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REGULAR ARTICLE

Establishment of an immortalized cell line derived from the pupal ovary of *Mythimna separata* (Lepidoptera: Noctuidae) and identification of the cell source

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Abstract

Determining the source of primary cells is conductive to enriching sufficient cells with immortal potential thereby improving the success rate of establishing cell lines. However, most of the existing insect cell lines are established by mixing and fragmentation of explants. At present, the origin of cell lines can only be determined according to the cultured tissues, so it is impossible to determine which cell types they come from. In this study, a new cell line designated IOZCAS-Myse-1 was generated from pupal ovaries of the migratory pest *Mythimna separata* by explant tissues to derive adherent cultures. This paper mainly shows the further descriptive information on the origin of primary cells in the process of ovarian tissue isolation and culture. Phospho-histone H3 antibody-labeled cells with mitotic activity showed that the rapidly developing somatic cells in vivo gradually stopped proliferation when cultured ex vivo. The primary cells dissociated outside the tissue originated from the lumen cells, rather than the germ cells or the follicular epithelium cells. The results suggest that the newly established cell line IOZCAS-Myse-1 had two possible sources. One is the mutation of lumen cells in the vitellarium, and the other is the stem cells with differentiation potential in the germarium of the ovarioles. Moreover, the newly established cell line is sensitive to the infection of Autographa californica multiple nucleopolyhedrovirus, responds to 20-hydroxyecdysone and has weak encapsulation ability. Therefore, the new cell line can be a useful platform for replication of viral insecticides, screening of hormone-based insecticides and immunology research.

Keywords Lumen cell · Ovarian cell line · *Mythimna separata* · Autographa californica multiple nucleopolyhedrovirus (AcMNPV)

Introduction

The female reproductive system of insects is an organ for ovipositing, mating, and reproduction of new populations and is related to involving the reproduction of the next generation. Ovarian development is a limiting factor for population expansion of migratory insects. The study of insect ovarian development helps to elucidate the reproductive strategy of its population. Therefore, biological pesticides can be developed by inhibiting the development of female reproductive system to prevent the large-scale reproduction of chemical resistant pests and reduce the oviposition rate to control pests. Cell lines provide infinitely homogeneous sources, easy to manipulate, and do not contain endogenous hormones (Smagghe et al. 2009), which are excellent tools for screening alternative hormonal insecticides. However, ovarian-derived cell lines of migratory pests have not been

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previously established, limiting species-specific and tissue-specific screening. The anatomy, morphology, and ultrastructure of ovary have changed throughout the life of insects (Büning 1994). Understanding the process of ovarian development and oogenesis of migratory insects and establishing ovarian derivative cell lines will help identify new targets and develop new pest control agents.

At present, there is an urgent need for cell lines of specific important species, such as pollinated honeybees, locusts causing agricultural disasters, and planthoppers transmitting plant viruses. Either insects with various strains, researchers hope to use specific strains of cell lines for their research, such as *Drosophila melanogaster* and *Bombyx mori*. Or because most NPVs are infected and replicated only in limited species, cell lines of specific species are required for virus isolation and research. In addition, cell lines with specific functions, such as immune and phagocytic hemocytes, are also required (Chisa et al. 2004). Or specific tissue sources, especially midgut cell lines, are needed because they are targets for oral baculovirus infection or chemical pesticide toxicity (Zhou et al. 2020). However, behind these urgent needs, more technical breakthroughs are needed on the basis of clarifying the basic biological research of insect cells in vitro. The success of insect cell lines establishment is highly dependent on species of origin and tissues of origin, and especially whether there are appropriate media available for the cell types.

Many lepidopteran cell lines were established by culturing ovaries and have a clear tissue sources, but it is not clear which cell types these cell lines are derived from. In the early stage of primary culture, the cells in the proliferative phase, the cells with high survival ability in vitro, or cells with re-proliferative potential should be isolated and enriched to ensure the high quality of primary cells. Understanding the process involved in the origin of cell lines can not only enrich a large number of the primary cells but also help to establish cell lines of more insect species, specific tissues and cell types, so as to provide information on the application range of cell lines. The origin of ovarian cells in lepidopteran insects was reported as ovarian epithelial tissue (Mitsuhashi 1989). Stanley and Vaughn compared the short-term cultured and freshly dissected ovaries and concluded that the cells migrating from silkworm ovaries were follicular epithelial cells and intermediate-layer cells (Stanley and Kirkland 1968). This conclusion was related to the fragmentation treatment of explants in the primary culture method. At present, the method of ovary culture in lepidopteran insects is to take out the ovary tissue and cut into small segments less than 1 mm (Goodwin 1975; Mitsuhashi 2002). However, due to the mixed types of primary cells caused by ovarian fragmentation, it is difficult to determine whether these primary cells came from ovarian stem cells or differentiated germ cells, nurse cells, interstitial cells, follicular epithelial cells, or lumen cells.

At present, over 800 cell lines from more than 170 insect species have been established. However, more than half of these cell lines come from the culture of newly hatched larvae and embryos (Zhang et al. 2007). According to our statistics, there are 117 insect cell lines from reproductive organs, belonging only to 27 species of Lepidoptera from 7 families (Bombycidae, Geometridae, Lymantridae, Noctuidae, Papilionidae, Saturniidae, and Tortricidae) and 2 species of dipteran mosquitoes (Culicidae). The common armyworm *Mythimna separata* Walker (formerly known as *Pseudaletia separata*) is a model species for studying migratory pests in China, characterized by concentrated damage and overfeeding of larvae, which often cause outbreaks (Jiang et al. 2011). Trade-offs between the competing physiological demands of migration and reproduction in *M. separata* are coordinated by the "oogenesis-flight syndrome" (Jiang et al. 2011; Johnson 1969). However, due to its migration and replenishment characteristics, the ovarian development progress of *M. separata* pupae is different from that of lepidopteran representative species of *B. mori*, resulting in some differences in obtaining donor tissues during culture, which is directly related to the success rate of lines. Currently, there are only 10 cell lines established from *M. separata.* These cell lines are derived from embryos, larval fat bodies, and hemolymph (Li et al. 1998; Meng et al. 2017; Qiu et al. 1991; Shen and Liu 1983; Yu et al. 2000, 2003; Zheng 2010) (see Table 1). In addition, six cell lines derived from the embryos of a related species (*M. unipuncta)* have been reported to damage corn in North America (Wang et al. 1999). Currently, there is no available report in the Expasy Cellosaurus database (https://web.expasy.org/cellosaurus/) for cell lines derived from the ovaries of the pupal stage of *M. separata.*

The purpose of this study is to establish *M. separata* ovarian cell line and analyze the primary cell types obtained from explants adherent culture, so as to provide the basis for the subsequent research and application of this cell line. By analyzing the characteristics of the newly established cell lines, including cell size, growth rate, chromosome number, sensitivity to viruses, response to hormones, and immune-related encapsulation, their advantages and application value were expounded.

Materials and methods

Animal preparations and anatomy

The insects were fed with artificial diet at 25 ℃ in the laboratory of Institute of Zoology, Chinese Academy of Sciences, Beijing, China. The histological description of the ovary was based on phase-contrast microscopy of cultured materials. Ovaries of each stage of the common armyworm

Table 1 Cell lines established from the common armyworm *Mythimna* (originally *Pseudaletia) separata* Walker (Lepidoptera: Noctuidae)

Names of cell lines	Tissue sources	Duration of primary culture	Morphologies	Diameter	Population doubling time	Susceptibilities to *nucleopolyhedroviruses*	References
IOZCAS-Myse-1	The follicular epithelium of the pupal ovary	3 m	Mainly spherical	11.6 μm	34 h	50% (AcMNPV)	In this study
QAU-Ms-E-10	Embryo	6 m	Spherical and spindle-shaped	25.31 μm × 11.35 μm(spindle-shaped); 13.26 μm (spherical shape)	46 h	67.8% (MsNPV); 92.3% (AcMNPV)	Meng et al. (2017)
QAU-Ms-E-10C (Ms-E-10 subclone)	Embryo	-	Mainly spherical	14.4	42 h	67.8% (MsNPV); 94.2% (AcMNPV)	Meng et al. (2017)
QB-Ms-E-1	Embryo	6 m	Spherical and spindle-shaped	12.5 μm × 9.1 μm (spindle-shaped); 9.6 μm (Spherical shape)	44.9 h	56.6% (MsNPV); > 90% (AcMNPV)	Zheng (2010)
QB-Ms-E-2	Embryo	6 m	Spherical and spindle-shaped	18.4 μm × 9.6 μm (spindle-shaped); 10.3 μm (spherical shape)	46.6 h	53.6% (MsNPV); > 90% (AcMNPV)	Zheng (2010)
QB-Ms-E-3	Embryo	6 m	Spherical and spindle-shaped	21.2 μm × 8.2 μm (spindle-shaped); 10.3 μm (spherical shape)	46.5 h	49.3% (MsNPV); > 90% (AcMNPV)	Zheng (2010)
QB-Ms-E-4	Embryo	6 m	Spherical and spindle-shaped	17 μm × 8.7 μm (spindle-shaped); 9.9 μm (spherical shape)	47.1 h	62.5% (MsNPV); > 90% (AcMNPV)	Zheng (2010)
NEAU-MS-980312	Embryo	12 m	Spherical and spindle-shaped	-	47.8 h	62.1% (MsNPV)	Yu et al. (2003)
NEAU-Ms-921015	Embryo	-	-	-	-	Low	Yu et al. (2000)
NEAU-Ms-927311	Embryo	13 m	Spherical and spindle-shaped	-	58.8 h	> 40% (MsNPV)	Li et al. (1998)
NIAS-LeSe-11	Larval fat body	146 d	Spherical and spindle-shaped	20 μm	48 h	Susceptible to AcMNPV	Mitsuhashi (1983)
SIE-Ls-805	Larval hemocytes	-	-	-	-	AcMNPV, HaMNPV	Shen and Liu (1983); Qiu et al. (1991)

AcMNPV Autographa californica multiple nucleopolyhedrovirus, *MsNPV Mythimna separata* nucleopolyhedrovirus,—no data was mentioned in the literature

pupae were dissected, and their developmental stages of were determined based on the number of days after pupation. The age of insect is expressed in days from pupation. The anatomical tissue was placed in 24-well plates (Corning, USA, 3524) and photographed immediately or after a short period of culture.

Proliferation assessment using phosphor-histone H3 antibody labeling

The ovarian tissue of *M. separata* was divided into three parts—the distal germarium, the tubular vitellarium, and the expanded pedicel—and were cultured in 24-well plates (Costar, USA) for immunofluorescence analysis. After the samples were fixed by immunostaining fix solution (Beyotime, China, P0098-100 mL), the permeable solution PBST (PBS containing 0.1% TritonX-100) was added, and then the tissue was incubated in blocking solution (PBS containing 5% skim milk powder) to block the non-specific binding sites. The commercial Phospho-Histone H3 (Ser 10) Rabbit Monoclonal Antibody (Beyotime, China, AF1180) was used to replace the blocking solution, and then the secondary antibody Goat Anti-Rabbit IgG conjugated TRITC (Easybio, China, BE0115-100) was added. The cytoskeleton was stained with Actin-Tracker Green (Beyotime, China, C1033). Finally, the nuclei was stained with DAPI (Beyotime, China,

C1006). The images were collected by inverted fluorescence microscope (EVOS, USA, FW5012).

Isolation of ovariole and initiation of primary culture

The main medium was a mixture of 10% fetal bovine serum (FBS, without heat inactivation) (BI, Beit Haemek, Israel, 04–001-1) and 90% Insect-XPRESS™ (BioWhittaker, Lonza, Walkersville, MD, USA, 12-730Q) was used as the growth medium. The medium was supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) to prevent bacterial growth. In addition, the ratio of primary culture medium was 8:1:1 (Insect-XPRESS™ medium:FBS:the Insect-XPRESS™ medium saturated phenylthiourea solution).

Insects were immersed in 75% ethanol for 10 min for surface sterilization and then dried using absorbent paper. Ovarian tissues dissecting from the abdomen of the pupae were rinsed thoroughly with sterile Ringer's salt solution to remove hemocytes and fat body remnants (Mitsuhashi 2002) to ensure the unity and the integrity of the ovarian tissues. Then, primary culture medium of 1.0 mL was added into a T-25-cm^2 culture flask (Corning, NY, USA, 430,168), and the completely spread on the surface of the culture flask to prevent the surface tension of the liquid from destroying the tissue adhesion. Ovarian tissues rinsed with the primary medium were transferred into the above culture flask by tweezers. Five sets of ovaries were placed in each culture flask. Then, the culture flask was placed at 27 °C overnight and gently dropped into the growth culture medium the next day. Growth medium (1.0 mL) was added again on the second and third day.

Long-term hypoxia–reoxygenation treatment during primary culture

After 21 days of initial culture, the plug seal cap of the flask was replaced with the vent cap (Corning, 430,639). The cultures were then transferred to the anoxic environment in a tri-gas incubator (Thermo Fisher Scientific, Marietta, OH, USA, Forma 3131). The tri-gas incubator was filled with nitrogen to discharge the air, and the oxygen concentration is finally maintained at 5%. After hypoxia treatment at 27 ℃ for 9 days, the fresh medium was used to replace the medium in the flask and replaced with a plug seal cap.

Subculture

The first subculture was performed 3 months after hypoxic treatment. The contents of the primary culture was agitated and transferred into a new flask containing 2 mL fresh growth medium. After overnight, the medium containing suspended cells or dead cells was replaced with fresh growth medium of 5 mL. According to the growth rate of cultured cells, the passage interval from the initial subculture to the 10th generation ranged from 1 to 3 weeks. After subculture, 1/2 to 4/5 volume of the medium was replaced. The newly established cell line was designated as IOZCAS-Myse-1 (IOZCAS referring to Institute of Zoology, Chinese Academy of Sciences). In this study, the cell line is referred as Myse-1.

Morphology and size of Myse-1 cells

Images of cells obtained from the primary culture and the cell line were captured using a Leica inverted phase contrast microscope (Leica, Germany, DM2000) fitted with a charge coupled device (CCD) camera system (TOUPCAMTM, Suzhou, China, E3CMOS). Cell size was calculated based on a calibrated magnification factor. Eight replications were performed for each cell line, and the ratio between the round and spindle cells was calculated. Average cell dimensions were determined from measurement of 100 cells. GraphPad Prism 8 software was used for statistical analysis. The differences between generations and cell lines were evaluated by analysis of variance (ANOVA) and *t*-test. All tests were performed at a confidence level of 95%.

Population growth of Myse-1 cells

The cells in log phase were inoculated in 25-cm^2 culture flasks at a density of 2.0×10^5 cells/mL. The cells were cultured in 5 mL growth medium per flask at 27 ℃ for 7 days, and the culturing was done in four replications. The cells were counted by a hemocytometer, and cell population doubling time was calculated by the exponential formula described by Kuchler (Kuchler 1977).

Chromosome analysis

The chromosome analysis of the Myse-1 cell line was carried out in the 15th passage following an improved method of Takahashi (Takahashi et al. 1980). Specifically, the cells were incubated at a lower temperature (27 °C) in a low-osmotic solution for 30 min and stained with a lower concentration (5%) of Giemsa solution for 30 min. The cells on the slides were stained with 0.1% Giemsa for 30 min. Finally, the metaphase chromosomes were observed and selected under the microscope and the images were obtained.

Cell chromosome multiples were detected by flow cytometry. The hemocytes of the *M. separata* larvae were used as diploid control. Cells were collected and fixed overnight at 4 °C with 70% ethanol. Propidium iodide (PI) (0.1 mg/mL) was used for 30 min at 37 °C, and flow cytometry (BD Biosciences, East Rutherford, NJ, USA, LSR Fortessa) was used for detection after filtration.

Identification of the species origin of Myse-1

The species origin of Myse-1 cell line was determined by comparing the genetic profiles of the cells at passage 20 with *M. separate* pupa. DNA amplification fingerprinting technique employing polymerase chain reaction (DAF-PCR) was used for the comparison. Cellular aldolase was used as the molecular marker. PCR primer pair of aldolase enzyme was as follows: aldolase-F (5′-CCGGAGCAGAAGAAGGAGCT-3′) and aldolase-R (5′-CACATACTGGCAGCGCTTCA-3′) (McIntosh et al. 1996).

In addition, mitochondrial cytochrome oxidase subunit I gene (*mtCOI*) in Myse-1 and *M. separata* genomic DNA was amplified using primers Myse-*COI*-F (5′-CTGCTGGAGGAGGTGAT-3′) and Myse-*COI*-R (5′-ATAATGAAAATGAGCGACTACA-3′). PCR reactions were performed at 94 °C for 5 min for an initial denaturation, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and then a final extension at 72 °C for 10 min. PCR products were then separated using 1% agarose gel and the target band excised for direct sequencing by Sangon Biotech Co., Ltd., Shanghai, China. DNA sequences were analyzed using DNASTAR software package (DNASTAR, Madison, WI, USA). The mt*COI* fragment sequences obtained were submitted to the National Center for Biotechnology Information (NCBI) and assigned unique Accession numbers. In addition, sequences were compared with *M. separata* mt*COI* sequences available in NCBI DNA sequence database.

Infection with baculovirus

Cells in log phase were seeded in T-25 cm^2 culture flasks at a density of 4×10^5 cells per flask. Cells were then cultured in growth medium at 27 ℃ for 12 h to attach to the bottom of the flask. The culture medium was removed, and cells inoculated with the budded viruses (BV) of Autographa californica multiple nucleopolyhedrovirus (AcMNPV) at a multiplicity of infection (MOI) of 0.1. The culture flasks were gently shaken for 3 h on a vertical shaker for viral adsorption. Cells were rinsed with Ringer's salt solution after removal of the viral solution and cultured in 3 mL of fresh growth medium at 27 ℃. Infection of cells was observed under an inverted phase contrast microscope and photographed. Polyhedral formed within the cells were used as the index to calculate infection rate (Granados et al. 1994).

HR3 gene expression in Myse-1 cells treated with 20-hydroxyecdysone (20E)

Myse-1 cells (1×10^6 cells per flask) were inoculated in T-25 cm^2 culture flask. 20E (Aladdin, Shanghai, China) (10^{-2} mol/L) was prepared with 100% ethanol and diluted by fresh medium. After 3 days of culturing the cells, 20E was added into the above culture flask to achieve a final concentration of 10^{-6} mol/L. Notably, the final concentration of 100% ethanol was below 0.1%, which is non-toxic to the cells. Total RNA was extracted using TRIzol reagent (Gibco, USA, 15,596,018) and reverse transcribed into cDNA. Primers for nuclear hormone receptor *HR3* were as follows: Myse-*HR3*-F, 5′-GCCGAGCTCAAGCGACCAGTTCC-3′, and Myse-*HR3*-R, 5′-GTCCGCACAGTCCAGCCACATTTC-3′. PCR reaction conditions were as follows: initial denaturation at 94 ℃ for 3 min, one cycle; 35 cycles at 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 1 min; a final one cycle extension of 72 ℃ for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis with a concentration using Trans2K DNA marker (TRANSGEN, Beijing, China).

Encapsulation assays

Encapsulation assay was performed with the larval hemocytes, pupa ovarian primary cells from *M. separata* and Myse-1 cell line cultured in 24-well plates, respectively. Each group was supplemented with 100 μL of 1 mg/mL SP-Sephadex C-25 beads (Pharmacia Fine Chemicals, Sweden, 6017) suspended in Ringer's salt solution and incubated overnight on a shaker at 27 °C. The results were observed and photographed under Leica inverted phase contrast microscope.

Results

Structure and composition of ovary during pupal development of *M. separata*

In order to obtain the best donor, we first analyzed the morphology and structure of the ovary of *M. separata* pupae at different developmental stages. The pupal stage of *M. separata* lasted about 10 days at 25 ℃ (Wu and Guo 1964). For convenience of description, the development process of *M. separata* pupae was simply divided into early stage (1–3 days after pupation), middle stage (4–6 days after pupation), and late stage (7–10 days after pupation). A simple way to distinguish the mid-pupa and late-pupa is by comparing their external characteristics, the mid-pupa eyes obviously changed from light red to deep red. The ovary of the early pupae is small and difficult to dissect. A protective capsule coated on the outermost layer of the ovary is known as the ovarian sheath, which encloses the entire ovary including all parts of the ovary in a 1-day-old pupa. Effective primary cultured ovaries are taken from mid-pupa, that is, the eyes turned to medium red. The ovaries of this period are easily dissected and can be removed from the posterior abdomen of the pupa. But no ovarian sheath was found in the tissues dominated by ovarioles. The tissue we cultured is a part of the reproductive system taken from the mid-pupa of *M. separata* with a common oviduct, a pair of lateral oviducts and 4 polytrophic meristic ovarioles on both sides (Fig. 1a).

Then, according to the anatomical structure of different developmental stages of *M. separata*, combined with the function of other insects and previous studies, the definition and location of different ovarian cell types in different developmental stages of *M. separata* were determined. Each ovariole is divided into three regions from the anterior to the posterior end of the ovariole proper, namely, the germarium, the vitellarium and the pedicel (Fig. 1a). The germinal tips of each four ovarioles on both side occurred together within the ovariole sheath matrix. No terminal filament was observed (Fig. 1b, c), and this is different from *D. melanogaster*. The germarium located in the anterior end of the ovariole contains oogonia, accounting for a large proportion in the ovarioles of the early-pupa (Fig. 1b). In the germarium of the ovarioles of the early-pupa or mid-pupa, the germline stem cells, cystoblast cells and cystocytes were stably embedded in the thicker stroma matrix or intestitial cells (Fig. 1b). Obvious cystoblast cells in cystocytes have not yet formed (Fig. 1b). Morphologically pronounced somatic apical cells are found and germ cells build clusters around it (Fig. 1b). On the other hand, the ovarioles of the late pupae are fully matured and the germarium in this stage is shortened to almost invisible, and cystoblast cells in cystocytes were still enclosed in the matrix (Fig. 1c). Due to the thinning of the matrix, germ cells were not easily identified (Fig. 1c).

The vitellarium is significantly elongated during pupal development, constituting the main part of the ovary (Fig. 1a). A linear arrangement of follicles was observed (Fig. 1f, g), and the asynchronous nature of oogenesis was similar to that of the mature larvae and prepupa of the *Bombyx mori* (Swevers and Iatrou 1992). It was observed that the ovarioles in the whole pupal stage of *M. separata* were transparent and were in the previtellarium stage, and there was no vitellogenesis (Fig. 1a, g). Ovaries are internal organs which shelter germ cells during their development from their dormant stages as oogonia to fully differentiated oocytes and all somatic tissues of the ovary are derived from the mesodermal sheath (Büning 1994). Ovariole sheath consists of inner and outer layers (Fig. 1d). Ovariole sheath is composed of two layers of membrane and has the function of stretching, showing the characteristics of muscle cells: the inner layer (tunica propria or basal lamina) can fold and twitch in the outer layer (tunica extema) sleeve (Fig. 1d, e). The trachea supplying the ovary extends into the tunica extema of the ovarioles, visible in vitro and easy to remove. The inner layer of ovariole is thin and dense. It is considered that tunica propria maintains the stability of ovarian elongation structure, hemolymph physical screening, and chemical selectivity of macromolecules that play a hormone role in vitellogenesis (Büning 1994; Van De Bor et al. 2015). Intermediate-layer cells (lumen cells) occur between the two layers (Fig. 1d,

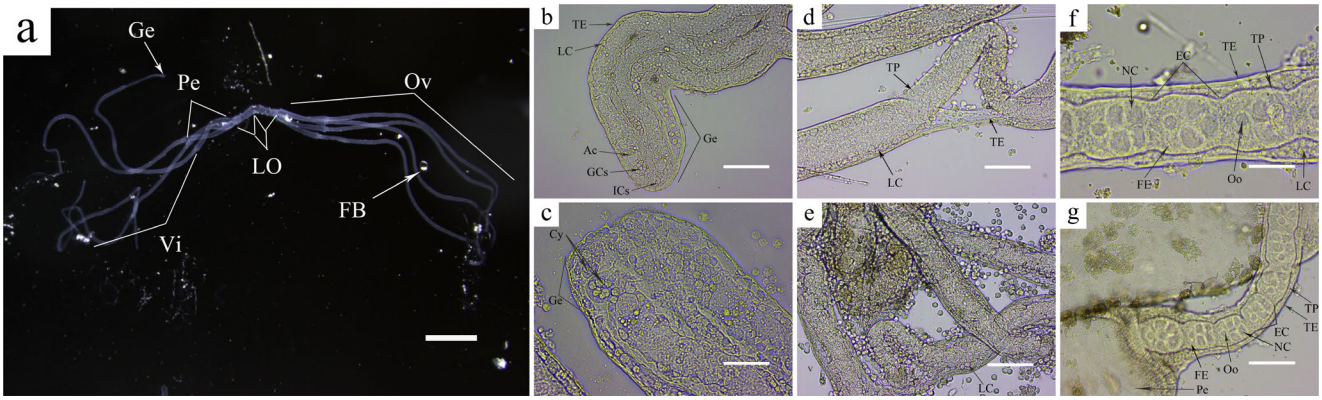

Fig. 1 Ovarian structure and ex vivo culture of *Mythimna separata* pupae. A large number of primary cells were isolated from lumen cells in the vitellarium of ovarioles. **a** The whole ovary of a single pupa of *M. separata* was removed under stereomicroscope, including a common oviduct (not shown in the picture), a pair of LO and 4 polytrophic meristic Ov. Each ovariole consists of the Ge, the Vi, and the Pe. Scale bar = 1 mm. **b** In the early-pupa, the germarium of the ovariole is long. The GCs from clusters around the larger AC were stably embedded on the thicker stroma matrix or ICs. CCs in Cy and no terminal filament was observed. Scale bar = 100 μm. **c** The germarium of ovariole in late-pupa was short, and only CC in Cys were obviously observed. Scale bar = 50 μm. **d** The cells migrated from the crevasse within 24 h of plating came from the LCs between the TE and the TP. Scale bar = 100 μm. **e** Seven days after culture, a large number of free cells were observed around the ovarioles affixed to the bottom of the culture flask. Scale bar = 100 μm. **f** The ECs/follicles wrapped in the TP. The NCs occupy half the size of the EC, and the cuboidal FE cells encased the small round and transparent Oo. Scale bar = 50 μm. **g** No cells dissociate from the Pe of ovarioles. In the Vi of the broken ovariole, the LCs between the TE and the TP are clearly visible. The number of LCs did not decrease in the middle and late stage of development. Scale bar = 100 μm. AC apical cell, CC cystoblast cells, Cy cystocytes, EC egg chambers, FB fat body, GCs germ cells, Ge germarium, ICs intestitial cells, GS germline stem cell, LO lateral oviduct, TP tunica propria, Ov ovarioles, TE tunica extema, Pe pedicel, Vi vitellarium, LC lumen cell, Oo oocyte, NC nurse cell, FE follicular epithelium

e). Lumen cells spread throughout the vitellarium of the ovariole, from the germarium to the junction of the end chamber with the pedicel (Fig. 1b, d, f). A large number of lumen cells were observed in the early, middle, and late stages of pupae, and no degeneration was observed throughout the pupal stage (Fig. 1c, e, g). Each follicle inside tunica propria is composed of an oocyte, several anteriorly placed nurse cells, and the surrounding somatic follicular epithelial cells (Fig. 1f, 1g, 2f). In the 3-day-old pupae, differentiation of oocytes and nursing cells can be seen in the region of the ovariole near pedicel. At the mid-pupa on the 4th day after pupation, the outline of the eggs was observed next to the pedicel (Fig. 1g), but no follicles were formed next to the germarium (Fig. 1c). In the middle and late stages, the follicles formed by round oocyte, nurse cells of different sizes, and follicular epithelial cells wrapped in both of them arranged closely throughout the vitellarium (Figs. 1f, g and 2f). Since the division cycle of Lepidoptera is highly constant, with $n = 3$ (eight siblings) (Büning 1994), we speculate that there are seven nurse cells in each follicle. Differentiated cuboidal follicular epithelial cells were observed in the follicle (Fig. 1f). During this period, lumen cells and follicular epithelial cells appear simultaneously (Figs. 1f and 2f), which can be easily distinguished under the light microscope. Due to the tight entrapment of the tunica propria, oocytes, nurse cells, and follicular epithelial cells did not show outgrowth from the ovariole ex vivo (Fig. 1f, g). In summary, these results showed that the ovaries isolated from the mid-late pupae developed completely. The main part of the ovariole is the germarium and the vitellarium, which can provide enough primary cells for further culture.

The origin and cell type of a large number of isolated single primary cells

In order to evaluate the source of primary cells, we isolated the mid- and late-stage ovaries and conducted short-term ex vivo culture. Cells were dissociated from ovarioles on the second day after in vitro culture (Fig. 1d). After 4 days of culturing, a large number of cells were attached to the surface of ovarioles and outgrowth of the tissue was observed. At the same time, a considerable number of single primary cells were obtained (Fig. 1e). Comparison of the pupal ovaries of the three stages cultured in vitro showed that primary cells can be isolated in each stage. Notably, the number of isolated primary cells increased significantly with increase in tissue biomass of the developing ovary and a considerable

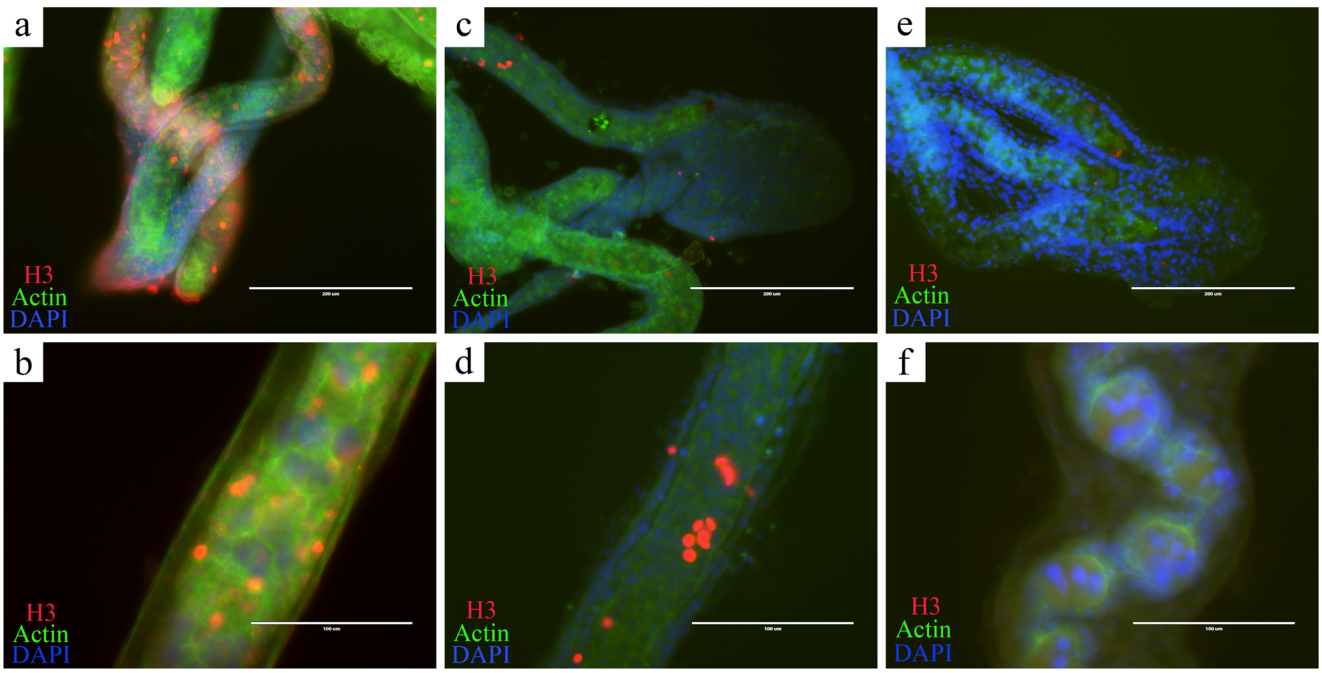

Fig. 2 The proliferative cells of pupae ovaries of *M. separata* decreased gradually after being cultured ex vivo, immunolabeled with phospho-histone H3 (serine 10) antibody. Ovariole develops rapidly in vivo (0 day), and many cells are undergoing mitosis **a**, **b**. The number of mitotic cells was decreased significantly after cultured for 2 days **c**, **d**. After 10–14 days of culture in vitro, almost no cells still mitosis **e**, **f**. Mitotic cells are mainly somatic cells that surround the oocyte and the nurse cell. Histone H3 binding to phosphor-histone H3 (Ser 10) antibody shows red with goat anti-rabbit IgG, TRITC conjugated secondary antibody. The microfilament combined with the Actin-Tracker Green showed green. The nuclei were stained with DAPI and showed blue. Scale bar = 100 μm in **b**, **d**, **f**; scale bar = 200 μm in **a**, **c**, **e**

number of primary cells were obtained from the ovariole of the middle and late pupae (Fig. 1e). Almost all the free cells originated from the lumen cells in the vitellarium of ovarioles (Fig. 1d, e).

Throughout the culture flask, the suspended intact tissue gradually decayed and died over time, and no single cell was separated from the tissue (not shown in the figure). This is consistent with the description of Mitsuhashi (Mitsuhashi 1989). However, tissue adhesion on the bottom wall of culture flask may lead to rupture of the ovariole sheath (Fig. 1d). In some specimens with the stroma and ovariole sheath degeneration, the tunica propria was clearly visible. This phenomenon was shown by analyzing the cells remaining attached to the bottom of the culture flask after removing the explants. Although cells were seen on the surface of the pedicel, the lateral oviduct and the common oviduct, these cells were mainly attached to the tissue surface and gradually died after in vitro culture (Fig. 1g).

Lumen cells are located at the vitellarium of ovariole between the tunica extema and the tunica propria that surrounds the germ cells. Lumen cells grew from the ruptured or dissected areas of the ovariole sheath (Fig. 1d, e). In the late-stage of ovarian development, lumen cells and follicular epithelial cells appear simultaneously (Figs. 1f, and 2f), so they are two different cells. Because of being tightly wrapped by tunica propria, oocytes and nurse cells did not grow out of the egg chamber (Fig. 1f, g). Analysis under optical microscope showed that although the lumen cells are attached to the surface of the ovarian muscle sheath of the cultured explants, the outer dense layer of the tunica propria that surrounds the germ cells was intact (Fig. 1d). Because of being tightly wrapped by tunica propria, the oocyte and nurse cells did not show outgrowth (Fig. 1f, g). Therefore the possibility of primary cells coming from germ cells and follicular epithelial cells were excluded on the basis of anatomical process and the culture process (Fig. 1d, f). In the early and middle stages of the ovary, epithelial cells have not yet differentiated in the egg chamber, so free single primary cells exclude the possibility from follicular epithelial cells. In summary, we believe that most of the primary ovarian cells of originate from the lumen cells of the ovariole vitellarium.

Mitotic activity of ovariole cells cultured ex vivo

In order to investigate the origin of proliferation cells, we analyzed the location and type of proliferation cells at different stages in vivo and with the prolongation of culture time using mitotic markers (phosphorylated histone H3). The results showed that there were more Phosphorylated histone H3 positive cells in vivo (Fig. 2a, b), but phosphorylated histone H3-positive cells decreased significantly after 2 days of cultured ex vivo (Fig. 2c, d), and even difficult to be detected after 10–14 days (Fig. 2e, f). In vivo, most of the rapidly developing cells in the pupal ovariole vitellarium are in mitosis, transcriptional activation and chromosome condensation. The nuclei of phosphorylated histone H3-positive cells were small and located on the periphery of the ovariole (Fig. 2b, d). Coupled with the reasons for the high specialization of nurse cells and oocytes, it is speculated that these proliferative cells are not oocytes and nurse cells, but somatic cells. After 2 days and 10 days of culture ex vivo, no positive proliferative cells were found even in the terminal germarium (Fig. 2c, e). Cells no longer proliferate continuously even if they are not dissociated from the tissue (Fig. 2d, f). This indicates that cell proliferation occurs in vivo, but after culture ex vivo, cell proliferation decreases significantly.

Reproliferation of primary cells after hypoxia-reoxygenation

In this study, 60 female pupae were used as materials, and 12 vials of effective culture were obtained simultaneously. Among them, 6 flasks were used for hypoxic inducement and 6 flasks for direct normoxia culture. After the first subculture, the cell states of cultures treated with hypoxia showed significant differences compared with non-hypoxic-treated cultures. Most cells in non-hypoxic-treated culture floated in the medium and gradually died within 20 days after the first subculture (Fig. 3b). On the other hand, cells cultured under hypoxic conditions remained active after the first passage. However, only one culture flask after the first passage showed an increase in the number of cells, presenting multiple clumps.

Successful subculture process

The successful culture initiated on June 15, 2018. The ovaries used for culture were obtained from *M. separata* of 5–8 days after pupation. Cell migration occurred within 24 h after the culture was initiated (Fig. 1d). The cells were at first densely distributed around the explants. Then, the cells gradually moved to the surrounding area, and finally distributed over almost the entire area of the flasks within 20–30 days. The ovariole contraction were lasted for more than 30 days, and the lumen cells were squeezed out of the intermediate layer, resulting in a continuous increase in the number of individual cells outgrowth around the tissue (Fig. 1e). If the primary cells at the bottom of the culture flask were directly passaged, no matter how many cells were accumulated, almost all the cells transferred to the new culture flask would die within 1 to 2 weeks after the first subculture, while the cells in the original culture flask would not die so quickly. Finally, most of the expectations of cultivation stop there.

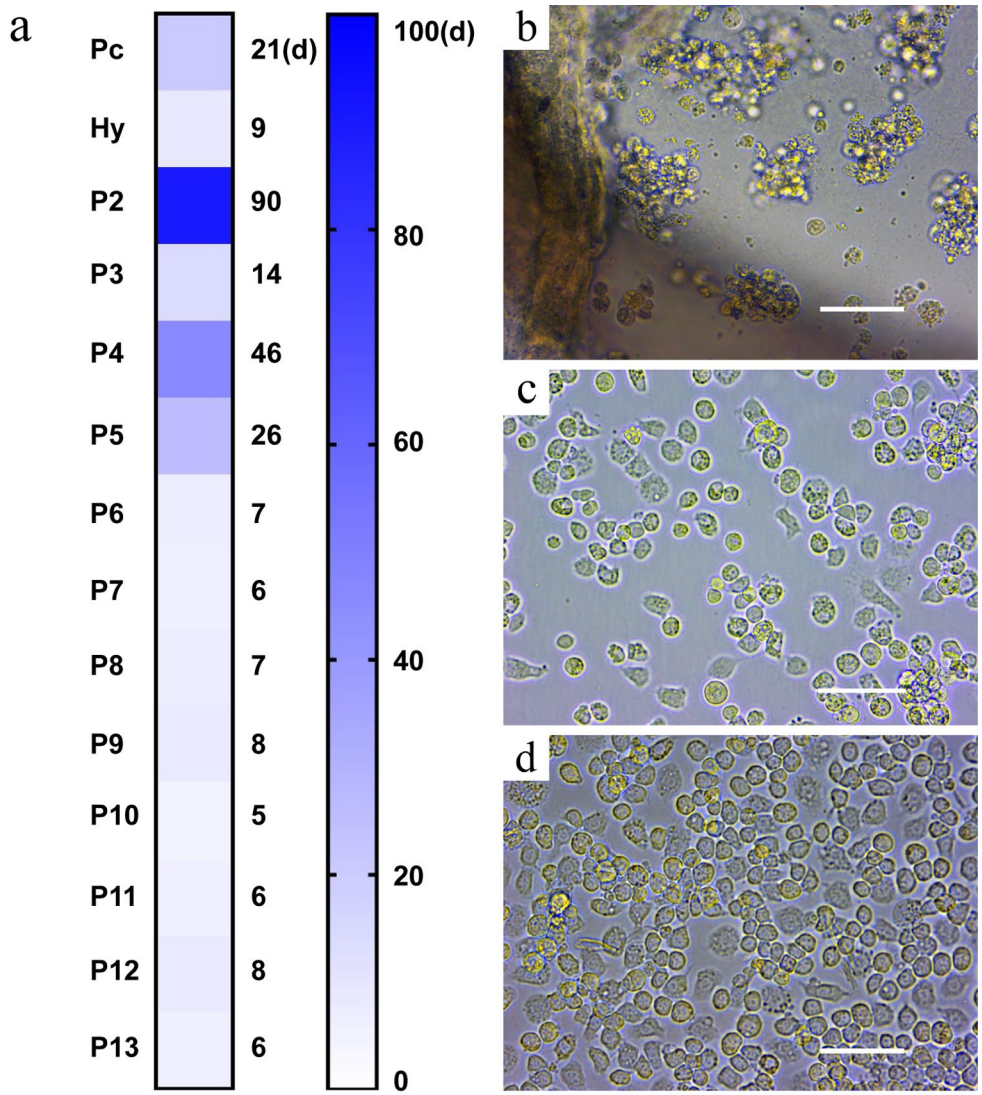

Fig. 3 The process of primary culture and cell line generation. **a** The lifespan of primary cultured and isolated young cell line. The depth of color indicates the time interval (days) from the initiation culture to successive subculture and passage. Pc primary culture, Hy under hypoxia condition, P2–P13 passage 2 to passage 13. **b** Cells without hypoxia treatment gradually died after the first subculture. **c** The fifth generation of Myse-1 cells showed spherical and spindle cell. **d** The 30th generation Myse-1 cells showed predominantly spherical cell. Scale bar = 50 μm

The second subculture was performed 10 days after the first passage. However, the proliferation was significantly reduced for a considerable period (Fig. 3a). Subsequently, for a long time, the cells attached to the bottom of flask formed aggregates again, and active multiplying of small cells groups occurred. The interval durations between the first five generations were random and irregular (Fig. 3a). For example, the longest interval needs 90 days. Cell growth was stable at the sixth generation (Fig. 3a, c). These cells formed a cell line capable of continuous passage which we named IOZCAS-Myse-1 (Myse-1) cell line. The cell line was routinely sub-cultured on a weekly basis using a ratio of cell suspension to fresh medium of between 1:2 and 1:5 based on cell concentration. Currently, this cell line has undergone more than 50 successive passages (Fig. 3d).

Myse-1 was identified to be homologous to *M. separata* species

After DAF-PCR analysis, the DNA fragment patterns of Myse-1 was similar to that of the ovary of *M. separata*, but different from that of other cell lines currently preserved in out laboratory (Fig. 4a), such as IOZCAS-SpexII (from *Spodoptera exigua*) (Zhang et al. 2006), QAU-Se-E-3 (from *Spodoptera exigua*) (Su et al. 2016), IPLB-Sf21 (from *Spodoptera frugiperda*) (Vaughn et al. 1977), and BTI-Tn-B1-4

(also named High Five, from *Trichoplusia ni*) (Granados et al. 1994), indicating that Myse-1 was derived from *M. separata*.

To further confirm the origin of Myse-1 cell line, partial nucleotide sequences of mtCOI were sequenced. A 473-bp PCR product was amplified from Myse-1 and pupae ovaries of *M. separata* (Fig. 4b). Sequence alignment results showed that the Myse-1 sequence (MT021760 in NCBI) was 100% identical with that of *M. separata* (MT021759 in NCBI). These results further indicated that Myse-1 was derived from *M. separata*.

Morphology, growth, and chromosome characteristics of Myse-1

The morphology of the isolated primary cells was consistent, but the cells after re-proliferation were more diversified. With the increase of passages, the cells with rapid growth gradually overtook and replaced the cells that were not proliferating. Subsequently, homogeneous cells were stably amplified in vitro (Figs. 3c and 5a). The morphology and size of Myse-1 was uniform after the 10^{th} passage (Fig. 5a) and the smaller cells were dominant (Fig. 5b). Obviously, Myse-1 cells showed a significantly smaller size compared with the size of IPLB-Sf9 cells (from *Spodoptera frugiperda*) (Hink et al. 1991), BCIRL-Hz-AM1 cells (from *Helicoverpa zea*) (McIntosh and Ignoffo 1983) and BTI-Tn-B1-4 cells (also named High Five, from *Trichoplusia ni*) (Granados et al. 1994) (Fig. 5c). However, the size of Myse-1 cells was not statistically different from that of S2 cells (also named SL2, from *D. melanogaster*) (Schneider 1972) (Fig. 5c). Spherical cells (91.1%) were predominant among the various cell types of Myse-1 (Fig. 5a). These were approximately 11.6 μm in diameter at the 40th passage (Fig. 5b). The size of the spindle-shaped cells (8.9%) ranged from 13.5 to 23.1 μm (mean 16.9 μm) in length, and from 6.5 to 13.5 μm (mean 9.9 μm) in width (Fig. 5a). Myse-1 had a lag phase of 1–2 days after subculture (Fig. 5d). Its population doubling time was 39.8 h. Moreover, the highest cell density in the stationary phase was 3.2×10^6 cells/mL.

The Myse-1 cell population is mainly tetraploid

Typical lepidopteran chromosomes morphology and distribution were observed during the metaphase chromosomal spreading of Myse-1 cells at the 15th passage (Fig. 5e). Lepidopteran chromosomes are difficult to count accurately because of their small size and large number. Therefore, it is difficult to count accurately under optical microscope, but the chromosome number of Myse-1 cells can be counted more than 100, which was presumed to be tetraploid. Further, we used flow cytometry to detect cell cycle. Cell chromosome multiples were detected by flow cytometry. Cell cycle was divided G0/G1 phase (diploid), S phase (2–4 ploidy) and G2/M phase (4 ploidy) according to DNA content. Myse-1 has more S phase cells (Fig. 5g) than that of larval hemocytes (Fig. 5f), indicating that more cells are proliferating in the cell line (Fig. 7). With the hemocytes of the *M. separata* larvae as the diploid control, the first fluorescence intensity peak of hemocytes was about 40 at 360 V (Fig. 5f). The first fluorescence intensity peak of Myse-1 was about 80 (Fig. 5g), which was twice that of diploid control. Therefore, Myse-1 was determined as tetraploid.

Myse-1 cells can support AcMNPV replication

In order to determine whether hypoxia will affect the sensitivity of the resulting cell lines to the virus, and for the

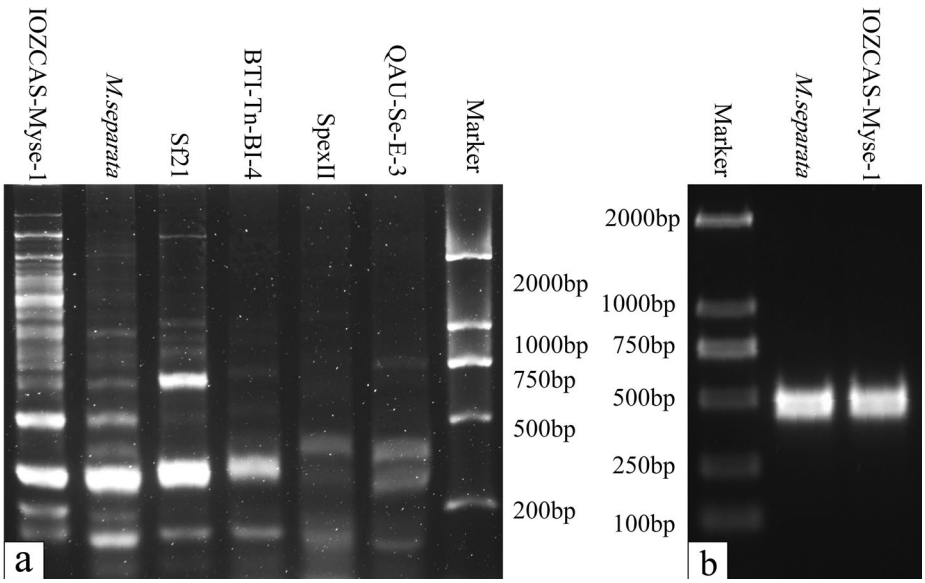

Fig. 4 Identification of Myse-1. **a** The DAF-PCR profiles of Myse-1 cell line and its host exhibited similarities. Cell lines Sf21 (IPLB-Sf21), BTI-Tn-B1-4, Spex II (IOZCAS-SpexII), QAU-Se-E-3, and S2 were used for comparisons. A slightly different profiles were observed in the other cell lines. Primer pair used was aldolase-F/aldolase-R. **b** PCR product (473 bp) of the mitochondrial-encoded cytochrome c oxidase subunit I gene (*CO I*)

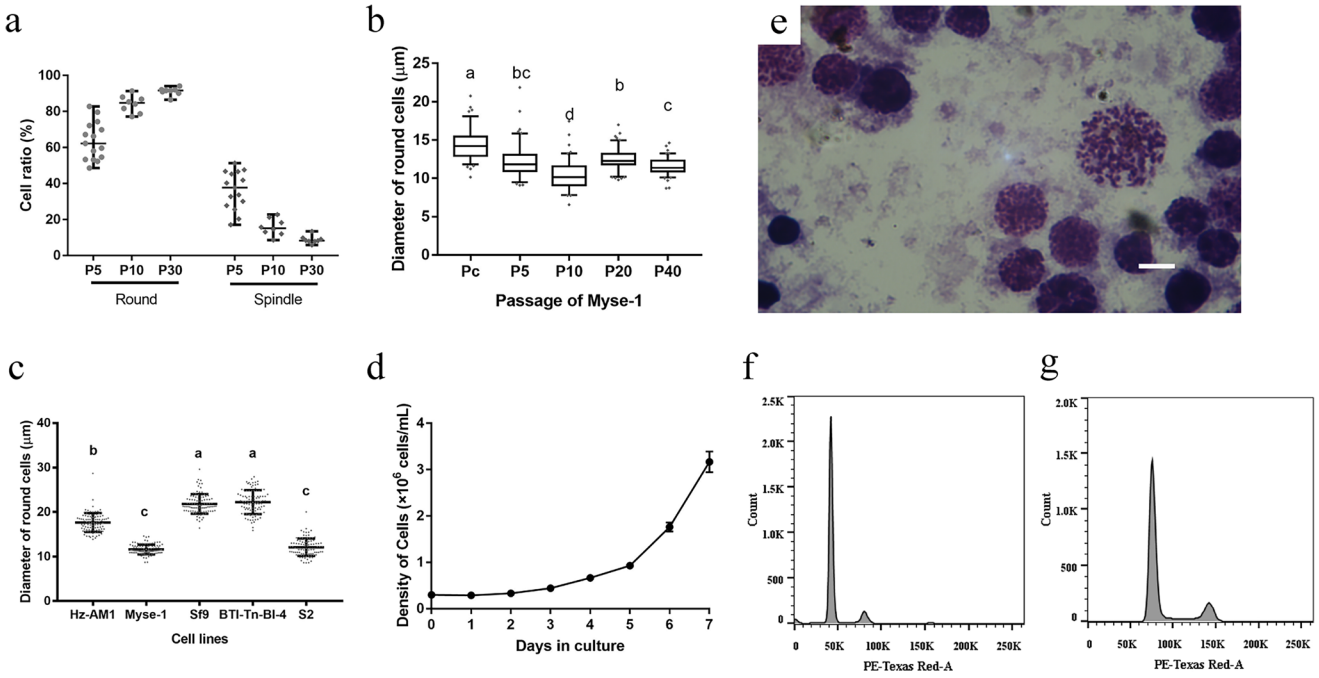

Fig. 5 Characteristics of Myse-1. **a** Myse-1 cells were more spherical from passage 5, 10, to 30 (62.3%, 83.2%, and 91.1%, respectively). **b** The cell size was consistent after the 10th generation. **c** Myse-1 cells were significantly smaller than Sf9 (IPLB-Sf9), Hz-AM1 (BCIRL-Hz-AM1), and BTI-Tn-B1-4 cells. Diameter of Myse-1 cells had no significant difference with S2. **d** Growth curves of Myse-1 cells cultured at 27 ℃ for 35 generations in INSECT-XPRESS medium supplemented with 10% FBS. Myse-1 showed a lag phase of 1–2 days after subculture. Error bars indicate the standard deviation of four independent trials. **e** Typical chromosome spread at passage 10 showed that the cells were tetraploid. **f** Primary ovarian cells of *M. separata* chromosome multiples were detected by flow cytometry. **g** Myse-1 cells chromosome multiples were detected by flow cytometry

future application of cell lines in pathology, we inoculated AcMNPV. Cytopathogenic features like bulging cells and enlarging nuclei with numerous occlusion bodies (OBs) were observed at the anaphase of infection (Fig. 6a). Myse-1 cells were susceptible to AcMNPV, and the infection rate of Myse-1 cells was 54%, 6 days after post-infection, so Myse-1 was moderately susceptible to AcMNPV. Therefore, in order to obtain higher cell line infection rate, it is necessary to further clone and isolate the cell line. However, Myse-1 cells were not susceptible to another baculovirus named PsunGV-Ps (*Pseudaletia unipuncta granulovirus*, *M. separata* infected with PsunGV-H) (figure not shown). As far as we know, there is no cell line capable of supporting granulovirus (GV) replication and showing granules by now.

Determination of hormone receptor HR3 gene expression in cell lines treated with 20E

In order to investigate whether the new cell line can respond to hormone stimulation, semi-quantitative RT-PCR was performed to investigate the expression level of the nuclear hormone receptor gene *HR3* after 20E treatment. The amplified band using the designed *HR3* primers was 412 bp. *HR3* gene expression was detected after treatment with 20E (10^{-6} mol/L) for 3 h, and the expression level of *HR3* gene increased steadily within 6–24 h (Fig. 6b).

Myse-1 cells have a weak encapsulation capability

In order to compare whether the primary cells derived from lumen cells and the established cell lines have phagocytic characteristics and blood cell characteristics, beads were used for simulated encapsulation test. The encapsulation reaction begins when clusters of cells attach to the surface of a bead. When part of the surface or the entire surface is covered by multilayer cells, the beads are thought to be encapsulated. The encapsulation assays showed that only the beads of hemocytes group were tightly encapsulated by multilayer cells (Fig. 6c), presenting a positive encapsulation response. While the groups of primary cells and Myse-1 cells only a few cells adhered to the beads, suggesting that no strong encapsulation reaction occurred (Fig. 6d, e). These results indicated that the hemocytes had stronger encapsulation ability compared with the primary cells and Myse-1 cells.

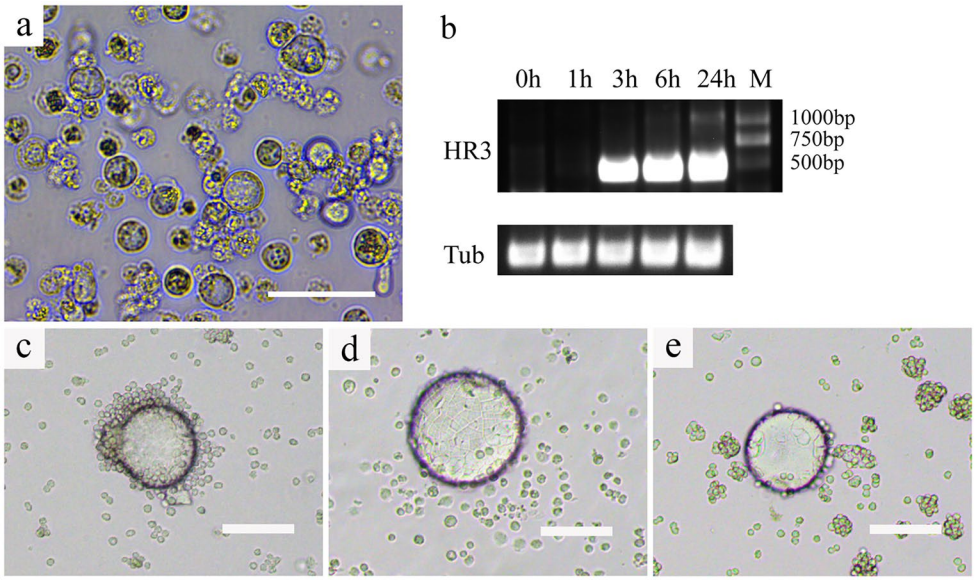

Fig. 6 **a** Cytopathology of Myse-1 infected with AcMNPV showing presence of occlusion bodies. Typical cytopathogenic effects such as enlarged nuclei with numerous occlusion bodies formed in the nuclei at anaphase of infection were observed. Scale bar = 10 µm. **b** After treatment with 20-hydroxyecdysone for 3 h, the expression of *HR3* gene was detected by semi-quantitative RT-PCR, and the results showed that expression level increased steadily. The amplified band by the designed *HR3* primer was 412 bp, and the primer Tub was used as the internal reference gene. Scale bar = 50 µm. **c** *M. separata* hemocytes had a strong wrapping effect on the beads. Scale bar = 100 µm. **d** *M. separata* primary cells had a weak encapsulation effect on the beads. Scale bar = 100 µm. **e** Myse-1 cells had a weak encapsulation effect on the beads. Scale bar = 200 µm

Discussion

Stanley and Vaughn (1968) argues that cell source is often intertwined with the optimal donor age and is a stage that contains the most qualified cells (Stanley and Vaughn 1968), but Mitsuhashi (2002) mentions that the ovarian developmental stage is not an important source of primary cells (Mitsuhashi 2002). So far, many insect cell lines have been established using pupal ovaries, mostly from Lepidoptera insects. At present, many ovarian cell lines have been established using *B. mori*, but most of them come from the ovary of larvae (Grace 1967; Khurad et al. 2006, 2009; Pan et al. 2010; Quiot 1982; Sudeep et al. 2002) rather than pupa. Although *B. mori* is a model insect in Lepidoptera insects, the ovary culture of silkworm was used in the early stage of cell line establishment in vitro, but the optimal development stage of donor insects was not typical. According to Stanley and Vaughn's research (1968), ovaries of the *B. mori* begin to develop in the mature larval stage, the ovary developed fully in the middle and late pupae, and most of the developing cells degenerate after yolk deposition, which makes it difficult to culture cell lines successfully when the ovary has been fully developed (Stanley and Kirkland 1968). Therefore, we believe that the ovaries of silkworm larvae may be more suitable for culture, whereas, unlike the silkworm imago which rarely feed, *M. separata* adults require replenishment to facilitate ovarian development and reproduction. This characteristic survival mode restricts maturation of ovaries in the pupal stage of *M. separata*. Ovaries of pupae used for establishing the new cell line were established because they develop significantly through frequent mitosis at this stage. Although different life habits of *M. separata* and *B. mori* lead to the use of ovaries at different development stages of insect for in vitro culture, their ovarian development is similar to the cell types used for successful culture. This is consistent with Stanley and Vaughn's study, so the stage of donor development should be selected according to the developmental characteristics of the ovary.

Mitsuhashi believed that oocytes cultured in ovaries neither migrate nor proliferate (Mitsuhashi 1989), which is consistent with our observations. In addition, we also found that oocytes and nurse cells did not dissociate or proliferate in vitro due to the entrapment of the basement membrane. In *D. melanogaster*, the basement membranes (BMs), also known as basal lamina (Fessler and Fessler 1989), are composed of self-assembled collagen IV (Col IV) and laminin networks essential for the stability of BM. Nidogen/entactin and perle can crosslink laminin and Col IV network, and increase its stability and control the structural integrity of the whole membrane. In adult ovaries of *D. melanogaster*, somatic follicular cells produce Col IV and assemble into oocyte BM network via Crag/Rab10/PI-dependent mechanism (Van De Bor et al. 2015). Previous studies have reported that primary cells from young ovaries originated from

follicular epithelium or the intermediate cells (Grace 1958; Jones and Cunningham 1960; Mitsuhashi 1989; Stanley and Kirkland 1968), but the definition of follicular epithelium and lumen cell types in lepidopteran ovary is still inconsistency and confusing. Compared with *D. melanogaster*, there are only a few reports on the development of ovarian follicular epithelial cells in lepidopteran. Most studies have explored the role of follicular epithelial cells in vitellogenesis and eggshell formation. In this study, the definition and location of follicular epithelial cells of *M. separata* were determined with reference to the internal morphology of other lepidopteran insects, such as *B. mori* (Stanley and Kirkland 1968; Yamauchi and Yoshitake 1984), *Diatraea saccharalis* (Santos and Gregorio 2002), *Diatraea saccharalis* Fabricius (Santos and Gregorio 2006), *Spodoptera frugiperda* (Alves et al. 2014), *Pieris napi* (Mazurkiewicz-Kania et al. 2019), and also based on the follicular cell development of *D. melanogaster* (Michael 1974; Büning 1994) and mosquitoes (Mazurkiewicz and Kubrakiewicz 2008).

Tunica extema refers to the extracellular material located between the outer sheath of the ovariole and follicles (Cruickshank 1973). Follicular epithelial cells are cells located inside the tunica propria, covering the combination of nursing cells and oocyte. The studies using somatic cell development of *D. melanogaster* ovary has shown that it is difficult for follicular epithelial cells to go through mitosis after stage 6 (Duhart et al. 2017; Finegan et al. 2019; Kolahi et al. 2009; Peterson et al. 2015). Cuboidal epithelium cells become polyploid in stages 7–10 and are referred as the main epithelium. The main epithelium subsequently underwent morphological changes and migration, forming cuboidal epithelium over the oocytes and squamous epithelium over the nurse cells (stage 9) (Dobens and Raftery 2010). In this study, follicular epithelial cells were found inside the ovarian sheath, and the protective layer of tunica propria was not destroyed during culturing of cells. In addition, the early pupae were used for in vitro culture, and only lumen cells were isolated after ovarian adherent culture. At this time, the follicular epithelial cells were not differentiated. Therefore, based on the above two reasons, we believe that the isolated primary cells are not follicular epithelial cells. In a study of *B. mori*, immunocytochemistry experiments have also showed that BmHR3 existed in the nuclei of follicular epithelium, i.e. the cells where the *ESP* gene is expressed, rather than in the trophoblasts or the oocyte in *B. mori* (Eystathioy et al. 2001; Swevers and Iatrou 2003). However, the follicular epithelial cells referred to in *B. mori* seem to refer to the cells located on tunica propria on the ovarian basement membrane, and we believe that these cells are evenly distributed muscle cells, rather than columnar epithelial cells wrapped in oocytes or squamous epithelial cells wrapped in nurse cells. In this study, after 20E stimulation of Myse-1, the ecdysone-responsive early gene *HR3* was up-regulated.

Lumen cells are simply described in several insects. The cells in the germarium and vitellarium of the ovarioles sandwiched between tunica extema and tunica propria are called lumen cells (*Hyalophora cecropiu*) (King and Aggarwal 1965; Santos and Gregorio 2002), intermediate layer cells (*B. mori*) (Stanley and Kirkland 1968), hemocytes (*Solenobia triquetrella*) (Brunold 1957), or macrophages (*Ephestia Kiihniella*) (Cruickshank 1973; Salt 1967). The study of Salt (1967) in the *Hyalophora cecropiu* has shown that these cells were thought to come from hemocytes which have penetrated the sheath via the pores (Salt 1967). Cruickshank (1973) believed that the lumen cells may come from the cells between the sheath and the tunica of the germarium, and found that they had phagocytosis (Cruickshank 1973). Nakahara et al. (2010) used the hemocyte precursors in the hematopoietic organ (HPO) in vivo and in vitro culture system and found that HPO of *B. mori* could release granulocytes, plasmatocytes, and oenocytoids, in which plasmatocytes had the potential to differentiate into oenocytoids (Nakahara et al. 2010). Spindle-shaped plasmatocytes could produce encapsulation reaction. Circular granulocytes have the function of phagocytosis and encapsulation in response to exogenous material stimulation. Both have adhesive ductility. Both have adhesive ductility. Although the isolated primary cells were spindle-shaped and round, which were consistent with the above two in morphology, our results showed that compared with hemocytes, primary cells and cell lines had poor encapsulation characteristics. Studies have also shown that lumen cells are macrophages and have the function of secreting substances to repair tunica propria at its torn points caused by ovariole growth to the moment of egg deposition (Cruickshank 1973). Therefore, the relationship between lumen cells and hemocytes deserves further study.

The lumen cells are not fixed and can move in and out of the two layers (Fig. 1d). In addition, after tissue adherent culture for a period of time, lumen cells continue to dissociate from the incision, whereas follicular epithelial cells are wrapped in the tunica extema and do not migrate out of the tunica extema. Furthermore, morphological analysis showed that isolated cells had characteristics of epithelial cells rather than those for fiberblastoid cells. Therefore, the primary cells are not follicular epithelial cells. Due to lack of molecular markers in non-model organisms, some easy-to-use molecular markers should be developed to quickly identify sources and distinguish these cells from other cells. In addition, lumen cells and intermediate cells described the same kind of cells, but different from interstitial. The interstitial cells derived from mesodermal cells exist in the germarium, encasing the germline stem cells (oogonia), cystoblasts, and oocytes. Interstitial cells develop from some

somatic cells intermingling with germ cells in early ovary anlagen. The proliferation of intestitial cells is rarely found.

Based on the morphological findings, we speculate that the continuous increase of primary cells observed in the early stage of culture was divided into two steps or a mixture of both cases. Firstly, with the increase of culture time in vitro, some cells continued to migrate out of the tissue, resulting in the observed increase of free cells. This feature can be attributed to the contraction and convulsions of skeletal muscles surrounding each ovariole, resulting in extrusion of lumen cells between the outer ovariole sheath and inner tunica propria from the ruptured site. Ovarian contraction can sometimes last for more than one month in vitro. Mitsuhashi (2002) mentioned that the migrated and proliferated cells had different sizes, and the initial proliferation rate was usually low. Some migrated cells may proliferate through mitosis and form colonies of multiplied cells (Mitsuhashi 2002). However, we believe that adhesion of extracellular matrix can make cells together and form clumps. We believe that the second visible increase in free cells is the cytoplasmic division of migrating cells. The DNA synthesis of cells in vivo have been doubled before culture, and the free primary cells that had passed the G2/M checkpoint would continue to divide in vitro. However, the result of reduced hormones or cytokinesis in the culture environment is that the next DNA proliferation will not take place, and cells will enter the quiescent phase (G_0), but will not die soon. Proliferation does not continue after these cells were directly transferred of into a new culture flask. Then, they gradually differentiate or undergo apoptosis and die.

There are many types of cells in the anterior germarium of the ovariole, including oocytes, cystoblasts, as well as cells that are differentiating or in a pre-differentiated state, such as interstitial cells and pre-follicular cells. These types of cells, such as pre-follicular cells and stem cells, reportedly retain the characteristics of embryonic cells in a state of rapid proliferation and division (Büning 1994). So far, the number of primordial germ cells invading the ovary has been low in all stem cells studied (Büning 1994). However, the few stem cells have a limited mitotic differentiation program, which results in a fixed number of oocytes per ovariole. Stem cells rest during reproductive life time, and their differentiation and mitosis produce siblings, one of which behaves as the stem cell and the other one is as the cystoblast (Büning 1994). Each cystoblast undergoes a genetically fixed synchronous division cycles, which leads to the incomplete cytokinesis to form interconnected cells clones (King and Biining 1985). There is always continuity between these nests and some smaller nests or individual cells in the germarium. It is difficult to separate them from interstitial cells and somatic cells. Fewer tools available to mark specific cells in lepidopteran insects, coupled with fewer cells, make it difficult to isolate and culture. Therefore, whether these stem-like cells are the source of our cell line remains to be further determined.

The ability of insect cells to establish cell lines is limited by the poor repeatability of primary cell transformation. In this study, the survival and division ability of primary cells in the hypoxia group after the first subculture was stronger, but there was no solid evidence to support the phenomenon that environmental hypoxia lead to cell immortalization. It needed to be further verified and illustrated. Moreover, this method does not affect the infection of the established cell line to the nucleopolyhedrovirus, and the new cell line can also react to hormones.

An ideal gas environment is a necessary condition for cell survival in vitro. Unlike mammals cultured in carbonate buffer system, invertebrate cells with medium using a phosphate buffer system do not need carbon dioxide (CO_2). Insect cells are cultured in a sealed cell culture flask under atmospheric oxygen environment (Lynn 2002; Mitsuhashi 2002). In our recent study, we found that hypoxic stress may lead to immortalization of insect cells. This phenomenon was first discovered in the establishment of *S. exigua* cell lines, and later similar experiments using *H. armigera* also showed consistent results (unpublished data). The exploration of stress conditions such as the severity and duration of hypoxia and their correlation with the success rate of establishing cell lines will be described elsewhere. But the reason is unclear. Cells need to receive and interpret extracellular signals to regulate cellular responses such as proliferation, survival and differentiation. Hypoxic microenvironment is an important factor in tumor formation and transformation of tumor cells from aerobic metabolism to anaerobic glycolysis (Hanahan and Weinberg 2011). What is the relationship between hypoxia and cell immortalization in insect cell culture, and the process of hypoxia promoting cell immortalization, needs more research to reveal its intrinsic link and molecular mechanism. Therefore, future in vitro studies may benefit from a series of physiologically related oxygen concentrations and various cellular responses in tissues (Ferguson et al. 2018). Notwithstanding its limitation, this study does suggest that this hypoxic induction method has the potential to be a new generation of establishing non-model insect cell lines.

In summary, this study provides a new reference approach for the establishment of ovarian cell lines (Fig. 7). In addition, we further refined the results reported by Stanley and Vaughn (Stanley and Kirkland 1968), indicating that lumen cells and follicular epithelial cells are two different types of cells, and lumen cells are the main source of primary ovarian cells. Moreover, the immortalization process of the isolated lumen cells was explored, and a cell line derived from the pupal ovaries of *M. separata* was established. The cell line comprised smaller tetraploid cells. Moreover, the newly

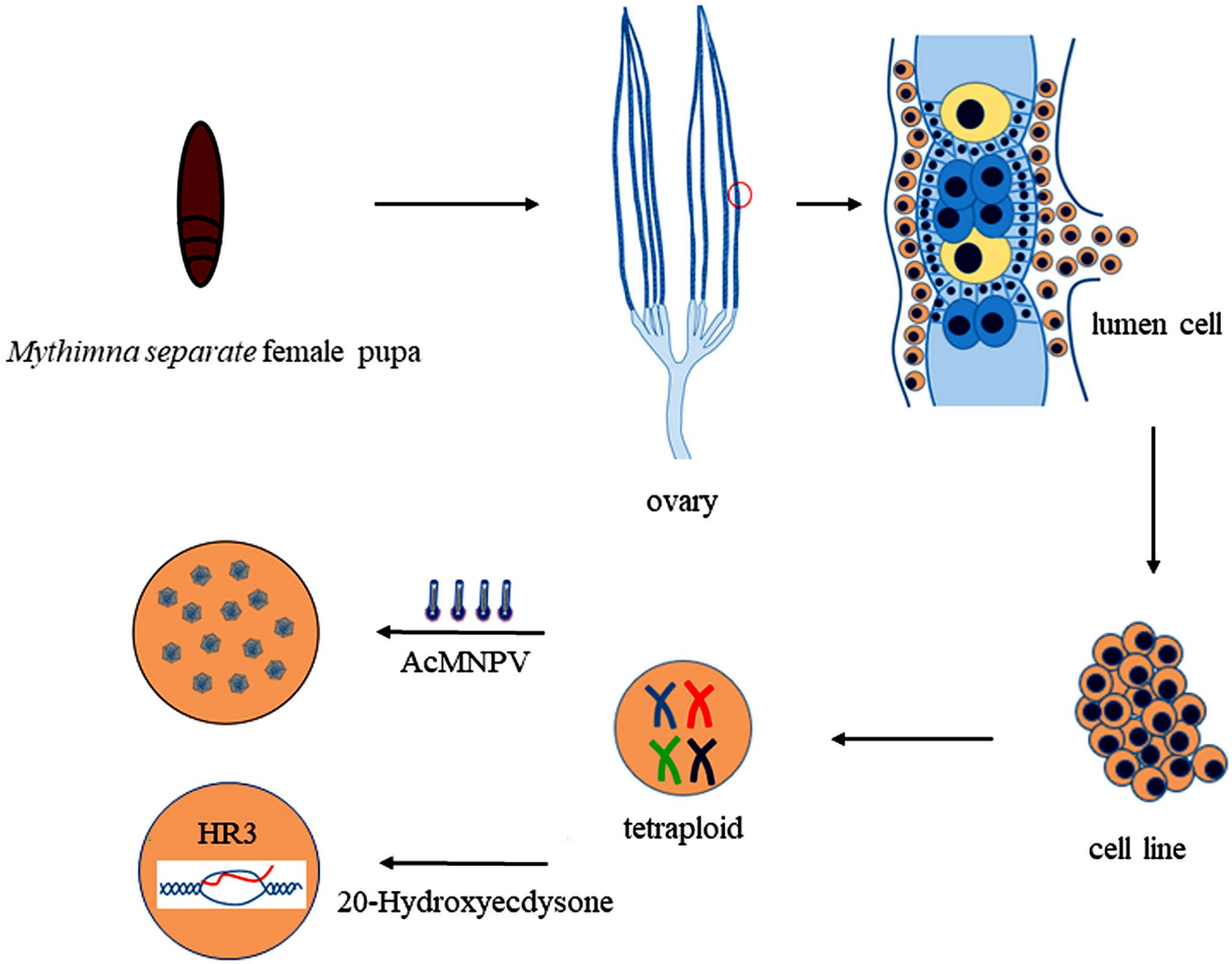

Fig. 7 The process of establishing ovarian cell line from pupae of *Mythimna separata*

established cell line is sensitive to the infection of AcMNPV, responds to 20-hydroxyecdysone and has weak encapsulation ability, implying that it can support large-scale production of certain baculoviruses or can be used as a vector for insecticide screening.

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Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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