle cells would activate the apoptosis pathway in the whole egg chamber (Fig. 3).

Intriguingly, ecdysteroid signalling has long been known to be necessary in mid-oogenesis of *D. melanogaster* [31,32] (see also above),

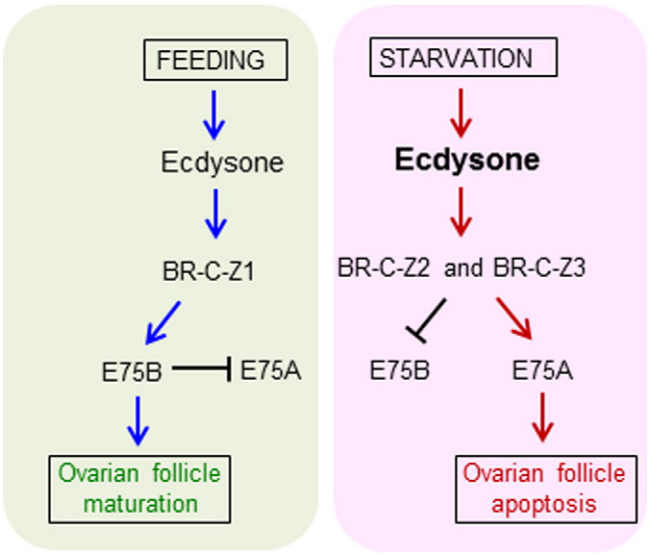

Fig. 3. Action of ecdysone response genes regulating apoptosis at stage 8 egg chambers in *Drosophila melanogaster*. Under correct feeding, current production of ecdysone induces BR-C-Z1 and E75B expression, and E75B suppresses E75A expression, thus ovarian follicle maturation proceeds. Under starvation, ecdysone is produced at higher levels, which induces the expression of BR-C-Z2 and BR-C-Z3 that suppress E75B and activate E75A expression so E75A, which triggers apoptosis. Drawn with data from Terashima et al. [29] and Terashima and Bownes [30].

whereas the results of Bownes' group point to a pro-apoptotic function of ecdysteroids in the same period. However, the two opposite effects might depend upon a threshold level of ecdysteroid concentration: if levels are below the threshold, then the network of genes regulating the progression towards egg maturation is activated at appropriate levels, whereas if the concentration exceeds the threshold then the alternative gene network that leads to apoptosis is activated [30].

6. Border cell migration

The border cells in *D. melanogaster* consist of two non-motile polar cells and four to eight migratory outer border cells that group at late stage 8, when the Jak/Stat signalling pathway in neighbouring follicle cells is activated (Fig. 4). Jak/Stat signalling activates expression of the transcription factor Slbo in border cells, which regulates a number of factors that specify border cells and are needed for migration [33]. Border cells begin to migrate at early stage 9 and reach the oocyte at stage 10 (Fig. 4), where they will later contribute to formation of the micropyle, an important chorion structure that allows sperm entry into the egg.

If ecdysone signalling is impaired, then border cells are specified but do not migrate [34,35]. Moreover, a significant increase in ecdysone occurs in follicles during mid-oogenesis, and EcR-B1, one of the three EcR isoforms that contributes to ecdysone reception, exhibits a burst of expression just before the onset of border cell migration, with ecdysone signalling then steadily intensifying (Fig. 4) [35]. As stated above, the functional receptor of ecdysone is a heterodimer of EcR and Usp in which ecdysone binds to EcR. However, another important player in the context of border cell migration is the co-activator Taiman, a bHLH-PAS transcription factor that is recruited into the receptor complex [34]. If Taiman is constitutively expressed at stage 8, then ecdysone signalling is precociously induced, thereby suggesting: 1) that sufficient ecdysone must be present at this stage, and 2) that there must be a repressor that prevents ecdysone signalling in this context. Using a genetic screen to find genes blocking border cell migration, Montell and co-workers [35] identified the transcription factor Abrupt as a repressor of ecdysone signalling in this process. Abrupt belongs to a family of transcription factors containing a BTB domain and a zinc-finger motif, and is present in all follicle cells prior to stage 9, although its concentration decreases in border cells when migration begins (Fig. 4). These experiments elegantly showed that Abrupt binds to Taiman directly through the BTB domain of Abrupt and the bHLH domain of Taiman, and that this interaction is essential to suppress ecdysone signalling. In the transition to stage 9, Abrupt starts to vanish due to a combination of EcR and Jak/Stat signalling, whereas ecdysone signalling increases (Fig. 4) [35]. Although the immediate targets of ecdysone that stimulate border cell migration are yet to be identified, we can speculate that classical early genes of the ecdysone cascade, such as E74, E75 or BR-C, may also play a role in this process.

With regard to upstream ecdysone production, a recent report published by Schüpbach and co-workers, again in *D. melanogaster*, showed that mutations affecting shadow and phantom genes, which encode enzymes of the ecdysone biosynthetic pathway, impair border cell migration when the whole follicular epithelium of an egg chamber is mutant, even when the associated germline cells (nurse cells and the oocyte) are wild-type. Production of ecdysone specifically by the follicular epithelium appears necessary to trigger border cell migration as neither the germline, nor the neighbouring egg chambers, nor the surrounding haemolymph seem to provide the amounts of ecdysone necessary to exert this role [36].

7. Final roles of ecdysone before vitellogenesis

Before vitellogenesis, the ovary and fat body must be set up to receive the hormonal vitellogenic signal that will trigger production of vitellogenin or yolk proteins, mainly in the fat body, and uptake thereof in the growing ovarian follicle. In contrast with most insects where vitellogenesis is regulated by juvenile hormone [4], mosquitoes and flies regulate this process with ecdysone [3,5,6,8], whereas pre-vitellogenic activation of fat body nucleoli for ribosomal RNA production in these groups, as well as growth and differentiation of the follicular epithelium, appear to be under the control of juvenile hormone [3]. In the panoistic ovary of the cockroach *Blattella germanica*, pre-vitellogenesis indeed starts in the last nymphal instar [37] when, amongst other processes, follicle cells proliferate intensively [38], thus approaching the optimal cell number that will be needed in the adult stage to provide the optimal shape and volume of the basal ovarian follicle before vitellogenesis. RNAi experiments depleting EcR-A transcript levels in the last nymphal instar resulted in impaired proliferation in the follicular epithelium (Fig. 5), thereby indicating that EcR-transduced ecdysone signalling is involved in this proliferative process in panoistic ovarioles [38]. Conversely, vitellogenesis in these EcR-A-depleted specimens was apparently normal, but choriogenesis was prevented, as expected in a species where chorion formation is ecdysone-dependent [39].

8. Concluding remarks and perspectives

Practically all data currently available concerning the role of ecdysteroids and the description of oogenesis, both generally and especially at a molecular level, come from *D. melanogaster*. As such, no comparisons can be made between different insect groups, which might be of great interest as regards reconstructing the evolutionary transition from panoistic to meroistic ovarioles, with the increasing complexity that this transition represents in morphological and regulatory terms. The evolution of germarium stem cell niches might also be of great interest. Stem cells could have appeared when niches acquired the ability to sequester, preserve and control undifferentiated embryonic cells with the necessary cellular properties [40]. The study of less modified stem cell niches from meroistic ovarioles in more basal insect species might help to reconstruct the evolutionary history of germarium stem cell niches. Earwigs such as *Opisthocosmia silvestris*, which possesses a morphologically simple germline stem cell niche comprising only terminal filament cells and several structurally uniform escort cells, may be especially interesting in this sense [41]. However, the study of germarium stem cell niches opens up much broader horizons. The peculiar

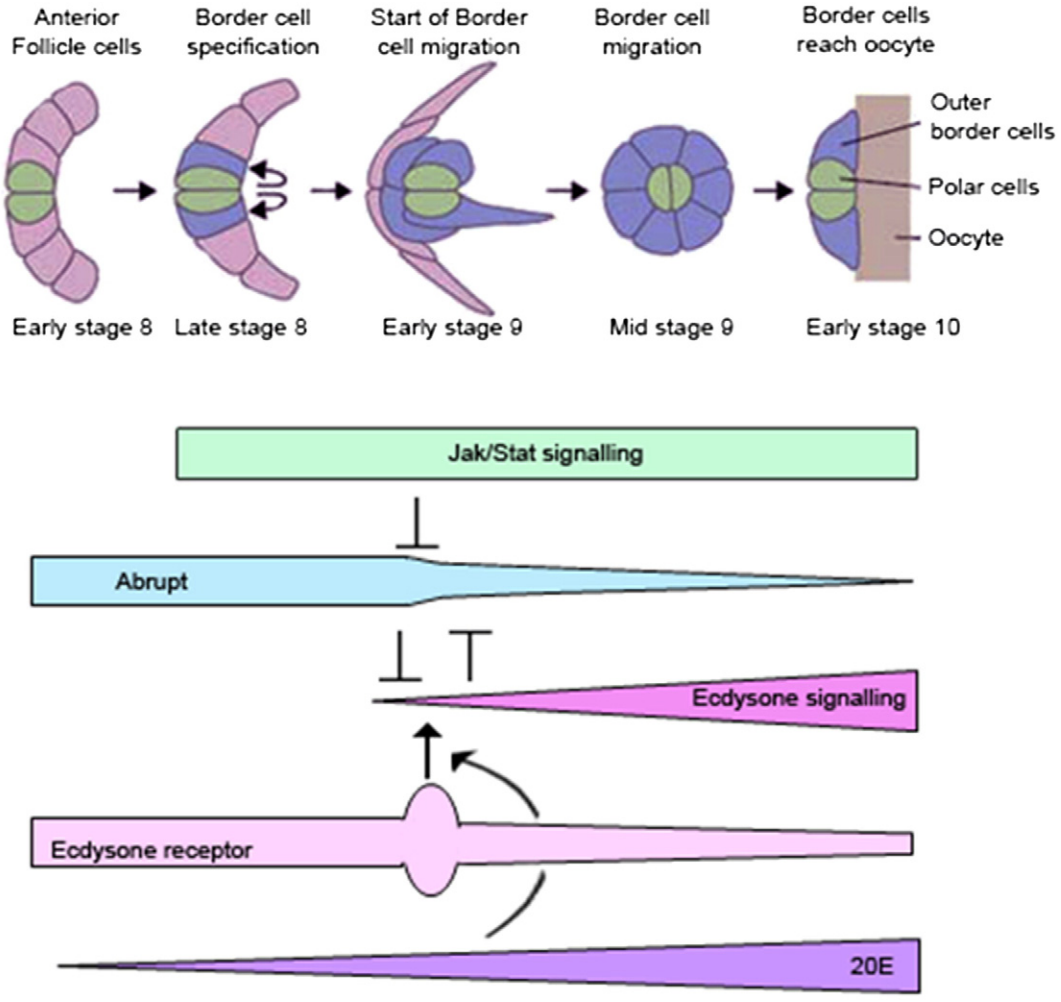

Fig. 4. Border cell migration in *Drosophila melanogaster*, and signals involved. Border cells form from anterior follicle cells in response to a Jak/Stat activating signal from polar cells (curved arrows). After delamination, the border cells cluster migrates until they reach the oocyte. Ecdysone signalling controls the start of border cell migration, and initiation of ecdysone signalling in border cells requires removal of Abrupt. Reduction of Abrupt is triggered by Jak/Stat signalling and reinforced by ecdysone signalling. In turn, ecdysone signalling appears to become activated by a combination of reduced Abrupt levels, a pulse of high ecdysone receptor expression, and increasing levels of 20-hydroxyecdysone. From Godt and Tepass [43], with permission.

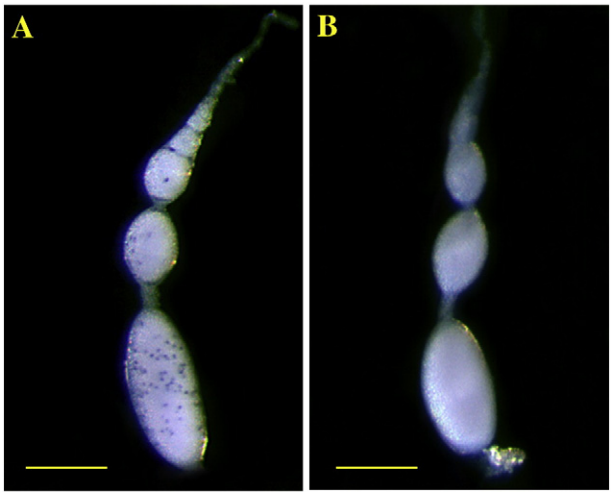

Fig. 5. Effect of EcR-depletion by RNAi on the proliferation of the follicular epithelium in the last nymphal instar of *Blattella germanica*. Insects were RNAi-treated and then pulsed with 5-bromo-2′-deoxyuridine (BrdU); ovaries were dissected and stained to reveal BrdU incorporation 2 h later, which reflects proliferating cells [38]. A: Ovariole from a control specimen, showing abundant BrdU-labelled cells in the basal, sub-basal and even in more immature ovarian follicles of the vitellarium. And B: ovariole from a specimen with depleted EcR-A expression showing no BrdU incorporation. Scale bars: 200 μm.

characteristics of the niche allow keeping stem cells undifferentiated but also allow growth and differentiation in response to external signals, in addition to accomplish self-renewal. Therefore, the mechanisms underlying these processes in the stem cell niche point to fundamental insight in many fields in both basic science and applied fields related to biomedicine. In light of this, and as Spradling and co-workers have suggested, we can wonder whether a more in-depth understanding of stem cells might be used to develop a cell-based process for repairing malformed, damaged or ageing tissues, for example [40]. We can safely predict that the study of the niche regulatory mechanisms and signals, comparing the insect germarium with other systems, will unveil similarities that are likely to be of paramount importance in biology and biomedicine, and the study of the ecdysteroid–steroid input might be crucial to understanding the regulatory mechanism as a whole and the details of the circuitry that produce the different outputs, keeping the stem cells undifferentiated, allowing stem cell grow and differentiation or accomplishing stem cell self-renewal.

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