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ISOLATION OF STEM CELLS FROM THE MID GUT EPITHELIUM OF *CULEX PIPIENS* MOSQUITOES (DIPTERA: CULICIDAE)

By

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Abstract

Stem cells are undifferentiated cells that remarkable ability to self-renew and produce differentiated cells that support normal development and tissue homeostasis. The authors isolated spherical stem cells from the midgut epithelium of the late third larval instars of *Culex pipiens* mosquitoes. *In vitro*, cultivated epithelial stem cells were able to proliferate and differentiate in fresh culture for 48 hr in absence of 20- hydroxyecdysone (20-E). The stem cells were the most common cells in the initial culture. One hundred of epithelial stem cells had been cultured. After 24 hr became 132 cells, 78% was undifferentiated stem cells and 8.3% &13.7% was enterocytes and goblet cells respectively. This ratio slightly decreased after de 48 hr to be 74% undifferentiated stem cells, 4.5 % enterocytes and 5.6 % goblet cells. After 72 hr the undifferentiated stem cells were not able to proliferate .The most differentiated goblet and enterocytes survived few days then died.

Keywords: Midgut, Stem cells, Tissue culture, Mosquitoes**.**

Introduction

Culex (*Culex*) *pipiens* L. 1758 (Diptera: Culicidae), the northern house mosquito, is a common pest in urban and suburban areas and one of the most widely distributed mosquitoes in the world (Vinogradova, 2000). The recent spread of West Nile virus has focused attention on this mosquito, which is considered one of the principal vectors of the disease (Turell *et al*, 2001). *Culex pipiens* is also a vector of St Louis encephalitis (Beaty and Marquardt, 1996), Japanese encephalitis viruses (Weng *et al*, 2000), Rift Valley fever (Turell *et al*, 1996) and *banchrofti Filariasis* (Soliman, 1995). Understanding vector-pathogen interaction is fundamen-tal to the development of disease control strategies.

The mosquito midgut epithelium is a simple monolayer of polarized columnar epithelial cells throughout which other cell types are occasionally interspersed (Billingsley, 1990). Although the cellular composition of the midgut epithelium is currently controversial (Shahabuddin, 2002), probably only three morphologically distinct cell types are present: columnar epithelial cells (enterocytes), endocrine cells and regenerative cells (Baton and Ranford-Cartwright, 2004, 2005). Regenerative cells are small pyramidally shaped cells sparsely scattered throughout the basal region of the midgut epithelium, between adjacent midgut epithelial cells, which terminate within the basal half of the midgut epithelium (Hecker *et al*, 1971; Hecker, 1977). By analogy to morphologically similar cells found in the midguts of other insects, regenerative cells are presumed to be a population of un-differentiated precursor stem cells capable of proliferating and developing into functionally mature midgut epithelial cells (Hecker *et al*, 1971). However, despite their nominal description, the function of so-called ‘‘regenerative’’ cells are unproven: no evidence has previously been published that these cells are mitotically active within the midgut epithelium of mature adult female mosquitoes (Hecker *et al*, 1971; Hecker, 1977; Weaver and Scott, 1990).

In case of most insect lines, cell cycle parameters have not been systematically

examined. An exception is the *Drosophila* KC cells, which have a generation time of about 19 h. In KC cells, G2 was the longest phase of the cell cycle while G1 was short (Dolfini *et al*, 1970). In contrast, in *Aedes albopictus* C7-10 cells, G1 was the longest cell cycle phase (duplicated) after low dose (10^{-6}M) treatment with 20-E. Overall proliferation of C7-10 cells was inhibited (Gerenday and Fallon, 1996). Using flow cytometr, they verified that over a 20 hour period a decrease in the number of the proportion of cells in S within 4-6 after exposure to 20 E, a transient increase in G2 was followed by the accumulation of more than 70% of the cell in the G1, consistent with the decrease in the proportion of cells in S and G2 (Gerenday and Fallon, 2004).

Jiang and Edgar (2009) provide evidence that adult midgut precursor (stem) cells in larval *Dr. melanogaster* are stimulated to proliferate by an epidermal growth factor receptor (EGFR) signaling pathway. In the early larval instars, the surrounding midgut visceral muscles produce the protein Vein, a weak EGFR ligand, which stimulates a low level of adult midgut precursor cell proliferation via a paracrine pathway. Subsequently, two other ligands, Spitz and Keren, are synthesized by the stem cells themselves; these provide autocrine signals that augment the paracrine signal. The combination of signals leads to a higher level of proliferation in the late larval period.

The present approach was based on the ultra structural observations had been made by Baldwin and Hakim (1991). They stated that stem cells are not attached to other cells by separate junctions and that they rapidly proliferate to become the major cell type in the midgut early in the molt. In contrast, mature goblet and the enterocytes remained anchored to one another by separate junctions at this time. So when pieces of midgut from *pharate* fourth-stage larvae were cultured *in vitro* for approximately one week, stem, goblet and columnar epithelial cells tended to fall away from the muscle and tracheal framework to lie in a random distribution on the bottom of the culture vessel. In mixed culture, in the presence of the insect molting hormone 20-E and of growth factors generated by co-cultured fat body, stem cells in the culture entered mitosis and differentiated into new columnar and goblet cells (Sadrud-Din *et al,* 1994; Loeb,1994).

This study aimed to describe isolation and culture of stem cells from the midgut of *Cx. pipiens.* Also, to investigate if the midgut stem cell can multiplicate *in vitro* in absence of 20-E and soluble fat body factors.

Material and Methods

Mosquito sample collection: *Cx. pipines* Mosquito samples were obtained from breeding habitat in Suez Governorate. Mosquito larvae were collected by sampling from various larval habitats and reared in sectary to obtain adults for morphological identification using taxonomic keys (Gad, 1963; Harbach, 1985).

Insect dissection: Before Dissecting the third larval instars, bench sterilized by soap, ethanol, & sodium hypochlorite and the larvae surface sterilized by immersing 2 min each in aqueous solutions of 20% detergent (Tween, Promega) and 0.1% p-hydrobenzoic acid methyl ester (Sigma, St Louis, MO). Third Larval instars were then transferred to the sterile water and dissected (Fig.1) immediately (Sadrud-Din *et al*, 1996).

Cells isolation: Midguts were isolated from larvae using the dissecting solution (NaCl 130 mM, KCl 4.7 mM, and CaCl2 1.9 mM, pH 7.2) by means of pins, the end of the abdomen removed. The midgut remains attached to the head and can be cut and allowed to fall into a dish. If the peritrophic membrane protrudes from one end, it can be pulled out with contents. The guts twice rinsed with dissecting solution and LPS (NaCl 178 mM, KCl 4.3 mM, CaCl2 4.3 mM, NaHCO3 3.8 mM, 0.5% gentamicin, 0.01% antibiotic–antimycotic solution, pH 6.5). The midguts placed in a plastic dish

(50-mm diameter) filled with LPS after cutting into small pieces then stirring once and then let sit 2-10min. Midguts transferred to another dish and let sit 2-3min, the same step repeated again. The midgut epithelium does not adhere to the plastic bottom of tissue culture dishes. Consequently, adherent cells such as muscle or Phagocytes are left behind when cells are transferred from one dish to the next. Stem and mature cells generally lie immediately above the bottom of the base of the dishes, then added 70-µm-pore-size filter basket in 50-mm Petri dish and filled half way with LPS, then midgut added and stirred for 1–2 h on an orbital or rotating stirrer.

Cell cultivation: The filter basket removed from the Petri dish, placed in media and freed cells into sterile conical bottom test tubes, then centrifuged at 400×g for10 min. Decant LPS, washed with 1-ml fresh LPS, stirred tube to distributed cells in LPS, and then centrifuged again. Repeated, but this step for the second time and used fresh growth medium in absence of 20-E and the growth factor generated by co-cultured fat body. By gentle manual swirling of the plate every 15 min over a 45-60 min period, loosely associated stem cells were liberated into the medium. The gentler the swirling, the fewer non-stem cells were released. Non-stem cells comprised less than 0.5% of the free cells observed after this process. Intact tissue fragments were then removed from each well using forceps. Free cells were centrifuged at 400g for 5 min and, after removing the supernatants, cells were resuspended in approximately 10 ml of culture medium and distributed evenly into the several wells used in each experiment. The final volume of culture was 500µl. Cultures were incubated at 25°C in an air environment, and counted at zero time and 24 h intervals using inverted microscope (Sadrud-Din *et al*, 1996).

Results

The media that have been developed was good and allowed us to isolate the stem cells from the midgut epithelium of the *Cx. pipiens* mosquitoes. The midgut stem cells were able to proliferate and differentiate to enterocytes and goblet cells, which closely approximate these same processes in vivo (Fig. 2). Conditioned growth medium was not used, 20-E or fat body growth factor. One hundred undifferentiated stem cells cultured in a fresh growth medium were to investigate if factors released from the midgut tissue fragments would be enough to proliferate and differentiate these cells. The authors' knowledge of stem cell functions and controls also derived from studies of stem cells systems in cultures *in vitro* (Hakim *et al*, 2010) and Transmission Electron Microscope examination of the midgut epithelium (*in vivo*) of the third larval instars of *Cx. pipiens* (Wassim *et al*, 2014 in press). After 24 h the same culture was containing 132 cells, 103 were un-differentiated stem cells as well as differentiated 11 enterocytes and 18 goblet cells. This meant that proliferation of 100 stem cells proceeded quickly through the first 24 h with division rate 35%. The total no. of proliferated stem cells became 8.3% enterocytes and 13.7% was goblet cells. After the second 24 h, the proliferation and differentiation of stem cells of the culture slightly decreased to be 4.5% enterocytes and 5.6% goblet cells (table 1). Proliferation of stem cells proceeded very slowly after 72 h. The percentage of differentiated each type of the proliferated and differentiated cells was zero. The differentiated enterocytes and goblet cells survived a few days then died. Undifferentiated stem cells fluctuated between 74 %and 78% of the total no. of cells and approximately equal the primary cultivated stem cells numbers (Tab.1).

Larval enterocytes and goblet cells cultured in vitro recognized by their mor-phological similarity to their counterparts in vivo (Fig. 2). The goblet cells are elongate cells with a large central vacuole. The goblet cells have a characteristic pear shape that

permits their identification. The enterocytes have a brush border at its apical surface and in folded plasma membrane at its base. In midgut, the stem cells lie among the bases of the goblet cells and enterocytes and above the basal lamina. They are not tightly bound to the goblet or enterocytes with mechanical strength. The stem cells are spherical in shape and have central nuclei. The stem cells are the most common cells in the initial culture (Fig. 2). The most differentiated goblet and enterocytes survived few days then died. Other remained as stem cells.

Table 1: Investigating, counting and differentiating of isolated cells from midgut of *Culex pipiens* larvae during different time interval

Time interval	Total # of cells	Type and numbers and % of total isolated Cells				
		Stem cells			Columnar cells	Goblet cells
Post Cultivation		Total # of stem Cells	*D. stem Cell #	Und. Stem Cell #	Columnar Cell #	Goblet Cell #
24h	132	103(78%)	-	103(78%)	11(8.3%)	18 (13.7%)
48 h	178	160(89.88%)	27 (15.17%)	133(74.73%)	8(4.5%)	10 (5.6%)
72 h	150	150(100%)	81(54%)	69 (46%)	-	-
96 h	13	13(100%)	13(100%)	-	-	-
1 week	71	71(100%)	61(85.9%)	10(14.1%)	-	-

*D. Stem = divided stem cell, Und. Stem = undivided stem cell

Discussion

The midgut, the second largest organ in the insect body, is the entry site for harmful bacteria, viruses, and toxins, as well as for food and water. This organ consists of a simple or pseudostratified epithelium, which is supported by a basement membrane, striated muscle, and tracheoles. For holometabolous insects, the structure and function of the midgut vary by stage of development. Growth, development and cell death are coordinated by endocrine and paracrine controls. Understanding of these processes and their implications for insect biocontrol is essential (Hakim *et al*, 2010). Stem cells are responsible for mid gut growth and development and cell death are coordinated by Paracrine and endocrine. The cell contact -based regulation of stem cell activity determines how much growth and differentiation can occur. There are unipotent stem cells exhibit limited capabilities to the formation of one cell type. Stem cell of the insect mid gut give rise to larval goblet and enterocytes when grown in larval culture conditions (Loeb and Hakim, 1996). The results showed that cultures enriched to approximately stem cells gave rise to few mature goblet cells 8% and enterocytes 13.7%. Spread out cells was the earliest indication of differentiation of either cell type. The presence of spherical cells with a neck region preceded the appearance of a mature goblet cell.

The ratio of undifferentiated stem cells remained approximately constant through the 48 h post cultivation. The results agreed with Morrison *et al*. (1977) who explained that stem cell function as a reservoir of cells usable for growth or repair where large numbers of cells need to be generated quickly a "symatric" pattern of growth occurs in which stem cells divided into two daughters. Both of which can then divided. In this growth process, the stem cell number increases geometrically. The number of cell produced is two. At the other extreme is an asympatric pattern of cell division where after each division one daughter begins the bath toward differentiation and the other remains a dividing stem cell. In this developmental scheme the number of the stem cells remains the same. Another explanation for the constant ratio of cultivated stem cells is the growth factors released from the tissue fragments conditioned the growth medium. These factors were required for proliferation and differentiation of the cultivated stem cells. Stem cells, detached from the tissue

fragments, began to proliferate and differentiate into new goblet and enterocytes. Baton and Ranford-Cartwright (2007) verified that in case of injuring the midgut epithelium, enterocytes rupture, releasing its contents though an apocrine process. These secreted contents include cell organelles and the growth factors. At the same time, the midgut stem cells proliferate to replace the damaged cells. If few midgut enterocytes die, the midgut can heal and a new enterocytes formed *in vivo.*

However, the present study did not use 20-E and the growth factor, the midgut epithelial stem cells were able to proliferate and differentiate *in vitro*. Our results came together with Gerenday and Fallon (2004) who revealed that the 20-E has no effect on the growth of plated cell line C7-10 from *Ae. albopictus* after 24 h .The no. of treated cell line with 20-E (10^{-6} M) depressed after 2 days relative to controls. Previous studies (Gerenday and Fallon, 1996) using autoradiography showed that by 10-12 h after treatment with 20E, the percentage of cells in the S phase of the cycle decreased sharply. Gerenday and Fallon (2004) verified these observations using flow cytometry. Cells under various treatments were collected from suspension cultures at 2 h intervals for 20 h, fixed, and processed for flow cytometry as the G1 and G2 peaks decreases noticeably in the 20E-treated cells, relative to controls. In control cells, S was maintained at a steady level of approxima-tely 40% and G1 was maintained at about 50%. The remaining approximately 10% of the cells were in G2/M. The proportion of cells in S declined sharply within 4-6 h after 20E treatment and by 7 h after treatment, the proportion of cells in G2 showed a transient compensatory increase, suggesting that a proportion of the cells underwent mitosis after exposure to 20E. By 12 h after 20E treatment, more than 70% of the cells were in G1 and only 15% of the cells were in S. During the next 8 h, we noted little change in the proportion of cells in S and G1. The substantial decline in the proportion of cells in S by 12-15 h after 20 E treatments would be associated with a decreases in cyclin A. The progression of the cell cycle is regulated by several proteins, including cyclins, cyclin A- dependent kinases, and kinase inhibitory proteins. Homologous of many of these signaling molecules have been described in *Drosophila* (Edgar and Lerner, 1996).

In Lepidoptera, larval growth is episodic. Midgut epithelial stem cells rapidly proliferate immediately prior to each molt (Baldwin and Hakim, 1991). They differentiate into enterocytes and goblet cells during the molt while intercalating between polyploidy, the previously matured enterocytes and goblet cells enlarge the midgut. After the last larval molt, insects enter metamorphosis (Baldwin *et al*, 1996; Hakim *et al*, 2001). In this new environment, stem cells differentiate into a simple cuboidal epithelium (Hartenstein, 1993; Tettammati *et al*, 2007; Uwo *et al*, 2002). Within primary cultures, these events occur simultaneously and result in a semi stable balance between stem cell proliferation, differentiation to goblet, enterocytes and death of these mature cells (Loeb and Hakim, 1996). The mature cells, which possess maturation factors, normally do not die in vivo to release these factors. Rather, other mechanisms for release of these factors must occur (Goto *et al*, 2001).

The present results approved that the isolated stem cells are mitotic ally active within the midgut epithelium of *Cx. pipiens* mosquitoes *in vitro* and entered mitosis. These stem cells were able to proliferate and differentiate into new enterocytes and goblet cells through 24-48 h post cultivation in fresh growth medium without using 20-E or fat body growth factor. Hakim *et al.* (2010) stated that, it is unclear how one can mesh the molecular studies on *Dr. melanogaster* with studies on Lepidoptera cell cultures. Whereas ecdysteroids and -arylphorin could be driving the gene activations seen in the fly. Paracrine factor released by adjacent

muscle cells are not present in the primary midgut cultures and have not been studied in culture systems. Furthermore, larval *Dr. melanogaster* adult midgut precursor cells do not increase their rate of proliferation prior to each molt (Jiang and Edgar, 2009) as do Lepidoptera. Clearly, more data are needed before a complete picture of the factors controlling stem cell proliferation in the larval midgut. Local-acting growth and differentiation factors have been identified and purified from condition medium CM(Midgut Differentiation Factors, MDF1 and MDF2) and from hemolymph of *L. dispar* (MDF3 and MDF4) (Loeb and Jaffe, 2002; Loeb *et al*, 1999). They were identified by ability to replace CM in purified stem cell cultures. Although the site of synthesis of the MDFs has not been identified in vivo, antibodies to MDFs stain larval enterocytes in vivo and in vitro, suggesting molecules with similar epitopes are present in the cells (Goto *et al*, 2001; Loeb *et al*, 2004).

Conclusion

The isolated mid gut epithelial stem cells of *Cx. pipiens* are mitotically active *in vitro*, able to proliferate and differentiate into new enterocytes and goblet cells through 24-48 h post cultivation without using 20-E or fat body growth factor. Extensive work on cell cycle machinary of the Egyptian mosquito species, which genes are regulating this cycle is ongoing to understand the role of cyclins A and B in progression of mosquito cell cycle.

References

Baldwin, KM, Hakim, RS, 1991: Growth and differentiation of the larval midgut epithelium during molting in the moth, *Manduca sexta*. *Tissue Cell* 23:411-22

Baldwin, KM, Hakim, RS, Loeb, MJ, Sadrud-Din, SY, 1996: Midgut develop-ment. In: Biology of the Insect Midgut, ed. MJ Lehane, PF Billingsley. London: Chap-
man and Hall

Baton, LA, Ranford-Cartwright, LC, 2004: *Plasmodium falciparum* ookinete invasion of the midgut epithelium of *Ano-pheles stephensi* is consistent with the Time Bomb model. Parasitol. 129:663-76.

Baton, LA, Ranford-Cartwright, LC, 2005: How do malaria ookinetes cross the mosquito midgut wall? Trends Parasitol. 21: 22-8.

Baton, LA, Ranford-Cartwright, LC, 2007: Morphological evidence for prolifera-tive regeneration of the *Anopheles stephensi* midgut epithelium following *Plasmodium falciparum* ookinete invasion. J. Invertebr. Pathol. 96:244-54.

Beaty, BJ, Marquardt, WC, 1996: The Biology of Disease Vectors. University Pre-ss of Colorado, Newt.

Billingsley, PF, 1990: The midgut ultra-structure of haematophagous insects. Annu. Rev. Entomol. 35:219-48.

Dolfini, S, Courgeon, AM, Tiepolo, L, 1970: The cell cycle of an established line of *Drosophila melanogaster* cells in vitro. Experientia 26:1020-1.

Edgar, BA, Lerner, CF, 1996: Develop-mental control of cell cycle regulators: a fly's perspective. Science 274:1646-52.

Gad, AM, 1963: Insects of medical impor-tance. Unpublished lecture notes (in Arabic); Research Institute of Medical Entomology, MOH. Cairo, Egypt**.**

Gerenday, A, Fallon, AM, 1996: Cell cycle parameters in *Aedes albopictus* mosquito cells. *In-vitro* Cell. Dev. Biol. Anim. 32: 307-12.

Gerenday, A, Fallon, AM, 2004: Ecdy-sone-induced accumulation of mosquito cells in the G1 phase of the cell cycle. J Insect Physiol. 50, 9:831-8.

Gordon, JI, 1993: Understanding gastro-intestinal epithelial cell biology: lessons from mice with help from worms and flies. Gastroenterology 104, 3:15-324.

Goto, S., Takeda, M, Loeb, MJ, Hakim, R S, 2001: Immunohistochemical detection of a putative insect cytokine, midgut differen-tiation factor 1 (MDF-1) in midgut columnar cells of *Heliothis virescens*. Invert. Reprod. Dev. 40:117-24.

Hakim, RS, Baldwin, KM, Loeb, M, 2001: The role of stem cells in midgut growth and regeneration. *In-vitro* Cell Dev. Biol. Anim. 37:338-42.

Hakim, RS, Baldwin, K, Smagghe, G, 2010: Regulation of midgut growth, deve-lopment and metamorphosis. Ann. Rev. Entomol. 55:593-608.

Harbach, RE, 1985: Pictorial keysto the genera of mosquitoes, sub-genera of *Culex* and the species of *Culex* (*Culex*) occurring in south-western Asia and Egypt,with a note on the sub-generic placement of *Culex deserticola* (Diptera: Culicidae). J. Mosq. Sys. 17, 2:83-107.

Hartenstein, V, 1993: Atlas of Drosophila Development: Cold Spring Harbor, NY: Cold Spring Harbor` Lab. Press.

Hecker, H, Freyvogel, TA, Briegel, H, Steiger, R, 1971: Ultrastructural differen-tiation of the midgut epithelium in female *Aedes aegypti* (L.) (Insecta, Diptera) imagines. Acta Trop. 28:80-104.

Hecker, H, 1977: Structure and function of midgut epithelial cells in Culicidae mosque-toes (Insecta, Diptera). Cell Tissue Res. 184: 321-41.

Illa-Bochaca, I, Montuenga, LM, 2006: The regenerative nidi of the locust midgut as a model to study epithelial cell differentia-tion from stem cells. J. Exp. Biol. 209:2215-23.

Jiang, H, Edgar, BA, 2009: EGFR signa-ling regulates the proliferation of *Drosophila* adult midgut progenitors. Development 136: 483-93

Loeb, MJ, Hakim, RS, 1996: Insect midgut epithelium in vitro: an insect stem cell syst-em. J. Ins. Physiol. 42:1103–11

Loeb, MJ, Jaffe, H, Gelman, DB, Hakim, RS, 1999: Two polypeptide factors that pro-mote differentiation of insect midgut stem cells in vitro. Arch. Insect Biochem. Physiol. 40:129-40.

Loeb, MJ, Jaffe, H, 2002: Peptides that elicit midgut stem cell differentiation iso-lated from chymotryptic digests of hemo-lymph from *Lymantria dispar* pupae. Arch. Insect Biochem. Physiol. 50:85-96.

Loeb, MJ, Coronel, N, Natsukawa, D, Takeda, M, 2004: Implications for the func-tions of the four known midgut differentia-tion factors: an immunohistologic study of *Heliothis virescens* midgut. Arch. Insect Biochem. Physiol. 56:7-20.

Micchelli, CA, Perrimon, N, 2006: Eviden- ce that stem cells reside in the adult *Droso- phila* midgut epithelium. Nature 439:475-79.

Morrison, SJ, Wandycz, AM, Hemmati, HD, Wright, DE, Weissman, IL. 1997: Identification of a lineage of multipotent hematopoietic progenitors. Development 124, 1929-39.

Ohlstein, B, Spradling, A, 2007: Multi-potent *Drosophila* intestinal stem cells specify daughter cell fates by differential Notch signaling. Science 315:988-92.

Park, MS, Takeda, M, 2008: Starvation suppresses cell proliferation that rebounds after refeeding in the midgut of the American cockroach, *Periplaneta america-na*. J. Insect Physiol. 54:386-92.

Sadrud, DS, Hakim, RS, Loeb, MJ, 1994: Differentiation of stem cells from the midgut of the insect *Manduca sexta*, occurs in vitro in the presence of a factor from mature larval midgut cells. *In-vitro* Cell Dev. Biol. Anim. 30A:106.

Sadrud-Din, SY, Loeb, MJ, Hakim, RS, 1996: In vitro differentiation of isolated stem cells from the midgut of *Manduca sexta* larvae. J. Exp. Biol. 199:319-25.

Soliman, BA, 1995: Comparative exsheth-ment of microfilariae of *Wucheraria ban-crofti* in certain mosquito species. J. Egypt. Soc. Parasitol. 25, 1:207-12

Shahabuddin, M, 2002: Do *Plasmodium* ookinetes invade a specific cell type in mosquito midgut? Trends Parasitol. 18:157-61.

Tettamanti, G, Grimaldi, A, Casartelli, M, Ambrosetti, E, Ponti, B, 2007: Programmed cell death and stem cell differentia-tion are responsible for midgut replacement in *Heliothis virescens* during prepupalinstar. Cell Tissue Res. 330:345-59.

Turell, MJ, Presley, SM, Gad, AM, Cope, S, Dohm, D, *et al*, 1996: Vector competence of Egyptian mosquitoes for Rift Valley fever virus. Am. J. Trop. Med. Hyg. 54:136-9.

Turell, J, O'Guinn, MJ, Dohm, DJ, Jones, JW, 2001: Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J. Med. Entomol. 38: 130-4.

Uwo, MF, Ui-Tei, K, Park, P, Takeda, M, 2002: Replacement of midgut epithelium in the greater wax moth, *Galleria mellonela*, during larval-pupal moult. Cell Tissue Res. 308:319-31.

Vinogradova, EB, 2000: *Culex pipiens pipiens* Mosquitoes: Taxonomy, Distribution, Ecology, Physiology, Genetics, Applied Importance & Control. Sofia-Moscow: Pensoft.

Wassim, NM, Soliman, BA, Husien, MI, Metwaly, HG, 2014: The role of epithelial stem cells in fate of peritrophic membrane in *Culex pipiens* mosquito (Diptera: Culicidae). In Press.

Weaver, SC, Scott, TW, 1990: Peritrophic membrane formation and cellular turnover in the midgut of *Culiseta melanura* (Diptera: Culicidae). J. Med. Entomol. 27: 864-73.

Weng, MH, Lien, JC, Lin, CC, Yao, CW, 2000: Vector competence of *Culex pipiens molestus* (Diptera: Culicidae) from Taiwan for a sympatric strain of Japanese encepha-litis virus. J. Med. Entomol. 37:780-3.

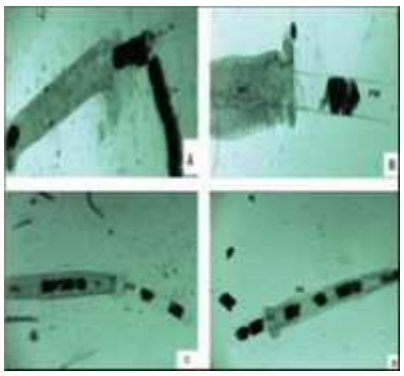

Fig 1: Light micrograph showing mosquito larval midgut dissection

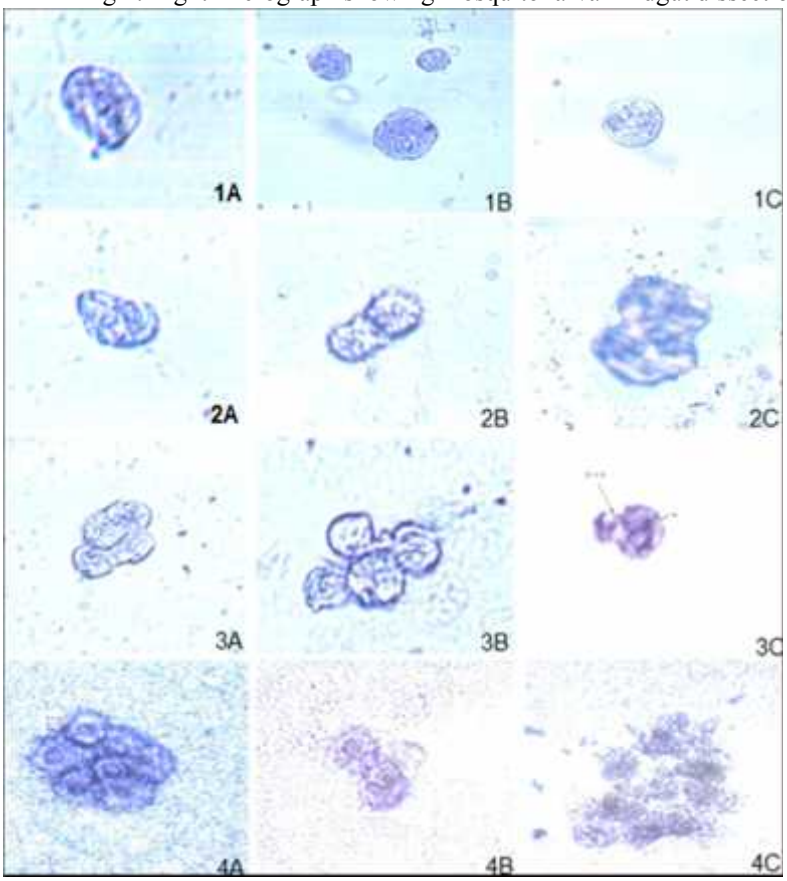

Figure 2: Inverted microscope images of stem cells cultivated from larval *Cx. pipiens* midguts (1B,1C) at 0h, (1A,2A, 2B, & 2C) 24h. Post cultivation, (3A, 3B, & 3C) 48h. Post cultivation, (4A, & 4B) 72hrs. Post cultivation, & (4C) 168h. Post cultivation. (1A & 1B 100X, rest 200X).1A: shows different midgut cells (rounded stem cells, columnar cells, pear-shaped goblet cells).1B: shows 3 stem cells (on right solitary cell, on left one stem cell entered division having 2 nuclei and inclusion on membrane, & last one enlarged stem cell with terminal nucleus). 1C: shows 1 enlarged stem cell with terminal nucleus through division. 2A: shows different midgut cells (stem cells solitary dividing). 2B: shows 1 stem cell (completely divided into 2 cells each with nucleus and membrane attached). 2C: shows 2 stem cells (each entered division, with 2 nuclei included in membrane). 3A: shows 2 stem cells began division (each with 2 nuclei in opposite ends, membrane inclusion increased to separate), 3B: shows cluster of 4 small stem cells (2 stem cells divided into 4 cells). 3C: shows 2 completely stem cells still attached (right one entered another division with 2 nuclei). 4A: shows cluster of 5 stem cells (3 with 2 nuclei each in opposite ends and membrane inclusion increased to separate & 2 cells solitary). 4B: shows 2 stem cells attached together (lower 1 entered division with 2 nuclei and inclusion in membrane). 4C: shows cluster of 8 stem cells.