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Drosophila neural stem cells in brain development and tumor formation

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Abstract

Neuroblasts, the neural stem cells in *Drosophila*, generate the complex neural structure of the central nervous system. Significant progress has been made in understanding the mechanisms regulating the self-renewal, proliferation, and differentiation in *Drosophila* neuroblast lineages. Deregulation of these mechanisms can lead to severe developmental defects and the formation of malignant brain tumors. Here, we review the molecular genetics of *Drosophila* neuroblasts and discuss some recent advances in stem cell and cancer biology using this model system.

Keywords: neural stem cells, neuroblasts, *Drosophila*, cancer stem cells, brain tumor

The central brain of *Drosophila* is a highly complex neural structure composed of tens of thousands of neurons that are inter-connected into intricate neural circuitry. All of these neurons (and a small subset of glial cells) are generated during development by a surprisingly small number of neural stem cells that are called neuroblasts (Urbach and Technau, 2004; Doe, 2008; Knoblich, 2008; Egger et al., 2008; Reichert, 2011; Homem and Knoblich, 2012). Each hemisphere of the *Drosophila* central brain is the product of approximately 100 neuroblasts, which are specified during embryogenesis from the cephalic neuroectoderm (Urbach and Technau, 2004). During development, most of the neuroblasts undergo two rounds of proliferation; during embryonic development they generate the neurons of the larval brain, and during post-embryonic development they give rise to the adult-specific neurons of the mature brain (Hartenstein et al., 2008).

The balance between self-renewal and differentiation of neuroblasts and their progeny is tightly regulated, and deregulation of this process often results in profound pathological defects during brain development (Doe, 2008; Knoblich, 2008; Egger et al., 2008; Reichert, 2011; Chang et al, 2012; Homem and Knoblich, 2012). Notably, mutations in key regulatory genes can result in dedifferentiation of their lineal progenitors and uncontrolled proliferation of neuroblasts, which leads to the formation of lethal brain tumors (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a; Lee et al., 2006b; Wang et al., 2006). Recent neurogenetic analyses of tumorigenesis in *Drosophila* have established a firm link between impaired neuroblast division and brain tumor formation (Caussinus and Gonzalez, 2005).

In this review, we consider the molecular genetic mechanisms by which the ensemble of neural stem cells generates the circuitry of the *Drosophila* brain during normal development. We focus on the recently discovered type II neuroblasts, which generate transit amplifying intermediate

progenitors and give rise to about one-fourth of the total number of neural cells in adult *Drosophila* central brain, and discuss how the lineage progression is regulated in these neuroblast lineages. Furthermore, we discuss recent advances using *Drosophila* neuroblasts in analyzing how deregulation of these developmental mechanisms can lead to neural stem cell overproliferation and tumorigenesis.

EMBRYONIC AND POSTEMBRYONIC DEVELOPMENT OF *DROSOPHILA* NEUROBLASTS

Drosophila neuroblasts are formed in the neuroectoderm during early embryonic development around stage 9 (Fig. 1A) (stages of embryogenesis according to Campos-Ortega and Hartenstein, 1997). Initially, clusters of neuroectoderm cells express proneural genes, including genes of the *achaete-scute* locus and *daughterless* (Fig. 1A). All of these genes encode transcription factors containing a DNA-binding basic helix-loop-helix (bHLH) motif (Campos-Ortega, 1993; Goodman and Doe, 1993). Shortly afterward, single neuroblast cells are determined within each cluster through Notch/Delta signaling (Fig. 1A). This process, usually referred to as lateral inhibition, inhibits the expression of proneural genes in all cells within a cluster except the neuroblast (Campos-Ortega, 1993; Goodman and Doe, 1993). Specified neuroblasts then delaminate from the neuroectoderm and start to divide along their apical-basal axis (Goodman and Doe, 1993).

In the central brain, most neuroblasts undergo two rounds of proliferation during normal development (Fig. 1B) (Hartenstein et al., 2008). During embryonic development, neuroblasts divide only a limited number of times to generate a small number of neurons that make up the larval brain (Fig. 1B). At the end of embryogenesis, most neuroblasts enter a quiescent phase.

Near the end of the first larval instar, these silent neuroblasts exit the quiescent stage and reinitiate proliferation (Fig. 1B). This second phase of proliferation continues throughout larval development and results in the production of the numerous adult-specific neurons that comprise the mature brain (Fig. 1B) (Hartenstein et al., 2008). Neuroblasts entry into quiescence is controlled intrinsically by Hox proteins and temporal identity factors (Tsuji et al., 2008). Neuroblasts exit from quiescence is regulated by extrinsic dietary nutrients through a fat-body to glia to neuroblasts relay mechanism (Sousa-Nunes et al., 2011).

Like other types of stem cells, an important feature of *Drosophila* neuroblasts is their ability to divide asymmetrically to self-renew and generate a large number of more differentiated progeny (Doe, 2008; Knoblich, 2008; Egger et al., 2008; Reichert, 2011). Although the division of many types of stem cells, for instance the germ line stem cells, depends on the interaction between the stem cells and their surrounding microenvironment (called stem cell niche) (Li and Xie, 2005), the proliferation of neuroblasts seems to be controlled primarily by cell-intrinsic mechanisms acting in several crucial steps. These steps are the establishment of an apical-basal polarity before neuroblast division, the asymmetric localization of cell fate determinants during neuroblast division, and the proper segregation of these determinant proteins into only one of the two daughter cells (Knoblich, 2008; Neumüller and Knoblich, 2009; Homem and Knoblich, 2012). Essential cell fate determinants in *Drosophila* neuroblast lineages include Brain tumor (Brat) (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a), Numb (Rhyu et al., 1994; Spana et al., 1995), and Prospero (Pros) (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995); their segregation into the differentiating progeny, but not into the self-renewing neuroblasts, is most important during the brain development and will be further discussed below.

TYPE I AND TYPE II NEUROBLASTS IN *DROSOPHILA* BRAIN

Surprisingly, the complex central brain (suparesophageal ganglion) of *Drosophila* is derived only from approximately 200 neuroblasts (~100 per hemisphere of the brain) (Urbach and Technau, 2004). The majority of the neuroblasts are type I neuroblasts that proliferate in a rather simple way (Fig. 2); they divide asymmetrically to give rise to a renewed neuroblast and a smaller ganglion mother cell (GMC), which further divides only once to produce two daughter cells that later differentiate into neurons or glia (Fig. 3) (Doe, 2008; Knoblich, 2008; Egger et al., 2008; Reichert, 2011). Another type of neuroblast, the type II neuroblast, has been recently discovered in the *Drosophila* brain (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008). Unlike the type I neuroblasts, only eight type II neuroblasts are present at the posterior brain of each hemisphere (Fig. 2). Six of them are formed at the dorsomedial edge (referred to as DM neuroblasts in some publications) and the other two localize at a more lateral position (Fig. 2) (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008).

In the *Drosophila* brain, a typical type I neuroblast can give rise to a lineage consisting of approximately 100-150 neurons (Fig. 2). In contrast, each type II neuroblast can generate a lineage that contains 400-500 neurons (Fig. 2) (Bello et al, 2008). Such an amplification of proliferation is achieved through a specific population of self-renewing intermediate progenitor cells called INPs (intermediate neural progenitors) (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008). When a type II neuroblast divides, it gives rise to a neuroblast and an INP cell both of which can carry out self-renewing asymmetric divisions (Fig. 3). Each new-born immature INP cell undergoes a stereotyped maturation process which involves the sequential expression of specific developmental control genes (Fig. 3). Once mature, INPs can undergo further rounds of asymmetric cell division (in a similar fashion to the type I neuroblasts) to self-

renew for a limited time and to give rise to GMCs, each of which divides once to generate two post-mitotic cells (Fig. 3). As a result, a significant increase in the number of neural cells occurs in the type II neuroblast lineages (Fig. 2) (Bello et al., 2008; Bowman et al., 2008; Boone and Doe, 2008).

Indeed, neurons derived from the eight type II neuroblasts constitute approximately one-fourth of the adult brain. They comprise diverse neuronal types, exhibit extraordinary complex projection patterns, and arborize in almost all the neuropile regions of the central brain (Yu et al., 2013; Ito et al., 2013). Such diversity might be specified by sequential expression of an array of various transcription factors called temporal identity factors in both type II neuroblasts and INPs (Bayraktar and Doe, 2013). Furthermore, recent clonal analyses suggest that type II neuroblasts generate sibling INP lineages consisting of a series of morphologically similar but temporally divergent neurons (Wang et al., 2013). Interestingly, during post-embryonic development, programmed cell death occurs in many cells in the type II neuroblast lineages to counter-balance the amplified proliferation. This elimination of excess neurons appears to be essential for the correct neuropile structure in the adult brain (Jiang and Reichert, 2012). In addition to neuronal progeny, type II neuroblasts also give rise to glial cells (Fig. 2). At least two types of glial cells have been shown to have a type II neuroblast origin, namely central complex glia and optic lobe glia. This suggests that type II neuroblasts can be considered as multipotent neuroglial progenitors in the *Drosophila* brain (Izergina et al., 2009; Viktorin et al., 2011; Viktorin et al., 2013).

CONTROLLING LINEAGE PROGRESSION IN TYPE II NEUROBLASTS

As type II neuroblasts generate self-renewing INPs to amplify their proliferation, it is extremely important to ensure that their lineage progression is tightly controlled during development. For correct lineage progression, the self-renewal capacity of the neuroblasts must be maintained, the maturation of the immature INP cells must be ensured, the proliferation potential in mature INPs must be restricted, and the transit amplifying process must be terminated at the correct time point. Although the molecular control mechanisms involved in these processes remain largely unknown, recent discoveries have started to identify some of the regulators controlling the lineage directionality in the type II neuroblast lineages.

In the type II neuroblasts, one of the most important regulators identified to date is the Ets domain transcription factor Pointed (Pnt) (Zhu et al, 2011). One of its isoforms, PntP1, is specifically expressed in type II neuroblasts, where it is necessary for the specification of type II neuroblasts and the generation of INPs (Fig. 3). The function of this protein appears to be to suppress the expression of Asense (Ase), as loss of PntP1 results in a reduction or elimination of INPs and ectopic expression of Ase (Fig. 4) (Zhu et al, 2011). Normally, and in contrast to the type I neuroblasts, the proneural transcription factor Ase is not expressed in type II neuroblasts, and for this reason they were sometime called PAN (posterior Asense negative) neuroblasts (Fig. 3) (Bowman et al., 2008). Ectopic expression of Ase in type II neuroblasts leads to a reduction of INPs, and eventually results in lineages consisting of much fewer progeny, presumably by converting type II neuroblasts into type I neuroblasts (Bowman et al, 2008).

Similar to type I neuroblasts, type II neuroblasts express the bHLH-O transcription factor Deadpan (Dpn), and the cell fate determinants Brat and Numb (Fig. 3). However, a third cell fate determinant protein Pros is not expressed in type II neuroblasts (Fig. 3) (Bello et al, 2008; Bowman et al, 2008). The immediate progeny of type II neuroblast division are immature INPs,

which lack the expression of both Ase and Dpn (Ase^- imm. INPs) (Fig. 3). During the subsequent maturation process, they express first Ase (Ase^+ imm. INPs) and later express Dpn to become mature INPs (Fig. 3). In neuroblasts, Dpn acts together with members of the *E(spl)* locus to maintain the self-renewing potential of these cells (Fig. 4) (Zacharioudaki et al., 2012; Zhu et al., 2012).

A second important regulator of self-renewal in neural progenitors is the Zn finger transcription factor Klumpfuss (Klu) (Berger et al., 2012; Xiao et al., 2012). Like Dpn, Klu expression is found in both type I and type II neuroblasts and in the mature INPs, but not in the immature INPs (Fig. 3). In *klu* mutant brains, type II neuroblasts are progressively lost due to premature differentiation, while overexpression of *klu* causes dedifferentiation of immature INPs and accumulation of numerous ectopic neuroblast-like cells (Berger et al., 2012; Xiao et al., 2012). Thus, downregulation of *klu* expression seems to be required for the transition from immature to mature INPs (Fig. 4) (Berger et al., 2012).

Another Zn finger transcription factor Earmuff (Erm) is expressed in the mature INPs and plays a role in restricting the proliferation potential of these INPs (Fig. 3) (Weng et al., 2010). Although the initial maturation of INPs appears normal in *erm* mutants, the mature INPs gradually dedifferentiate back into a neuroblast state, which is functionally indistinguishable from normal type II neuroblasts. Erm may function by activating Pros to limit proliferation and by antagonizing Notch signaling to prevent dedifferentiation (Fig. 4) (Weng et al., 2010).

Recent genome-wide transgenic RNAi analyses have begun to reveal other novel genes that are involved in controlling the self-renewal and differentiation of neuroblasts (Neumüller et al., 2011). Using the well-established GAL4/UAS binary system and a whole genome transgenic RNAi collection, more than 17,000 RNAi lines (corresponding to 89% of the *Drosophila* genome)

were screened for an abnormal brain phenotype. Their study used a driver line (*insc-Gal4*) that is expressed in both type I and type II neuroblasts, to target the knock-down of candidate genes in all neuroblasts. In total, 620 genes were identified as potential regulators of *Drosophila* neuroblast self-renewal and differentiation. These candidate genes were further assigned to different functional subgroups including asymmetric cell division, neuroblast self-renewal, cell growth, and others (Neumüller et al., 2011).

In a subsequent study, an assay for isolation of large amounts of pure neuroblasts and differentiated neurons by FACS from *Drosophila* larval brains was developed and used in further transcriptome analyses based on mRNA sequencing (Berger et al., 2012). These analyses revealed a total of 3,532 genes that were differentially expressed in neuroblasts versus neurons of which 1702 (48%) were upregulated in neuroblasts. Among these, this study identified 28 genes encoding neuroblast-specific transcription factors (including *klu*) and proposed a hypothetical transcriptional network for neuroblast self-renewal based on these findings. Importantly, the RNA-seq data showed a tight correlation with the knock-down results from the genome-wide RNAi screen, thereby validating the results of both studies (Berger et al., 2012). The functions of the new candidate genes from these two large-scale genome-wide studies are now being verified using classical genetic methods, thus, these two studies represent a valuable resource for further investigation.

TUMORIGENIC OVERGROWTH INDUCED BY DEFECTIVE ASYMMETRIC CELL DIVISIONS

During asymmetric cell division of *Drosophila* neuroblasts, the three cell fate determinants Brat, Pros, and Numb localize to the basal cortex and are differentially segregated into only one of the

daughter cells (Knoblich, 2008). In these differentiating progeny, the proteins suppress the expression of neuroblast-specific genes to inhibit cell self-renewal, while at the same time initiating a differentiative program to induce cell differentiation (Fig. 4) (Knoblich, 2008). During the division of type I neuroblasts and mature INPs, all three proteins are inherited only by the GMCs. In the type II neuroblasts, Pros is not present and only Brat and Numb are segregated into the immature INPs. Asymmetric segregation of cell fate determinants is critical during the development of *Drosophila* brain, because mutations in genes encoding these proteins result in defective asymmetric cell division and lead to neuroblasts overproliferation and the formation of brain tumors (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a; Lee et al., 2006b; Wang et al., 2006).

Although the three cell fate determinants Brat, Numb, and Pros have been intensively investigated during the past decade, their exact functions in the proliferating neuroblasts are still not completely understood. Brat is a RNA-binding protein that appears to inhibit cell growth and ribosomal RNA synthesis (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a). Numb is a membrane protein and it acts as a suppresser in the Notch signaling pathway (Rhyu et al., 1994; Spana et al., 1995). Pros is a homeodomain containing transcription factor, which enters the nucleus of the GMC and transcriptionally regulate the expression of more than 700 target genes that may be necessary for neuronal differentiation (Fig. 4) (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Choksi et al, 2006).

Interestingly, overproliferation occurs mainly in mutant type II neuroblast lineages, which suggests that type II neuroblasts are more vulnerable for mutations in the cell fate determinant genes (Bowman, et al., 2008). Presumably, this is due to the presence of transit amplifying INPs in these lineages which may have a propensity to dedifferentiate into neuroblast-like cells.

Among these lines, Brat may also act by antagonizing the function of both Klu and β-catenin/Armadillo (Arm) to specify the identity of the INPs and to suppress the reversion of these progenitors into ectopic neuroblasts (Fig. 4). Reducing the activity of Klu or Arm can largely suppress the formation of supernumerary neuroblasts in *brat* mutant brains (Xiao et al., 2012; Komori et al., 2013). Numb / Notch signaling-regulated cell growth seems to involve the well-studied growth regulators eukaryotic translation initiation factor 4E (eIF4E) and dMyc (Fig. 4). Both proteins are up-regulated in the dedifferentiating progenitor cells in a Notch hyperactivation condition, and removal of either eIF4E or dMyc can strongly inhibit the overproliferation defect seen in these brains (Song and Lu, 2011).

The overgrowth phenotype observed in *brat*, *numb*, and *pros* mutants indicates that these genes can act as tumor suppressors during brain development. Indeed, the tumorigenic property of these mutant cells has been clearly demonstrated by transplantation experiments; mutant brain tissue transplanted into wild-type host flies can form malignant and metastatic tumors (Caussinus and Gonzalez, 2005). The tumor cells show apparent genome and centrosome instability and kill the host flies rapidly, and they can give rise to new tumors indefinitely upon re-transplantation (Caussinus and Gonzalez, 2005).

Like most of the other regulators of asymmetric cell division, the three cell fate determinants in *Drosophila* have homologues in vertebrates. The functions of these vertebrate homologues are less well-characterized, and only a few have been shown to be functionally conserved and play a similar role during mammalian neurogenesis (Petersen et al., 2002; Li et al., 2003; Dyer et al., 2003; Schwamborn et al., 2009). For instance, TRIM32, one of the mouse orthologues of *Drosophila* Brat, is required in dividing cortical progenitor cells for suppressing self-renewal and inducing neuronal differentiation during mouse brain development (Schwamborn et al., 2009).

Similarly, loss of function of these homologues can lead to brain malformation and early lethality (Li et al., 2003). However, a tumor suppressor function of the vertebrate homologues of *Drosophila* Brat, Numb, and Pros has not been described, and the link between mutations in the homologous genes and human brain tumors is still missing, probably due to functional redundancy of other tumor suppressors not yet identified (Schwamborn et al., 2009).

AN EMERGING MODEL TO STUDY STEM CELL-DERIVED BRAIN TUMORS

The cancer stem cell hypothesis proposes that some types of cancer consist of both tumorigenic and nontumorigenic cells, and it is a small fraction of stem cell-like progenitor cells, the "cancer stem cells", that are tumorigenic and have the potential to proliferate indefinitely, which eventually drive the propagation of tumor (Reya, et al, 2001; Magee et al., 2012). Although this hypothesis has been supported by an increasing number of studies on human cancers, it remains controversial due to the complexity of human cancers and the technical limitations in assays currently used to identify these cancer stem cells (Magee et al, 2012).

In recent years, *Drosophila* has become an emerging model for investigating the mechanisms underlying tumor formation (Read, 2011; Miles et al., 2011; Gonzalez, 2013). Following up on the discovery of malignant overproliferation in *Drosophila* brains and discs reported more than three decades ago by Gateff, (Gateff, 1978), recent experiments have now firmly demonstrated that overproliferating brain tissue in *Drosophila* can develop into lethal tumors upon transplantation (Caussinus and Gonzalez, 2005). In view of these findings, it seems that *Drosophila* neuroblasts might serve as an excellent stem cell model to test the "cancer stem cell" hypothesis. Of course *Drosophila* has other advantages suitable for such analysis such as powerful genetic and genomic tools, well-characterized cell markers, established driver lines

which allow more specific knock-down or overexpression of target genes in particular type of cells, and recently developed techniques like transgenic RNAi and FACS isolation of progenitor populations. Thus, further analysis of the known mutant genes and new candidate genes from large-scale genome-wide screens, should provide more insight into the mechanisms regulating the formation, progression, and hopefully even suppression of neural stem cell-derived brain tumors.

CONCLUDING REMARKS

Studies on *Drosophila* neuroblasts have already led to significant progress in understanding the mechanisms controlling neural stem cell specification, self-renewal, proliferation, and differentiation during brain development. The recent discovery and analysis of type II neuroblast lineages continues to provide important insight into both stem cell and cancer biology in this neurogenetic model. As mammalian neural stem cells often generate large numbers of neural progeny through transit amplifying intermediate progenitors, the molecular and cellular mechanisms identified in type II neuroblast lineages in *Drosophila* are likely to be evolutionarily conserved and might operate during the development of mammalian brains as well (Brand and Livesey, 2011; Homem and Knoblich, 2012).

The establishment of a *Drosophila* model to study neural stem cell-derived tumors is also relevant to human cancer biology. In current basic and clinical research, it remains a challenge to isolate cell-of-origin and cancer stem cells from human cancer, and to characterize the contribution of these cancer-initiating cells and cancer-propagating cells during cancer development (Visvader, 2011; Magee et al., 2012). The significant advantages of the *Drosophila* neuroblast model, as discussed in this review, may help to overcome some of the technical

obstacles encountered in mammalian cancer research. Progress made in the *Drosophila* model could therefore have notable implications for future prognosis and therapies in cancer biology, as well as in stem cell-based regenerative medicine (Gonzalez, 2013).

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FIGURE LEGENDS:

Figure 1. Embryonic and postembryonic development of *Drosophila* neuroblasts.

(**A**) Specification of neuroblasts during embryogenesis. *Drosophila* neuroblasts are formed in the neuroectoderm around stage 9. Initially, clusters of neuroectoderm cells express proneural genes (grey). Shortly afterward, single neuroblast cells (dark blue) are determined through Notch/Delta signaling, which is usually referred to as lateral inhibition, to inhibit the expression of proneural genes in all cells within a cluster except the neuroblast. (**B**) Two rounds of proliferation of neuroblasts. During embryonic stages, each neuroblast (NB, dark blue) divides only a limited number of times to self-renew and to give rise to a ganglion mother cell (GMC, purple), which further divides once to generate two neurons. The embryonic divisions only generate a small number of neurons (pink) that make up the larval brain. At the end of embryogenesis, most neuroblasts enter a quiescent phase. Near the end of the first larval instar, these silent neuroblasts exit the quiescent stage and reinitiate proliferation. This second phase of proliferation continues throughout larval development and results in the production of the numerous adult-specific neurons (light blue) that comprise the mature brain.

Figure 2. Type I and type II neuroblast lineages in *Drosophila* brain.

The central nervous system of *Drosophila* larvae consists of the optic lobe (OL), the central brain (CB), and the ventral nerve cord (VNC). In the central brain, most neuroblasts are type I neuroblasts that can give rise to a lineage consisting of a neuroblast (dark blue), a few ganglion mother cells (GMCs, purple), and approximately 100-150 neurons (light blue). In contrast, only

eight type II neuroblasts (brown) are present at the posterior brain of each hemisphere; six of them are formed at the dorsomedial edge and the other two localize at a more lateral position. Each type II neuroblast can generate a lineage that contains a neuroblast (brown), several immature intermediate neural progenitors (INPs, grey) and mature INPs (orange), GMCs (yellow), and 400-500 neurons (green) and a few glial cells (red).

Figure 3. Cell division of type I and type II neuroblasts.

Type I neuroblasts proliferate in a rather simple way. They divide asymmetrically to give rise to a renewed neuroblast and a smaller GMC, which divides only once to produce two differentiated daughter cells. When a type II neuroblast divides, it gives rise to a neuroblast and an immature INP cell, the latter undergoes a stereotyped maturation process which involves the sequential expression of specific developmental control genes. Once mature, INPs can undergo further rounds of asymmetric cell division to self-renew for a limited time and to give rise to GMCs, each of which divides once to generate two post-mitotic cells. The expression of some identified proteins in type I and type II lineages are indicated by different colors.

Figure 4. Factors regulating lineage progression in type II neuroblasts.

Some identified proteins and their functions in controlling the lineage progression in type II neuroblasts. See text for details.