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OVERVIEW OF MALPIGHIAN TUBULES DEVELOPMENT AND FUNCTION IN *DROSOPHILA MELANOGASTER*

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Introduction:

The Malpighian tubules are tubular structures that are free floating in the haemolymph and function as excretory and osmoregulatory organs of insects. These tubules function in the same way as human kidney and share common principles during development. The Malpighian tubules (MTs) are connected with their proximal ends attached to the alimentary canal at the junction between midgut and hindgut (Fig1). The Malpighian tubules are named after the Italian scientist Marcello Malpighi who is considered to be the 'Father' of both Histology and Comparative Physiology. Marcello Malpighi described numerous features of insects, including the Malpighian tubules.

In *Drosophila*, the hemolymph fluid enters the MTs at their distal end. This fluid flows within the tubule and empties into the insect hindgut. As the fluid flows through the tubule, the cells of the MTs take up useful nutrients from the fluid and transport the nutrients through the cell and into the hemolymph. Those substances that are toxic or unwanted stay with the urine for eventual excretion from the insect. The study of development of MTs during embryogenesis provides a useful system to investigate the basic mechanisms underlying epithelial morphogenesis and organogenesis.

Development of Malpighian tubules:

In *Drosophila*, the MTs development begins by budding from a single layered epithelium of hind gut primordium during embryogenesis, which is not invested with mesenchyme, and cease cell division relatively early in the development (Saker 1993). A number of genes have been identified which are involved in MTs development. The development of MTs is completed in different stages, such as:

(i) **Primordium specification:** The over lapping expression of genes like *tailless* (*tll*), *huckebein* (*hkb*), *forkhead* (*fkh*) and *wingless* during early embryogenesis from stage 5-10 at the posterior of the embryo, is required to establish and maintain the position of the proctodeum from which the tubules arise (Weigel *et al.* 1989, Gaul and Weigel 1990, Skaer 1993, Harbeck and Langyel 1995, Wu and Langyel 1998) (Fig 2).

(ii) **Bud evagination:** Two pair of MTs evaginate as buds at the junction of hindgut and posterior midgut under the influence of *Krüppel* (*Kr*) and *wingless* (*wg*) at stage 11 of embryonic development (Harbecke and Janning 1989, Skaer 1993). *Hedgehog* is also required for the completion of bud evagination (Hoch and Pankratz 1996) (Fig 2).

(iii) **Bud extension:** After bud evagination *Kr* regulates the expression of transcription factor Cut in a ring of cells in bud which results in cell shape changes. At the same time Kr, Cut and Wg plays an important role in directing the bud to extend cylindrically and become narrower both proximally and distally resulting in a crescent-shaped morphology during stage 12-13 (Fig 2).

(iv) **Tubule elongation:** After bud extension, a single cell is specified via the Notch pathway within the tubule primordial, called the tip cell. The tip cell expresses Kr and also secretes epidermal growth factor (EGF) which stimulates mitosis in neighboring cells and growth of tubules by addition of new cells. At the same time wingless is also required for cell division and morphogenesis of tubules (Skaer and Martinez 1992, Hoch *et al.* 1994, Herbecke and Lengyel 1995, Singh *et al.* 2007). At the end of stage 13 of embryonic development there are no further cell divisions and the circumference of each primitive tubule is of about eight cells (Janning *et al.* 1986, Skaer and Arias 1992). Once the cells stop dividing the tubular circumference is reduced to only two cells and the tubule enters the vicinity of the caudal mesoderm and interacts with a subpopulation of the caudal mesodermal cells (Fig 2 D). At this satge the surrounding mesodermal cells undergo a mesenchymal-to-epithelial transition and by the process of cell rearrangement, these mesodermal cells get recruited into the ectodermal bud cells at regular intervals which later form the stellate cells (SCs) (Fig 2 E) and the ectodermal cells form the principal cells (PCs). Later by tubule elongation the MTs are concomitantly extended further during stage 14-15 of embryonic development (Skaer 1993). The PCs and SCs also known as primary cells and secondary cells respectively and have their number consistent throughout life (Fig 1C). They are also known as type I and type II cells respectively. Thus, *Drosophila* MTs develop from two sources: hindgut primordium (ectodermal epithelia) and the visceral mesoderm (Denholm *et al.* 2003, Jung *et al*. 2005).

At stage 16, four renal tubules are formed with one pair of tubules spanning the posterior abdominal and terminal segment of embryo while the other pair extending forward into abdominal segments where the tubule loops back on itself so that the tips of both anterior tubules lie more posterior within the abdomen (Skaer 1993, Ainsworth *et al.* 2000, Delnholm *et al.* 2003, Jung *et al.* 2005). The mature tubules are made up of approximately 484 PCs and 110 ± 1 SCs divided in 4 tubules (Janning *et al*. 1986, Sozen *et al.* 1997). The number of PCs and SCs differ in anterior and posterior tubules.

In anterior tubules, number of PCs and SCs are 145 ± 0.9 and 33 ± 0.4 respectively while in posterior tubules the PCs and SCs number are comparatively less, which are 111 ± 1 and 22 ± 0.4 (Sozen *et al.* 1997).

Apart from theses two cell types, some other cells are chatercterized in the MTs, for example 'tiny' cells in the proximal part of MTs (including lower tubules and ureters) which could be homologs of myoendocrine cells of the ants *Formica* (Garayoa *et al.* 1994), which collect the urine in the renal duct and secrete neurohormones in the hemolymph (Sözen *et al.* 1997). Recent studies have shown that these tiny cells in the region of lower tubules and ureters might also be functioning as pluripotent adult stem cells and that an autocrine JAK-STAT signaling regulates the stem cells self-renewal (Singh *et al.* 2007).

Genetic mapping of tubule subregion by enhancer-trap analysis suggests that anterior and posterior MTs are divided into the initial, transition, main and lower segment and joined to the gut through the ureter (Fig 1 B, C) (Sozen *et al.* 1997).

Morphogenesis of Malpighian tubules :

Many genes are involved in complete morphogenesis of MTs. *hibris* (*hbs*) is expressed specifically in SCs during MTs development (Denholm *et al.* 2003). It is a member of immunoglobulin-like protein family and play role in cell-cell recognition and adhesion in myoblast fusion processes (Artero *et al.* 2001, Dworak *et al.* 2001, Dworak and Sink 2002). Therefore, it is believed that during MTs differentiation, Hibris mediates cell adhesion and rearrangement between PCs and SCs. Nephrin is the homolog of Hibris and expresses in the vertebrate kidney (Artero *et al.* 2001, 2006). CMS/CD2AP has been identified as an interacting partner of Nephrin in vertebrate. The CMS/CD2AP homologue in *Drosophila* is detected by computational analysis as CG11316 (www.flybase.bio.indiana.edu). The experiments with mice suggest that knock-out of CD2AP gene results in death due to kidney failure (Shih *et al.* 1999, 2001). Besides this, the Nephrin/CD2AP complex is able to bind to actin and to $p130^{cas}$.

Drosophila homologues for these mentioned factors involved in protein complexes have been identified but knowledge about their role in the MT development is less well understood. Other factors involved in cell recognition, cell migration and cell adhesion are members of immunoglobulin superfamily like Sticks and stones (Sns), Dumbfounded (Duf), SH3 domain-containning adaptor molecule like Myoblast city (Mbc) and multidomain protein like Rolling pebbles (Rols). Two different isoforms of rolling pebbles, Rols 6 and Rols 7 have been identified. Rols 7 play important role in muscle fusion while Rol 6 required for MTs development (Putz *et al.* 2005).

In addition to these genes, many other genes are described which have a role during MTs development. For example *faint sausage* (*fas*) is a cell adhesion molecule which is required during tubule elongation. It encodes extra cellular, immunoglobulin-like molecules (Lekven *et al.* 1998) which play a role in cell rearrangement. Fas also

plays a role in neuronal and exonal pathway formation. Another gene *walrus* (*wal*), is required for tubule bud evagination (Liu *et al.* 1999), *faint little ball* (*flb*) is essential for reception of a signal from the tip cell that promotes cell proliferation in MTs (Skaer 1989, Baumann and Skaer 1993, Kerber *et al.* 1998). *barren* (*barr*) and *three rows* (*thr*) both encode proteins necessary for segregation of sister chromatids throughout the embryo (D' Andera *et al.* 1993, Bhat *et al.* 1996).

Among the genes identified which affect MTs development, the largest number have been shown to affect tubule elongation which suggests that elongation is the most complex step in tubule morphogenesis and is regulated by a large number of genes. Many genes that affect cell proliferation may also affect tubule elongation such as *star, rhomboid, spitz, flb, pointed and sevenup* activated by the EGF signaling pathway (Skaer 1989, Bumann and Skaer 1993, Kerber *et al.* 1998). The genes *barr, thr, pimples* (*Pim*) *and string* (*stg*) affect mitosis and are required to provide a particular number and size of cells and their arrangement in MTs. It has been shown that there are many genes which are not expressed in the tubule but have role in its development, for example: *sog*, which antagonizes activity of the *BMP4* homolog *Dpp* (Francois *et al.* 1994; Harbecke and Lengyel, 1995), *raw* required for the cell intercalations involved in dorsal closure (Jack and Meytte, 1997). Similarly, *byn* and *srp* which encode transcription factors, are required for development of hind gut and posterior mid gut, respectively. Since they do not express in the MTs, but expresses in the early embryo from where the tubule arises, they may indirectly affect tubule development.

During MTs development the mechanism essential for stereotypic tract of MTs through the body cavity is still a mystery. However, studies on MT phenotype of *numb* mutants show that the tip cell and its siblings, both might play an important role in regulating the spatial arrangement of the growing tubules. Embryos that do not have either the tip cells or the sibling cells in elongating MT form the misrouted tubule through the body cavity with normally rearranged PCs (Ainsworth *et al.* 2000; Wan *et al.* 2000).

Function of Malpighian tubules :

The renal system occupies a critical and privileged role in the body which is involved in filtering the entire blood volume, not just for osmoregulation and elimination of waste, but also receives an early indication of immune, toxic and other insults to the body. Approximately every 2 hr, the turnover of human renal system (kidney) is equivalent to the entire extracellular fliud volume, by first filtering the extacellular fluid and then reabsorbing 99% of water from it. During this filtration the excess of solute and water, products of metabolism and filtered toxins that are not reabsorbed from the tubule lumen and are excreted out. Many of the solutes like organic acids and bases and foreign substances (many antibiotics) secreted into the lumen by epithelial cells are also excreted out (Beyenbach 2003).

In renal system of insects, tubular secretion is the only mechanism for passing solute and water to the tubule lumen, as there is no glomerular filtration. The renal

turnover of the extracellular fluid in insects is therefore accomplished by the epithelial transport mechanisms of secretion and absorption. Typically, the blind-ended distal segment of MTs secretes electrolytes, organic solute, water and proximal segments further down stream reabsorbs solute and water (Linton and Donnell 2000, O' Donnell and Maddrell 1995). Reabsorption continues in the hind gut and rectum (Spring and Albarwani 1993) *Drosophila* MTs can move their own cell volumes of fluid every 10 sec, so they offer an excellent model for the study of ion transport and excretion (Beyenbach *et al.* 2010, 2011, Dow 2009).

MTs are very robust tissues which continue to function for hours when removed from insect. This property of MT has been exploited to study the physiology of fluid secretion which was first introduced by Ramsay in 1953. After the measurement of control secretion rate, potential stimulators and inhibitors of transport can be added to observe their effects on fluid secretion. The knowledge of concentration and voltage difference is useful for distinguishing between active and passive transport. Transport is passive if it proceeds from high to low electrochemical potential and active, if it proceeds against the electrochemical potential via energy consuming pumps and pump-dependent transport systems.The initial segment is not able to secrete detectable fluid (Dow *et al.* 1994) whereas main and lower segments are responsible for fluid secretion and reabsorption respectively (Donnell and Maddrell 1995). The functions of MTs are performed mainly by two types of cells, PCs and SCs which number remain constant in different segments throughout life cycle (Fig 1C).

Four classes of transporters control the function of PCs, which are: 1. (Na^+-K^+)-ATPase (Lebovitz *et al.* 1989, Torrie *et al.* 2004) 2. Inward-rectifier K^+ channel (Evans *et al.* 2005) 3. V-ATPase (Day *et al.* 2008) 4. Alkali-metal/proton exchanger (Day *et al.* 2008). Together, these transporters and channels perform active transport (Dow *et al.* 1997) of ions from the basolateral (haemocoel) to apical (tubule lumen) surface.

The SCs have been proposed as the route for passive transport and water conductance via chloride channels (O'Donell *et al.*1998) and aquaporins (Spring *et al* 2007) respectively. Chloride is also thought to move paracellularly through the tight (in insects, septate) junctions, although the relative contribution of transcellular and paracellular routes may vary between insects. The Na^+ driven Cl^-/HCO_3^- exchanger (NDAE1) (Sciortino *et al.* 2001) and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) (Ianowski and Donnell 2004) are also present in tubules and may contribute to trans-epithelial chloride flux.

Tissues like MTs with such cellular and regional heterogeneity can exhibit complex control pathways that may be difficult to analyze. Secretion by the tubule is stimulated by peptide and amine modulators (Davies *et al.* 1997), and by treatments that raise intracellular level of secondary messengers cAMP (adenosine 3',5'-cyclic monophosphate), cGMP (guanosine 3',5'-cyclic monophosphate) or calcium ions (Dow *et al.* 1994b). *Drosophila* neuropeptide $CAP_{2b,}$ ELYAFPRV-amide (Huesmann *et*

al. 1995) and leucokinin IV (Davies *et al.* 1995) stimulate fluid secretion in MTs. CAP_{2b} stimulate fluid secretion via the activation of the nitric oxide (NO) signaling pathway and cGMP but not via cAMP (Davies *et al.* 1995, 1997). It has been observed that CAP_{2b} also has a cardio-stimulatory effect on insect myocardium which is mediate via a change in intracellular levels of inositol 1, 4, 5-trisphosphate (InsP3) (Tublitz 1988). It has also been shown that leucokinin IV stimulate chloride shunt conductance via intracellular calcium (Donnell *et al.* 1996), independent of cAMP/cGMP- mediated processes (Davies *et al.* 1996).

Thapsigargin, an inhibitor of the endoplasmic reticular Ca^{2+}-ATPase, causes an elevation in Ca^{2+} levels in many cell types (Thastrup *et al.* 1990) and like leucokinin IV, has been shown to increase both chloride shunt conductance (Donnell *et al.* 1996) and fluid secretion rates (Rosay *et al.* 1997) in *Drosophila* tubules. It also causes an elevation in basal cGMP levels which are not increased further on treatment with CAP_{2b} suggesting that CAP_{2b} mediated NOS activation may be a Ca^{2+}-dependent process. In Ca^{2+}-free medium, thapsigargin elevates Ca^{2+} ion only in the SCs but not in PCs, suggesting that principal cells do not contain a thapsigargin-sensitive intracellular pool (Rosay *et al.* 1997).

Insulin signaling pathway:

The function of MTs is also controlled by many signaling pathways, one of which is the insulin signaling pathway. Insulin-like peptides (ILPs) play role through the insulin signaling pathway and is evolutionarily conserved in animals and regulates growth, reproduction, metabolic homeostasis, stress resistance and life span. In *Drosophila*. seven insulin-like peptides (DILP1 to DILP7) are known, some of which are produced in the brain, others in fat body or intestine. In MTs, DILP5 is expressed in PCs and affects survival during stress. MTs primarily regulate water and ion homeostasis, but it also plays role in immune responses and oxidative stress. During desiccative, nutritional and oxidative stress the DILP5 signaling in MTs is controlled by *Drosophila* tachykinin peptide and its receptor DTKR. The expression of DILP5 levels in principal cells of the tubules are affected by stress and manipulations of DTKR expression in principal cells. Depletion of DTKR, DILP5 and the insulin receptor dInR in principal cells or mutation of *Dilp5* resulted in increased survival at either stress, whereas over-expression of these components produce the opposite phenotype. Therefore, stress induces hormonal release of DTK that acts on the renal tubules to regulate DILP5 signaling (Söderberg 2011).

Conclusions:

Though MTs are not homologs of human kidney, but they share common principle in development. They both show interaction between two distinct cell populations, ectodermal and mesodermal. Wnt pathway and signaling molecules like Kr-Glis2/Klf-6, Cut-Cux-1 that are involved in the induction of tubulogenesis and Hibris-Nephrin in cell differentiation seems to have been conserved throughout evolution (Denholm *et al.* 2003, Jung *et al.* 2005). In both these organs, one of the cell

populations undergo MET to integrate and become functional. The knowledge acquired from studies on development and function of *Drosophila* MTs is providing an insight into understanding the development and functions of human kidney. Moreover it is now becoming the model of choice to understand complex renal disorders.

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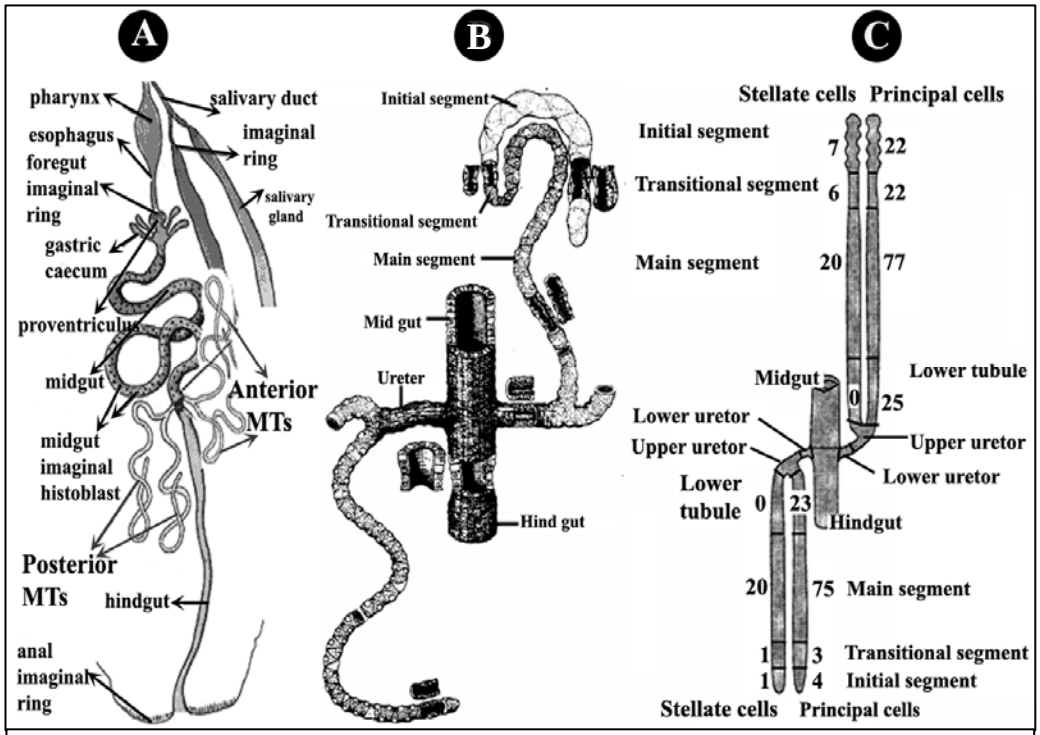

Figure 1. Structure of Malpighian tubules in *Drosophila melanogaster*

Two pair of Malpighian tubules (MTs) are anteriorly and posteriorly placed in the body cavity of larvae (A, red arrow), MTs are attached at the junction between mid gut and hind gut through ureter. Each tubule is divided into initial segment at most distal end and connected to the main segment by a short transitional segment. Following the main segment is the lower tubule. The two tubules of each pair are connected to the gut by ureter. The ureter is divided into upper and lower ureter (B), Number of principal cells and stellate cells remain fixed in each segment of MTs (C). Image modified from Sozen *et al.* 1997.

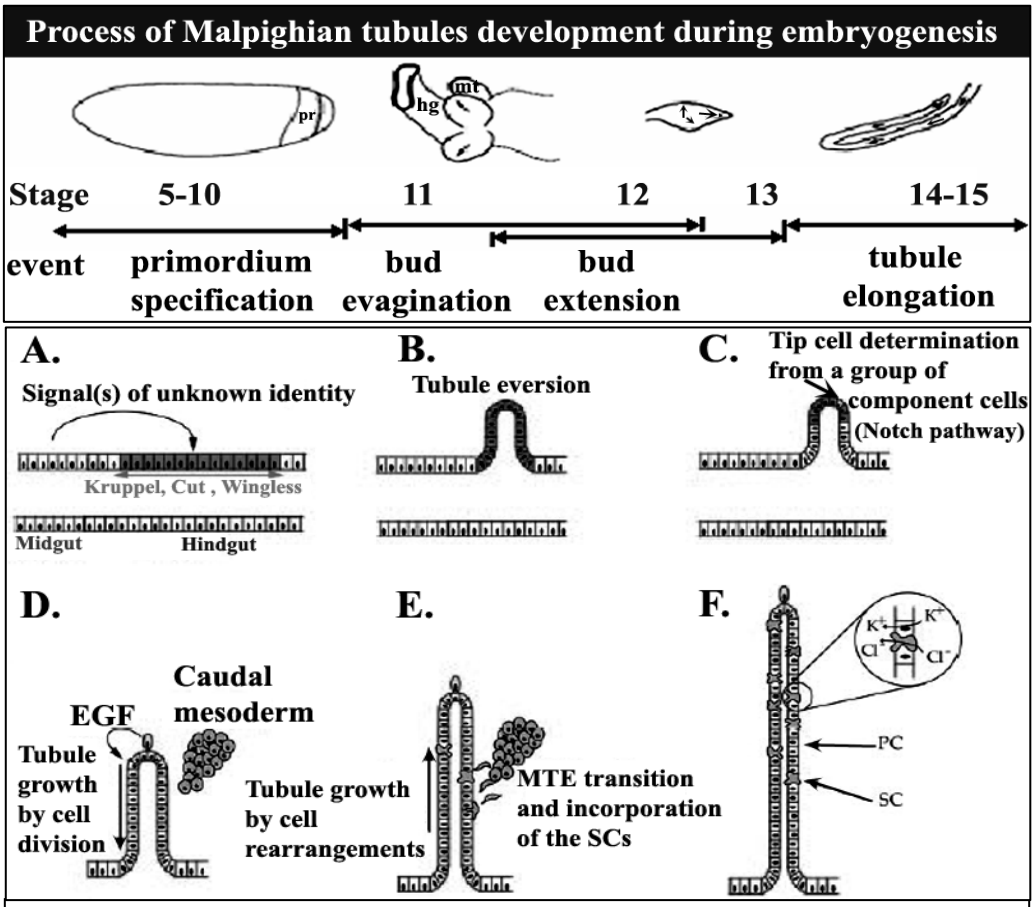

Figure 2. Process of Malpighian tubules development during embryogenesis

The overlapping expression of many genes at the posterior side of embryos established the portion of proctodeum, from which MTs arise by different developmental process like bud evagination, bud extension and tubule elongation.

During early embryogenesis the interaction of midgut (red) and hindgut (blue) anlagen, the signal of unknown identity activate the expression of Kruppel (Kr) in the hind gut to give rise to future PCs of Malpighian tubule. Kr triggers the expression of Cut in the hind gut (green). Transcription factors Kr and Cut along with wingless play role in tubule eversion (B). Moreover a new cell lineage called tip cell is produced from these cell population by activation of Notch (C). The specialized tip cell produce epidermal growth factor (EGF) and stimulate mitosis in neighbouring cells at the distal ends and tubule grow by addition of new cells. During development, tubule comes in vicinity of the caudal mesoderm (orange) (D). At this stage, the MTs cells stop dividing, and tubule growth proceed through extensive cell rearrangement and intercalation. The caudal mesoderm cells interacts with the tubule, individual cells undergo a mesenchymal-to-epithelial transition (MET) and progressively incorporate in to tubule epithelium as SC (E). By the end of embryogenesis, in mature tubule PCs and SCs specialized for different function (F). Image modified from Jung *et al.* 2005 and Liu *et al.* 1999.