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Asymmetric Cell Division

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Asymmetric Cell Division

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Preface

Symmetry is a source of stability but what remains symmetric does not evolve. On the other hand the breaking of symmetry is a source for change, the Universe survives on symmetry breaking, it was created symmetrically but today it is very asymmetric. At the very beginning an asymmetric event might have been at the origin of the matter-antimatter asymmetry. If the same amount of matter and antimatter were created we would not exist.

Symmetric causes can have asymmetric effects it is this loss between cause and effect that is called symmetry breaking, it is found everywhere: from the splash of a raindrop to the vibrations of stars (Stewart and Golubitsky 1992).

Symmetry breaking is also present universally in biological systems. It is not exclusive to any phyla nor is it exclusive to metazoans, indeed it has been described in unicellular organisms.

Louis Pasteur discovered that molecules could be left handed or right handed but only the former are utilized in living organisms. Pasteur thought life itself may be the result of asymmetry.

Cell division is a tool for renewal and maintenance but also a way for the cell to evolve. It is the asymmetry created during division that generates cellular diversity during the development of an organism. Without asymmetric division daughter cells would be exactly identical to the mother cell so that metazoans would not be viable.

After fusion of the gametes, asymmetric divisions lead to a multicellular organism with different functional cell compartments. Later in the adult organism cell regeneration from the stem cell pool can take place through asymmetric divisions, one daughter cell remaining a stem cell and the other committed cell going on dividing. The machinery that keeps the process of cell stem regeneration in check may become defective and be a cause of malignant growth.

Cancers were believed by Hansemann and Boveri to originate from a single cell that received an unbalanced set of chromosomes through the formation of pluripolar spindles. Asymmetric mitoses generating cells with significant sizes have also been observed in immortal cell populations and are thought to generate chromosomal imbalance that maintain the immortalized state (Kvitko et al 2006).

Because of the concept of semi-conservative DNA synthesis it was thought that after division daughter cells have the same genetic information, however, when events are analyzed at the level of individual cells it has become apparent that DNA is not distributed evenly between sister cells so that slight differences are created at each division. As described in this volume this creates a drift in cell function and is one of the mechanisms of aging of the organism.

Asymmetric divisions seem also to be responsible for aging in bacteria. Caulobacter crescentus differentiates into a sessile reproductive stalked cell attached to a solid substrate, which produces progeny swarmer cells. The reproductive output of stalked cells decreases at an accelerating rate with aging (Ackermann, Stearns, Jenal 2003). In Escherichia coli the cell that inherits the old pole exhibits a diminished growth rate, decreased offspring production, and an increased incidence of death (Stewart et al 2005). Saccharomyces cerevisiae is another unicellular organism where aging through asymmetric division has been extensively studied.

In this volume we have concentrated on less publicized organisms to describe this paramount regulatory mechanism in cell biology whose elucidation is necessary to understand the very basis of development, aging, and disease.

References

Ackermann M, Stearns SC, Jenal U (2003) Senescence in a bacterium with asymmetric division. Science 300:1920

Kvitko OV, Koneva II, Sheiko YI, Anisovich (2006) Hunting the mechanisms of sel-renewal of immortal cell populations by means of real-time imaging of living cells. Cell Biology International 29:1019–1024

Stewart EJ, Maddden R, Paul G, Taddei F (2005) Aging and death in an organism that reproduces by morphologically symmetric division. PLOS biology 3:0295–0300

Stewart I, Golubitsky M (1992) Fearful Symmetry, Penguin Books, England

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Asymmetric Cell Division in Plant Development

Renze Heidstra

Abstract

Plant embryogenesis creates a seedling with a basic body plan. Post-embryonically the seedling elaborates with a lifelong ability to develop new tissues and organs. As a result asymmetric cell divisions serve essential roles during embryonic and post-embryonic development to generate cell diversity. This review highlights selective cases of asymmetric division in the model plant *Arabidopsis thaliana* and describes the current knowledge on fate determinants and mechanisms involved. Common themes that emerge are: 1. role of the plant hormone auxin and its polar transport machinery; 2. a MAP kinase signaling cascade and; 3. asymmetric segregating transcription factors that are involved in several asymmetric cell divisions.

1 Introduction

Asymmetric cell division produces daughter cells with different fates. Distinct fate properties may be morphological or biochemical features or different progeny that cells produce (Horvitz and Herskowitz 1992). Ensuring the asymmetry of divisions at distinct locations and time points provides a commonly exploited solution to the fundamental problem of creating cell diversity in multicellular organisms. This cellular specialization generates the structural and functional cell types that make up tissues and organs during development.

Central to the process of asymmetric cell division is the question how a single cell can produce different daughter cells. A complication to this process is that the daughter cells generated upon division need not be morphologically dissimilar initially. Two distinct mechanisms are employed to generate asymmetric division. First, different daughter cells are generated due to differential inheritance of fate determinants as a consequence of unequal distribution of these factors in the mother cell. In this case, intrinsic factors determine cell fate. This scenario requires the mother cell to be polar at the onset of division leading to the immediate follow up question how the mother cell becomes polarized. Second, identical daughters are produced

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with initially equal developmental potential but fates diverge due to subsequent interaction with their surrounding environment. Now extrinsic cues determine cell fates. Whereas the intrinsic and extrinsic strategies appear very distinct it is easy to envisage how actual development may employ a combination of both. Note for example that in the intrinsic case the polarization of the mother cell before division may be under influence of external spatial information. The central question in both scenarios concerns the factors that cause daughter cells to obtain different fates.

Plant cells are surrounded by a cell wall restricting their movement. To cope with their sessile lifestyle plants evolved a remarkable developmental flexibility to detect and respond to changes in the environment by cell fate changes that ensure their growth and survival. Plant embryogenesis results in the formation of a seedling merely having the basic body plan with the shoot and root stem cell populations at apical and basal ends. A major specialization different from most other multicellular organisms is the lifelong ability of plants to develop new tissues and organs. As a result asymmetric cell divisions serve essential roles during embryonic and post-embryonic development to generate cell diversity. Orientation of cell division is an important aspect of plant asymmetric cell division to determine the position that of a cell in a growing tissue and so the positioning of new walls has significant effects on development. Mutants in which the normal cell division pattern is disturbed still form relatively normal and functional plants with their different tissues in the expected place but growth can be dramatically reduced (Torres-Ruiz and Jurgens 1994; Traas et al. 1995; Smith et al. 1996).

Despite very ordered cell divisions from embryogenesis onwards in some plants species (e.g. the model plant *Arabidopsis thaliana*) it is not lineage but positional information that determines cell fate (Poethig 1987; Furner and Pumfrey 1992; Scheres et al. 1994; van den Berg et al. 1995). This is consistent with the much more randomized division pattern in other species such as cotton, maize and rice (Pollock and Jensen 1964; Schel et al. 1984; Itoh et al. 2005). Nevertheless, the highly regular series of cell divisions during embryogenesis in *Arabidopsis* allows cell lineages to be traced back to their origin which presents a major advantage in developmental research.

The past years we have witnessed an explosion of data on gene and protein expression and auxin hormone accumulation from the initial stages of embryogenesis through to post-embryogenic development in higher plants using in vivo and in vitro systems. This review highlights selective cases of asymmetric division in plants and describes the current knowledge on fate determinants and mechanisms involved. I will focus the discussions on the model plant *Arabidopsis thaliana*. For the processes involved in the physical separation of a single cell into individual daughter cells and the function of the cytoskeleton the reader is referred to several recent reviews (Jurgens 2005; Konopka et al. 2006; Lloyd and Chan 2006).

2
Polarity and Orientation of Cell Division in Plants

Cell polarity is the development of asymmetry within a cell which can be monitored by physical changes in cell shape or localized distribution of molecular components. Cell polarity provides information for axis formation, patterning, growth and asymmetric cell division.

Asymmetric distribution of the plant hormone auxin is instrumental in regulating many polar growth and division responses at the tissue level (summarized in Dhonukshe et al. 2005) but is also implicated in specific cases of asymmetric cell division discussed here. Auxin is actively distributed within the plant by the combined action of AUX1 auxin influx carrier and PIN auxin efflux facilitators whose asymmetric subcellular localization has been correlated with the direction of auxin flow. Binding of auxin to Transport Inhibitor Response 1 (TIR1), an auxin receptor and subunit of an SCF-type ubiquitin ligase, promotes the degradation of a family of transcriptional repressors called Aux/IAA proteins. Aux/IAA proteins bind to Auxin Response Factor (ARF) proteins and inhibit the transcription of specific auxin response genes. Increased nuclear concentrations of auxin promote auxin binding to TIR1, causing the Aux/IAA proteins to associate with TIR1 and leading to their degradation by a proteasome-mediated pathway. The ARF protein is now free to activate transcription from its target promoter (reviewed in Jenik and Barton 2005). Studying the polar localization of PIN protein is aiding significantly in investigations on plant cell polarity and pinpointing the proteins involved (Xu and Scheres 2005).

Certain cell polarization events depend on ADP-Ribosylation Factor (ARF)-mediated vesicle trafficking to polarly localize Rho-related GTPases from plants (ROP). ROPs act as master switches in the transmission of various extracellular and intracellular signals and have classically been linked to the regulation of the cytoskeleton. ROPs control actin assembly and microtubule bundling through ROP-Interactive CRIB-motif (RIC) proteins and Wiskott–Aldrich syndrome protein family verprolin homologous/suppressor of cAMP receptor-actin related protein (WAVE/SCAR–ARP2/3) pathways (Gu et al. 2004; Burridge and Wennerberg 2004; Xu and Scheres 2005). In plants ARFs interact with Guanine nucleotide Exchange Factors (ARF–GEF) like GNOM/EMB30 for polar vesicle transport. *gnom* mutants display aberrant cell shape and abnormal orientation of cell division planes including the first division in the zygote (Sect. 4.1, Mayer U et al. 1993; Shevell et al. 1994; Geldner et al. 2003).

Another way to generate and maintain polarity may be altered sterol composition of the cell membrane which in yeast was shown to interfere with mating and may also be important for animal cell polarity (Bagnat and Simons 2002; Schuck and Simons 2004). A similar case has been made for plants as reported for the *Arabidopsis* mutant *sterol methyltransferase*

1/orc (smt1/orc), which is disturbed in the biosynthesis of plasma membrane sterols resulting in apolar distribution of cellular markers and aberrant division planes (Willemsen et al. 2003).

Clearly, disturbing the polarity of cells affects the orientation of the cell division plane. But how cell polarity is linked to division orientation in plants remains unclear. In animals the PARtitioning defective (PAR) proteins act downstream of polarization cues to stabilize polarity and they form the connection with the cytoskeleton to control asymmetric mitotic spindle positioning, determine the division plane and localize cell fate determinants to one side of the cell (reviewed in Wodarz 2002; McCarthy and Goldstein 2006). *PAR* genes were originally identified in a screen for mutants affecting the first asymmetric cell division of the *Caenorhabditis elegans* zygote and encode a diverse set of proteins consisting of Ser/Trh-kinases, PDZ-domain proteins and a 14-3-3 protein (Kemphues et al. 1988; Betschinger and Knoblich 2004).

Cell division is distinct in several ways in animals and plants. First, in animals, microtubule nucleation takes place at microtubule-organizing centers (MTOCs) such as the centrosomes associated with the poles of the mitotic spindle that determine the direction of chromosome segregation during mitosis. Higher plant cells lack discrete MTOCs but assemble highly ordered arrays of microtubules from nuclear polar caps that anticipate the mitotic spindle to coordinate cell division. Second, physical cell division or cytokinesis in animal cells involves inward constriction by an actinomyosin contractile ring that pulls in the plasma membrane whereas plant cell cytokinesis occurs at from the center toward the cell periphery. This process involves the "phragmoplast", the cytokinetic ring of the plant cell, consisting of antiparallel bundles of microtubules and actin that forms from the remains of the spindle between the two sets of chromosomes. The phragmoplast delivers vesicles to the plane of cell division forming the outward growing cell plate. Interestingly, the future site of division in plants is predicted late in G2 by a transient cortical preprophase band (PPB) of co-aligned microtubules and actin filaments encircling the nucleus whereas in animals the site of cytokinesis is selected after chromosome separation. Although on the surface animal and plant division appear very different, the involved mechanisms and protein conservation indicate a common basis to both types of division (Jurgens 2005; Lloyd and Chan 2006 and references therein).

3
Asymmetric Cell Divisions in Plant Development

Plant life starts with the formation of the zygote and its first division is asymmetric generating two daughter cells with different fate: a smaller apical cell and a large basal cell (Sect. 4.1) that will form different cell lineages.

The apical cell next divides vertically and will produce the majority of the embryo. The basal cell divides horizontally forming the extra-embryonic suspensor that connects the embryo to the maternal tissues. At the globular embryo stage of the uppermost cell from the suspensor is recruited to become the hypophysis. This cell then divides asymmetrically generating an apical lens shaped cell (Sect. 4.2) that is the progenitor of the root stem cell organizing cells, the quiescent center. Also embryonic are the oriented periclinal divisions that give rise to the progenitors for the three main tissues, epidermis, ground tissue and vascular tissue (Sect. 4.3). Radial patterning by asymmetric division continues post-embryonically throughout the life of plants in foci of cell division and development called meristems located at the tip of the root and shoot. In the vascular system phloem and procambium are established through asymmetric cell divisions of a set of progenitors. Other examples are the formation of epidermis and lateral root cap tissues and the formation of endodermis and cortex from single progenitor cells whereby the latter is the best-studied example of asymmetric division in plants (Sect. 5.1). All post-embryonic development has its origin in the stem cells located in niches in the heart of the shoot and root meristems. The function of the niche is to provide the microenvironment to keep the stem cells undifferentiated and as a consequence in asymmetric stem cell divisions (Sect. 5.2) to produce a daughter that remains stem cell and a daughter that will differentiate according to position as it moves out of the niche. As the plant develops new organs and specialized cell types are formed needed to deal with the outside world. Asymmetric divisions play a role in root branching (Sect. 5.3) as the root explores the soil in search of nutrient, and in the formation of stomata (Sect. 5.4), cells that regulate gas exchange from the aerial parts of the plant. Finally, when the plant reaches maturity and reproduction becomes an issue, asymmetric divisions are again employed to generate the male gametes completing the life cycle (Sect. 5.5).

4
Asymmetric Divisions in Embryogenesis

As different as the shapes and sizes of higher and lower plants appear, the early steps in embryogenesis show profound similarities. Embryos of higher plants develop deep inside maternal tissues and thus are difficult to use for experimental manipulation. Therefore research on embryogenesis has focused on genetics as an important tool. Lower plants such as the brown alga *Fucus* have free-living zygotes and have provided a valuable system to study asymmetric division (Fowler and Quatrano 1997; Scheres and Benfey 1999). Recently, however, a system has been developed to culture zygotic *Arabidopsis* embryos inside their ovules in vitro (Sauer and Friml 2004), greatly improving experimental accessibility while the normal embryogenesis program proceeds.

4.1 Division of the Zygote

Zygotic cell division is generally transverse and asymmetric in angiosperms (Lindsey and Topping 1993). Asymmetry is already evident in the egg cell itself prior to fertilization by means of polar localization of nucleus and vacuole according to the apical-basal micropylar-chalazal axis of the female gametophyte (Mansfield and Briarty 1991). After fertilization, the zygote elongates while remaining polarized and it divides asymmetrically to produce a smaller apical cell that will generate most of the embryonic tissues and a larger basal cell that will give rise to the suspensor (Fig. 1A). In *Arabidopsis* the pattern of cell division in early embryogenesis is extremely ordered. The smaller apical cell will divide longitudinally twice before the next division is transverse. The basal cell undergoes only horizontal divisions (Mansfield and Briarty 1991). The initial asymmetry in egg cell and zygote suggest a role for the maternal tissues in providing extrinsic cues to direct the asymmetric zygotic division, although it is unclear how instructive these polar morphological criteria are in directing the asymmetry of division and daughter fates.

Maize and tobacco zygotes produced by in vitro fertilization also develop into asymmetrical two-celled embryos that consist of a small cytoplasmic rich apical cell and a large vacuolated basal cell (Kranz et al. 1995; Okamoto et al. 2005; Ning et al. 2006). The maize zygotes can eventually develop into fertile plants (Kranz and Lorz 1993). These studies then suggest extrinsic maternal factors are not required for the asymmetric division of the zygote making a case for intrinsic regulation of asymmetry.

In vitro tobacco zygotes at elongation stage display apical polar distribution of arabinogalactan proteins (AGP) observed by way of epitope detection using monoclonal antibodies (Qin and Zhao 2006). Disturbing the biological activity of AGPs by application of β-D-glucosyl Yariv reagent (βGlcY, Knox 1997) increased symmetrical division of zygotes. AGPs are a diverse family of hydroxyproline-rich glycoproteins typically carrying >90% carbohydrates that play multiple roles in various processes associated with plant growth and development, including cell expansion, cell proliferation (Willats and Knox 1996; Nothnagel 1997; Shi et al. 2003). These results together with previous reports on AGP localization in embryogenesis (Pennell et al. 1991; McCabe et al. 1997) suggest AGPs may be involved in establishing and stabilizing polarity in the zygote as a first step towards asymmetric cell fate determination upon division.

Studies on differential gene expression in egg cells, zygotes, and later embryonic stages in tobacco, maize and wheat have identified a number of genes as candidate players in the asymmetric zygotic division (Okamoto et al. 2005; Sprunck et al. 2005; Ning et al. 2006). Some of the zygotic genes were already reported earlier for their expression or function in early embryogenesis, One of these was *WOX9* (*WUSHEL* related homeobox 9)

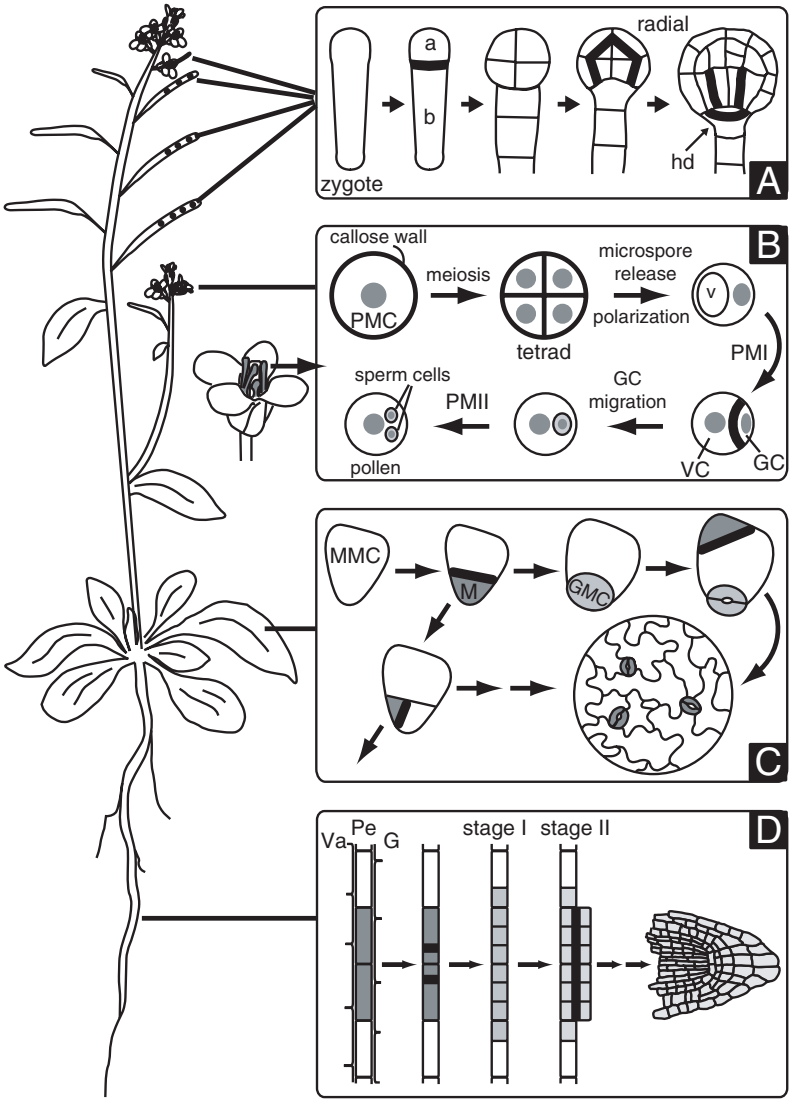

Fig. 1. A–D Sites of asymmetric cell division in plants. **A** Asymmetric division in embryogenesis takes place in the developing seeds in the siliques. The zygote divides in a smaller apical and larger basal cell. Radial divisions generate the epidermis, ground and vascular progenitors. Hypophysis division (hd) generates the lens shaped cell as progenitor of the QC. **B** Pollen development in the anthers of the male reproductive organs in the flower. Asymmetric division at PMI results in a vegetative and generative cell. **C** Asymmetric divisions during stomata development in leaves generate meristemoids. **D** Lateral root initiation starts with the specification of pericycle founder cells and their asymmetric division into flanking small cells. Thick black lines in panels A–D indicate the asymmetric division. See text for detailed descriptions

that in *Arabidopsis* was found to be expressed in the basal cell after zygotic division (see below, Haecker et al. 2004; Ning et al. 2006). Interestingly, a number of genes already expressed in the zygote were up regulated in the apical or basal cell (Okamoto et al. 2005; Sprunck et al. 2005; Ning et al. 2006). An important next step will be to determine whether and if any of these upregulated mRNA's or their products are polarly localized in the zygote and instrumental in asymmetric division and/or fate determination of daughter cells.

In animals homeodomain transcription factors specify distinct domains in the developing embryo (Krumlauf 1994). Plants also express homeodomain transcription factors and their specific expression domains are instrumental in specifying cell fates (Lu et al. 1996; Jeff et al. 1996; Mayer KFX et al. 1998; Abe et al. 2003). *WUSCHEL* (*WUS*) is the founding member of the *WOX* homeodomain gene family, and specifies the shoot stem cell organizing center (Mayer KFX et al. 1998). Expression of several *WOX* genes was found to mark cell fate changes during early embryogenesis. *WOX2* and *WOX8* are expressed in the egg cell and the zygote. After division of the zygote *WOX2* mRNA is detected in the apical cell complementary to *WOX8* mRNA expression in the basal cell. *WOX8* mRNA accumulation in the basal cell is joined by *WOX9* expression. Unfortunately, experimentation did not conclusively discriminate between asymmetric *WOX2* and *8* mRNA accumulation before zygote division or immediate after. However, never was overlapping expression observed in zygote daughters hinting towards intrinsic regulation of asymmetric division. Embryos mutant for *WOX2* showed a detectable phenotype displaying aberrant oblique divisions in the apical cell and its descendants suggesting a role for *WOX2* in apical cell specification. Surprisingly, later stage embryos recovered and eventually gave rise to fertile plants (Haecker et al. 2004). Genetic redundancy of the highly related *WOX8* and *9* genes may be the cause of the absence of mutant basal cell phenotypes.

Identification of the YODA (YDA) MAPKK kinase revealed a key role for MAP kinase signaling in asymmetric cell division of the zygote and establishing cell fate in its daughter cells (Lukowitz et al. 2004). In *yda* knock-out mutants the zygote does not elongate properly and the basal cell and its derivative show aberrant divisions. As a result these cells do not form a suspensor and are eventually incorporated in the embryo. Initial development of the apical cell lineage is normal indicating asymmetry of division is not completely abolished. Despite these defects, a proportion of *yda* mutants can develop into adult albeit severely dwarfed plants indicating YDA takes part in a number of developmental processes. Gain-of-function mutants suppressed embryo formation and in severe cases the zygote developed into a suspensor like cell file. This suggests YDA is down regulated in the apical cell lineage allowing embryo development. Although *YDA* mRNA accumulation appears ubiquitous in the tissues tested the expression is not resolved with cellular resolution. Interestingly, MAP kinase signaling

generally acts downstream of receptors activated by extracellular ligands. In that respect YDA activity during and after asymmetric division might represent the readout of extrinsic signaling.

Auxin distribution is associated in the first divisions of the plant zygote. In vitro cultured embryos were used to show that immediately after division of the zygote the auxin reporter *DR5::GFP* expression is pronounced in the apical cell and auxin accumulation was confirmed using anti-auxin antibody (Friml et al. 2003). This accumulation pattern was disturbed by application of the synthetic influx auxin derivative 2,4D or inhibition of auxin efflux by 1-*N*-naphthylphthalamic acid (NPA) or the vesicle trafficking inhibitor brefeldin A (BFA) implying the efflux machinery as the mechanism of auxin distribution. Specifically, in wild type embryos PIN7 was detected in the basal cell immediately after division of the zygote at the apical side facing the apical cell where PIN1 was expressed without detectable polarity. A low but reproducible fraction of *pin7* mutant and RNAi lines failed to establish the apical-basal auxin gradient and the stereotypical division pattern of the apical cell in some cases failing to establish the proembryo completely. As mentioned above GNOM/EMB30 controls the polarized vesicle trafficking and is required for PIN1 recycling (Steinmann et al. 1999; Geldner et al. 2003) but it may also direct polar PIN7 localization. *gnom*/*emb30* mutants display skewed or symmetric zygotic divisions but also later divisions are abnormal (Mayer U et al. 1993). Thus, *gnom* mediated asymmetric auxin accumulation appears to be essential for proper apical-basal axis specification and PIN7 acts as a player in establishing the initial embryonic auxin distribution. However, all these events occur after the zygotic division which by itself is already asymmetric and the auxin distribution may serve merely to reinforce or act in concert with polar inherited intrinsic factors to determine cell fate post-division.

In summary, the plant zygotic division shows the characteristics of an intrinsic asymmetric division. However, maternal input may be involved in polarizing the egg cell prior to fertilization and external factors appear important in fate stabilization of the generated daughters and their progenies.

4.2 Formation of the Lens Shaped Cell from the Hypophysis

Root formation is initiated at the boundary between the apical and basal cell lineage. When the pro-embryo derived from the apical cell reaches around 32 cells in size the uppermost cell of the basal lineage, the hypophysis i.e. the root founder cell, is specified and incorporated into the embryo. At this stage PIN1 becomes polarly localized in the provascular cells facing the hypophysis and PIN7 localization reverses to the basal side of the suspensor cells. These events correlate to the switch in polar auxin transport from up into the embryo to down towards the hypophysis and out of the

embryo. Next the hypophysial cell divides asymmetrically and generates a small lens-shaped cell that becomes the organizing (quiescent) center (QC) of the root meristem and a larger basal daughter that is the progenitor of the root cap tissue (Fig. 1A). The cells surrounding the QC will differentiate into stem cells for the various root tissues and in turn are dependent on the QC for their maintenance (Sect. 5.2).

The Ser/Thr protein kinase PINOID (PID) was shown to control PIN polarity (Friml et al. 2004) and *pid* mutants display post-embryogenic apical organogenesis defects similar to those of the *pin1* mutant (Bennett et al. 1995). Its function was traced to apical to basal shift of PIN protein localization leading to failure in establishment of local accumulation required for proper apical organ formation. Constitutive overexpression of *PID* during early embryogenesis leads to misspecification of the hypophysis, demonstrated by its abnormal transverse division and absence of root formation in seedlings. These defects were accompanied by complete basal to apical shift of PIN1 polarity and the establishment of an auxin maximum in the pro-embryo (Friml et al. 2004).

Thus auxin transport and signaling is implicated in hypophysial cell specification which in turn is required for its proper asymmetric division. Accordingly, mutants in the auxin response regulators *MP* and *BDL* fail to specify the hypophysis and do not form a lens-shaped cell eventually resulting in a lack of root formation (Berleth and Jurgens 1993; Hamann et al. 1999). BDL and MP interact in planta and overexpression of *MP* can complement the rootless *bdl* phenotype indicating BDL acts by inhibiting MP (Hardtke et al. 2004; Weijers et al. 2006). However, MP and BDL are not expressed nor move to the hypophysis and mRNAs accumulate in the lens-shaped cell only after its formation (Hamann et al. 2002; Weijers et al. 2006). Elegant experiments showed that BDL and MP activity is transiently required in the central provascular cells of the embryo for non-autonomous stable specification of hypophysis fate necessary for its asymmetric division and subsequent root initiation (Weijers et al. 2006).

WOX5 expression is initiated in the hypophysis at the time of its specification in the early globular embryo. After asymmetric hypophysis division *WOX5* is expressed in the lens-shaped cell only and expression is maintained in its descendents, the later QC, throughout the plant life (Haecker et al. 2004; Blilou et al. 2005). *WOX8* is also expressed in the hypophysis but expression ceases in the descendents upon division and becomes restricted to the suspensor. Neither *wox8* nor *wox5* single mutants displayed embryonic patterning defects (Haecker et al. 2004). Therefore their involvement in the asymmetry of division remains a question and it is possible that *WOX5* acts in a genetic redundant fashion e.g. with *WOX8*.

In analogy to zygotic division *WOX* genes may operate as intrinsic factors in asymmetric hypophysis division. In contrast, the role of auxin as extrinsic factor seems opposite to that in zygotic division and is required to

specify the hypophysis before its asymmetric division. Whether auxin plays a role in polarization of the hypophysis is not known.

4.3 Radial Patterning

Embryo tissue layers are patterned as concentric rings around the apical-basal axis. In *Arabidopsis* radial patterning starts in the octant stage embryo with fate establishment of the primary epidermis, ground and vascular tissue types established from the outside in. Initial periclinal asymmetric divisions set off the outer protoderm layer that will differentiate into epidermis (Fig. 1A). This event correlates with the restricted expression of homeodomain genes (Meristem Layer 1 (ML1) and Protodermal Factor 2 (PDF2) to this outer cell layer and their expression remains epidermal throughout development (Lu et al. 1996; Abe et al. 2003). Although both genes are expressed in the pro-embryo from the time that the apical cell is generated they have not been connected to the asymmetric division. ML1 and PDF2 expression is maintained in the above ground epidermis throughout development and they redundantly act in shoot epidermal differentiation. *pdf2ml1* double mutants are seedling lethal and display severe defects, e.g. with leaves lacking an epidermis (Abe et al. 2003).

Early globular embryos display additional asymmetric periclinal divisions forming the ground and provascular tissues (Fig. 1A). Around the triangular stage a next round of periclinal division separates the pericycle from the central provascular tissue (Scheres et al. 1995). Within this central domain a series of asymmetric divisions generate the precursors of the xylem and phloem cell lineages that later differentiate into the corresponding conductive vascular bundles that connect all parts of the plants. The xylem cell lineage forms an axis of cell files across the underlying QC cells. Cells flanking this axis go through a series of asymmetric divisions to form the phloem cell types (Fig. 2). Several genes have been identified that are required for proper radial patterning and differentiation of the vascular system but their role in the occurring asymmetric divisions remains unclear (Bonke et al. 2003; Scarpella and Meijer 2004; Carlsbecker and Helariutta 2005).

At the early torpedo stage the epidermis stem cells that surround the QC have divided asymmetrically and given rise to a layer of lateral root cap (Fig. 2C). Recently, two genes, *FEZ* and *SOMBRERO*, have been identified from a marker based screen in our lab that appear specifically involved in orienting the division plane of this asymmetric epidermis/lateral root cap stem cell division (V. Willemsen, unpublished).

Finally, asymmetric periclinal divisions in the daughters of the ground tissue stem cells give rise to the inner endodermis and outer cortex cell layer (Fig. 2B). This last set of asymmetric divisions has been studied in more detail mainly during post-embryonic development (Sect. 5.1).

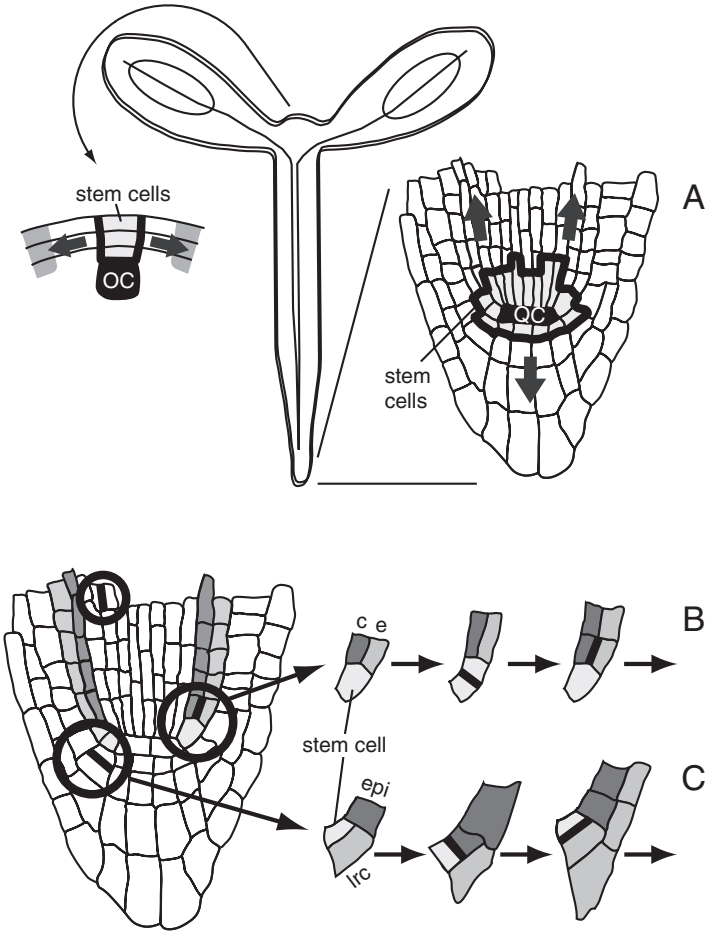

Fig. 2. **A–C** Asymmetric stem cell divisions and radial patterning. **A** Embryogenesis culminates in the seedling with the basic plant body plan. Stem cells in shoot and root meristem are maintained by signals from their organizing center (OC and QC, respectively). Asymmetric divisions retain the stem cells in the niche (light grey/thick line boundary). **B** Endodermis and cortex formation by asymmetric division of the ground tissue stem cell in the root meristem. **C** Epidermis and lateral root cap cells are generated by oriented asymmetric divisions of the epidermis stem cell. *Third circle* shows the position of asymmetric protophloem divisions. Thick lines mark the asymmetric division. See text for detailed descriptions

5
Post-embryonic Asymmetric Cell Division

Plant embryogenesis culminates in the formation of the mature embryo that represents the basic body plan of the plant. Post-embryonically the plant elaborates on this make up by continuously adding new organs during

its lifetime. Meristems provide the cells for repetitive formation of tissues and new organs allowing the plant to grow and develop. Consequently, certain asymmetric divisions observed during embryogenesis reoccur throughout development in defined positions as the plant grows, allowing these complex events to be studied post-embryonically.

5.1 Radial Patterning: Endodermis/Cortex Formation

As mentioned above, endodermis and cortex form as a result from the asymmetric periclinal divisions in the daughters of the ground tissue stem cells (Fig. 2B). Mutants in the *SCARECROW* (*SCR*) and *SHORT-ROOT* (*SHR*) genes encoding related transcription factors of the GRAS family produce only a single ground tissue layer due to the absence of the asymmetric periclinal division in the ground tissue stem cell daughter (Benfey et al. 1993; Scheres et al. 1995; Di Laurenzio et al. 1996; Helariutta et al. 2000). In *scr* mutants, the ground tissue has mixed endodermis/cortex identity indicating that *SCR* does not encode a fate determinant but that it controls asymmetric division (Di Laurenzio et al. 1996; Heidstra et al. 2004). The *shr* mutant ground tissue only displays cortex differentiation making it a candidate endodermis fate determinant (Benfey et al. 1993). However, SHR protein is transiently detected in both sister cells after asymmetric cell division (Nakajima et al. 2001) indicating SHR is a candidate extrinsic factor for post-division mediated asymmetry rather than an intrinsically segregated cell fate determinant.

SCR is expressed in the endodermis, the ground tissue stem cells, and the QC (Di Laurenzio et al. 1996), and expression of *SCR* is strongly reduced in the *shr* mutant background (Helariutta et al. 2000). Interestingly, *SHR* is not transcribed in the ground tissue but in the adjacent provascular cells and SHR protein moves in regulated and targeted fashion into the adjacent endodermis, ground tissue stem cells, and QC where it activates *SCR* (Helariutta et al. 2000; Nakajima et al. 2001; Gallagher et al. 2004). Recent identification of SHR target genes confirmed *SCR* as being one of its direct targets (Levesque et al. 2006).

Clonal deletion experiments demonstrated that *SCR* acts cell-autonomous in asymmetric cell division (Heidstra et al. 2004). Also, periclinal G2 activation clones that separate *SCR* to the outer cells leaving the inner cells devoid of *SCR* upon division still asymmetrically express the endodermis marker to the inner cells showing *SCR* is only transiently required to promote asymmetry. Interestingly, endodermis and cortex specific markers are maintained in their respective ground tissue layer upon clonal *SCR* deletion (Heidstra et al. 2004). This indicates that the *SCR*-mediated asymmetric cell division leads to immediate and stable separation of cell fates possibly involving as yet unknown differential chromatin states.

The asymmetric ground tissue division is complicated in the way that *SCR* induces an asymmetric periclinal division in the daughter of the ground tissue stem cell only, but both SHR and *SCR* are present in the entire endodermis. The involvement of extrinsic "top-down" signals from mature ground tissue cells to reinforce the asymmetric division was inferred from classical ablation experiments whereby ground tissue stem cells isolated from their daughters fail to perform their characteristic periclinal division (van den Berg et al. 1995). Clonal induction of *SCR* in the *scr* mutant however showed that all ground tissue cells are competent to perform the periclinal asymmetric division in the absence of mature endodermis and cortex acting as a patterning template. This result and the strict cell-autonomy of *SCR* action effectively rule out the need for top-down signaling to pattern the ground tissue (Heidstra et al. 2004). The reason that the ground tissue stem cell does not divide periclinally may be linked to non-cell-autonomous signaling from the QC that prevent progression of stem cell differentiation (van den Berg et al. 1997; Sabatini et al. 2003).

Ectopic *SHR* expression from the *SCR* promoter results in supernumerary cell divisions of the ground tissue and additional endodermis layers, suggesting that SHR can only move into the adjacent cell layer and limiting SHR expression is a mechanism to prevent continued activation of *SCR* and additional asymmetric periclinal ground tissue divisions (Nakajima et al. 2001). Rare periclinal ground tissue divisions occurring in *scr* mutants equally segregated endodermal fate markers, one of which was SHR:GFP protein, indicating that SCR is required for the asymmetry of the periclinal ground tissue division and fate separation by restricting SHR movement to the endodermis only (Heidstra et al. 2004). In accordance with this hypothesis, ectopically expressed SHR:GFP moves from the epidermis to the ground tissue in a *scr* mutant background but not in wild type (Sena et al. 2004).

The combined results support a model in which SHR protein moves from the vascular cells to the adjacent ground tissue layer where it induces *SCR* expression. Subsequently, *SCR* cell autonomously induces the asymmetric periclinal division in the ground tissue stem cell daughters because the active QC prevents this division in the ground tissue stem cells. Upon periclinal division rapid endodermis and cortex fate separation occurs and the cells become locked in their respective fates. To ensure a single periclinal ground tissue division, factors required for the execution of the periclinal division are segregated or degraded from the SHR and *SCR* expressing endodermal cells while *SCR* and SHR expression in the outer cortex cell is rapidly reduced. The function of SCR in the endodermis is to sequester SHR and prevent movement beyond a single cell file, thereby preventing the renewed induction of *SCR* in the cortex, which would lead to additional asymmetric divisions. This mechanism effectively limits asymmetric division to ground tissue stem cell daughters. Additional identification SCR downstream targets and analysis of both SHR and SCR targets for their involvement in fate determination and orienting cell division, respectively, may confirm the proposed functions.

5.2 Stem Cell Divisions

Stem cells typically reside in niches that provide the signals for their maintenance (Spradling et al. 2001). In plants the stem cell niche lies in the heart of the meristem (Fig. 2A), which defined as a group of cells that continuously divide. Here, stem cells asymmetrically divide to generate one daughter that will go through a few rounds of division before terminal differentiation and incorporation into the different tissues of the plant. The other daughter remains a stem cell. Growth and development of all the elaborate structures observed in adult plants both above and below ground have their origin in the shoot and root meristems.

In the shoot meristem a group of stem cells, three cell layers deep and defined through clonal analysis (Stewart and Dermen 1970; Furner and Pumfrey 1992; Irish and Sussex 1992), receives signals from the underlying organizing center that keeps the stem cells from differentiating (Fig. 2A). These signals act over several cell layers and their activity needs to be carefully regulated to maintain the balance between the stem cell pool and the surrounding differentiating cells. Genetic and molecular studies have revealed the homeodomain transcription factor WUS, the peptide ligand CLAVATA3 (CLV3) and the CLV1 receptor kinase as members of a feedback loop that maintains the size of the shoot stem cell population. WUS specifies the organizing cells that produce an unknown signal to maintain the overlying stem cells and induce CLV3 expression, which in turn signals back via the CLV1 receptor to restrict the domain of WUS expression (Schoof et al. 2000; Brand et al. 2000). Differentiating stem cell daughters move through a transition zone where they proliferate before entering the sites of organ initiation at the periphery of the meristem where groups of cells are simultaneously specified to constitute the different organ tissues.

In the root meristem laser ablation studies have identified the mitotically less active QC as a source for the stem cell maintenance signal (Fig. 2A) (van den Berg et al. 1997). Ablation of a single QC cell leads to differentiation of the contacting ground tissue stem cells into performing the asymmetric periclinal division generating endodermis and cortex, a process normally observed in its daughter cell (Sect. 5.1). Similarly, columella stem cells underlying the ablated QC cell accumulate starch granules, a characteristic of differentiated columella. Nearby intact QC cells were unable to rescue stem cell differentiation. In contrast to the shoot organizing center (OC) derived long range stem cell maintenance signal, the root QC apparently maintains the surrounding stem cells in a contact dependent fashion. However, recent experiments using plants with lowered *RETINOBLASTOMA-RELATED* levels showed an increase in stem cell layers surrounding the QC that remained under the influence of the QC for their maintenance (Wildwater et al. 2005) indicative of long range signaling similar to the shoot OC. Also, CLV3-type ligands can influence root meristem maintenance although their function appears not to affect the stem cells directly (Casamitjana-Martinez

et al. 2003; Hobe et al. 2003; Fiers et al. 2004). Thus, shoot and root stem cell maintenance signaling may not be so different and may well share conserved players yet to be discovered. The genes that are required to specify QC fate are the *SHR* and *SCR* transcription factors with auxin acting through the *PLETHORA1* and *2* transcription factors providing the spatial input (Sabatini et al. 1999, 2003; Aida et al. 2004). It appears that the role of *SHR* and *SCR* to specify QC fate is different from their function in asymmetric division of the ground tissue stem cell daughters. Transcript profiling of the QC has provided a long list of candidates that may be directly or indirectly involved in the stem cell maintenance (Nawy et al. 2005).

It is evident that in both shoot and root stem cell niches extrinsic factors are required to specify and maintain stem cell fate but the molecular nature of the signals remain elusive. In the shoot meristem CLV3 is considered a marker for stem cell fate and actively restricts its own expression domain (Schoof et al. 2000; Reddy and Meyerowitz 2005) but what determines that its expression is shut down in differentiating stem cell daughters is unknown. In the root meristem no stem cell specific markers have been reported so far. Marker analysis has shown that root stem cells and their daughters already display fate characteristics of the respective tissues formed that have been imposed during earlier asymmetric formative divisions. The extrinsic stem cell maintenance signal may suppress these intrinsic differentiation signals.

5.3 Lateral Root Initiation

Shoot and root branching allows the plant to explore and exploit the environment to optimize growth and development and guarantees its success as an individual in competing with surrounding plants. Shoot branches develop from dormant lateral meristems in the axils of leaves that originate from the shoot apical meristem. In contrast, lateral roots initiate from small groups of founder cells in the pericycle, the outermost layer of the vascular cylinder, opposite the xylem poles (in most species) indicating competence of only a limited set of cells (reviewed in Steeves and Sussex 1989). An ordered set of asymmetric divisions initiate the formation of a lateral root meristem that will subsequently grow out and generate the lateral root (Fig. 1D, Malamy and Benfey 1997; De Smet et al. 2006).

The first asymmetric founder cell divisions take place in the differentiation zone where normally cell division does not occur. However, in *Arabidopsis* it was shown that xylem pole pericycle cells remain competent to divide outside of the meristem (Dubrovsky et al. 2000; Beeckman et al. 2001). The *ABERANT LATERAL ROOT FORMATION* (*ALF*)*4* gene encodes a nuclear protein of unknown molecular function that is required to maintain the pericycle in a mitosis-competent state and as a consequence *alf4*

mutants do not initiate lateral roots (Celenza et al. 1995; DiDonato et al. 2004). Studies including other species indicate that founder cells may already be specified within the meristem (Mallory et al. 1970; Gladish and Rost 1993; Baum et al. 1998), but in the absence of suitable markers it is not yet possible to predict which pericycle cells along the length of the root are primed as lateral root founder cells.

Upon activation, two adjacent founder cells within the same cell file opposite the xylem pole undergo almost simultaneous asymmetric anticlinal divisions generating two short cells flanked by two longer cells (Fig. 1D, Laskowski et al. 1995; Dubrovsky et al. 2001; Casimiro et al. 2001). The asymmetric division is likely preceded by polar localization of founder cell nuclei moving towards each other to the site of the future division as reported in *Allium* (Casero et al. 1993). The two short daughter cells represent the center of the future primordium. The same set of asymmetric founder cell divisions occur in adjacent cells of the flanking pericycle cell files. The enhancer trap line J0121 specifically marks the three files of xylem pole pericycle cells with GFP expression (http://www.plantsci.cam.ac.uk/Haseloff, Casimiro et al. 2001). Subsequent anticlinal divisions occur in the founder cells until a set of eight to ten short cells is created that are similar in length. Following radial expansion the central short cells divide periclinal and asymmetric as observed by differential marker expression in the outer cell layer and generate primordia (Fig. 1D, Malamy and Benfey 1997).

Besides its implication in zygotic and embryonic root founder cell division, auxin plays a critical role in several steps during lateral root formation including the initiation of asymmetric founder cell division (reviewed in Malamy 2005; De Smet et al. 2006). First, the auxin response marker *DR5::GUS* stains the founder cells prior to their asymmetric division. Second, inhibition of auxin transport by NPA arrests lateral root development by blocking the first asymmetric anticlinal division of the founder cells but retain their xylem pole pericycle identity based on J0121 expression (Casimiro et al. 2001). Interestingly, the same effect is observed upon cytokinin application involving CRE1 mediated signaling (Li et al. 2006). Transfer to medium containing auxin induced asymmetric anticlinal divisions in xylem pole pericycle cells covering the entire root. This lateral root inducible system was used to connect auxin to cell cycle activation during lateral root initiation. The amount and direction of auxin flow in the roots was shown to determine the frequency and position of lateral root initiation sites along the apical-basal axis (Himanen et al. 2002, 2004). Similarly, auxin application on polar auxin transport mutants induces homogeneous proliferation in the whole pericycle and roots fail to produce actual lateral roots (Benkova et al. 2003; Geldner et al. 2004). Local production of ethylene by differentiating protoxylem was proposed to determine the site of lateral root initiation by locally inhibiting auxin transport resulting in an auxin peak in the founder cells (Aloni et al. 2006). Third, in the dominant *solitary-root 1* (*slr1*) mutant, the AUX/IAA protein IAA14 is stabilized,

strongly inhibiting the auxin response towards lateral root initiation. As a result, a partial block of asymmetric founder cell division and a total block of the periclinal cell division of the divided pericycle cells prevent the formation of lateral roots (Fukaki et al. 2002). *SLR1/IAA14* is expressed in the xylem-pole pericycle cells in mature root tissues corresponding to the site of lateral root initiation. Interaction between SLR1/IAA14 and the NPH4/ARF7 and ARF19 was shown indicating SLR1/IAA14 likely inhibits the activity of these ARFs. Consistently, the *arf7arf19* double mutant initially fails to produce lateral roots (Fukaki et al. 2005; Okushima et al. 2005; Wilmoth et al. 2005). Recently, the PICKLE (PKL) chromatin remodeling factor, identified from a *slr1* suppressor screen, was shown to be required for SLR1 mediated repression of ARF7 and ARF19 activity (Fukaki et al. 2006). Inducing anticlinal pericycle cell divisions by overexpression of *CYCD3;1* in *slr1* did not initiate lateral roots nor *plt1-1::GUS* expression at sites of future lateral root primordia (Aida et al. 2004; Vanneste et al. 2005).

The massive initiation of division in pericycle cells questions the concept of founder cells early in the meristem and argues in favor of xylem pole pericycle cell competence. Local extrinsic factors, involving auxin, then determine founder cell fate and initiate the asymmetric division and lateral root formation. Genomics approaches targeted specifically on the lateral root initiation have identified many genes and their functional analysis is required to reveal involvement in fate specification and asymmetric division (Himanen et al. 2004; Vanneste et al. 2005). One of the intriguing candidate targets found to be SLR1/IAA14-mediated auxin inducible is *WOX13*, a member of the *WOX* homeodomain gene family that are also associated with asymmetric division during embryogenesis (Vanneste et al. 2005).

5.4 Stomata

Stomata are specialized epidermal structures that consist of two guard cells around a pore acting as turgor operated valves to regulate gas exchange between the plant and its environment (Fig. 1C). Stomata are present in the epidermis of almost all shoot organs. Coordinated spatial patterning of stomata allows optimal regulation of CO_2 uptake, evaporation and temperature and overall stomata numbers are controlled by these environmental cues. Stomata on the leaf surface are embedded in fields of epidermal cells including trichomes with their accessory cells, and large jigsaw puzzle shaped pavement cells (Nadeau and Sack 2002b). As a rule, stomata are separated from each other by at least one cell (Sachs 1991). Both formation and spacing of stomata arises through specifically placed asymmetric division (Larkin et al. 1997; Croxdale 2000; Nadeau and Sack 2002b).

Development of stomata in *Arabidopsis* involves three types of precursor cells recognized predominantly by anatomical criteria. The meristemoid

mother cells (MMC) are specified from relatively small epidermal cells. The MMC divides asymmetric to generate a small triangular meristemoid and a larger sister cell. Meristemoids generally can divide up to three times asymmetrically, each time regenerating a smaller cell with meristemoid fate and a larger sister cell. These divisions can be considered stem cell-like divisions. The larger sister cell has a certain developmental plasticity. It may not divide and remain small or enlarge and become a pavement cell. It can become a MMC and divide asymmetrically to generate a satellite meristemoid. Or, it may divide symmetrically and generate daughters that inherit the developmental plasticity. Eventually meristemoids differentiate into guard mother cells (GMC) that divide symmetrically to produce the two guard cells around a pore that constitute the stoma (Fig. 1C). MMCs and meristemoids that are committed to divide are polarized with the nucleus and PPB located at the future site of division. (Zhao and Sack 1999; Nadeau and Sack 2002b; Lucas et al. 2006).

In the hypocotyl, stomata patterning is guided by the epidermal patterning genes that also direct the spatial formation of root hairs and trichomes (reviewed in Serna 2005). In contrast, the initial specification of MMCs in developing leaves appears quite arbitrary allowing adjacent MMCs to form. Also the first asymmetric division is randomly oriented and can produce contacting meristemoids indicating the absence of signaling between MMCs. However, to restore the one cell spacing rule, one of the meristemoids subsequently specifically divides away from the other. Alternatively, one of the meristemoids may become arrested. Similarly, neighbor cells next to a stoma, meristemoid or GMC may enter the stomatal pathway and become MMCs in which case the first asymmetric division is directed away from these cells (Fig. 1C). These so-called spacing divisions in neighbor MMCs and meristemoids likely involve extrinsic intercellular signaling on the position of a stoma, meristemoid or GMC in order to orient the plane of division (Sachs 1991; Geisler et al. 2000b; Lucas et al. 2006).

Thus, extrinsic factors may be involved in specifying the MMC whereas intrinsic factors may be separated by its asymmetric division generating the meristemoid daughter that in turn segregates the intrinsic fate determinants upon asymmetric division regenerating itself. However, the plane of division is under the influence of extrinsic intercellular signaling factors communicated by a nearby meristemoid, GMC or stoma.

Several genes are known to play a role in the intercellular signaling pathway. These are *STOMATAL DENSITY AND DISTRIBUTION 1* (*SDD1*, a putative subtilicin-like extra cytoplasmic protease), *TOO MANY MOUTHS* (*TMM*, a transmembrane leucine-rich repeat (LRR) receptor-like protein), *ERECTA* (*ER*) and its functional paralogues *ERL1* and *ERL2* (transmembrane LRR receptor-like kinases), and *YDA* (the MAPKK kinase described earlier, Sect. 4.1). Single knockouts in *SDD1*, *TMM*, *YDA* the triple mutant *ererl1erl2* all confer increased stomatal density

and clustering having more cells entering the stomata lineage and failing to orient the division planes to obey the one cell spacing rule (Yang M and Sack 1995; Berger and Altmann 2000; Nadeau and Sack 2002a; Von Groll et al. 2002; Bergmann et al. 2004; Shpak et al. 2005; Lucas et al. 2006). Based on the gene identities and their genetic interaction a working model was proposed in which TMM forms a receptor heterodimers with ER family receptor kinases. The complex interaction of TMM and ER family receptors suggests they work in different combinations to fine tune decisions on asymmetric division and fate determination (Shpak et al. 2005). SDD1 is required to activate the ligand for TMM/ER receptors, which signals to downstream MAPK cascades via YDA to repress stomata formation, regulate asymmetric division orientation and frequency (Bergmann et al. 2004; Gray and Hetherington 2004; Sack 2004; Serna 2004; Shpak et al. 2005). The proposed signaling cascade is in agreement with the observed expression patterns. *SDD1* is highly expressed in meristemoids and GMCs and *YDA* is expressed low uniformly throughout the plant (Von Groll et al. 2002; Bergmann et al. 2004). Initially, *TMM*, *ER*, *ERL1* and *ERL2* are uniformly expressed in the protoderm of developing leaves after which *ER* expression falls below detection in the differentiating epidermis. The expression of *ERL1* and *2* remains in all cells of the stomatal lineage with highest levels in the meristemoid very similar to *TMM* expression with the exception that *TMM* is not expressed in mature guard cells (Nadeau and Sack 2002a; Shpak et al. 2005).

The finding that the smaller daughter cell invariably becomes the meristemoid and meristemoids have never been observed to undergo a symmetric division suggested segregation of intrinsic factors (Geisler et al. 2000a; Nadeau and Sack 2003; Lucas et al. 2006). Candidate intrinsic factors are the partly redundant MYB transcription factor MYB88 and *FOUR LIPS* (*FLP*) that regulate GMC differentiation with *flp* mutants forming laterally aligned clusters of stomata (Yang M and Sack 1995; Lai et al. 2005). The *FAMA* basic helix-loop-helix (bHLH) transcription factor is another putative intrinsic determinant with *fama* mutants forming clusters of incompletely differentiated cells lacking stomata indicating its involvement in GMC differentiation (Bergmann et al. 2004; Ohashi-Ito and Bergmann 2006). With the discovery of the upstream acting *FAMA* paralogues *SPEECHLESS* (*SPCH*) and *MUTE* a group of bHLH genes was identified that are expressed and function in consecutive steps in stomatal development (MacAlister et al. 2007; Pillitteri et al. 2007). *spch* and *mute* mutants have no stomata with *spch* showing no signs of asymmetric divisions leading to meristemoid formation while *mute* forms meristemoids that undergo multiple rounds of asymmetric division without terminating into guard cells. Together with genetic interaction and ectopic expression data a model was proposed where SPCH initiates the asymmetric divisions towards stomatal development, MUTE constitutes the fate switch for meristemoid to GMC transition, and FAMA promotes guard cell identity.

This cascade is regulated by the intercellular signaling pathway to set the spacing and density of stomata (MacAlister et al. 2007; Pillitteri et al. 2007). *FAMA* comes from a genomics approach comparing *yda* with constitutively active Δ*N-YDA* plants (Bergmann et al. 2004). Seedlings homozygous for Δ*N-YDA* totally lacked stomata nor did they express a *TMM::GFP* marker for stomata precursors indicating *YDA* acts as a developmental switch similar to its role in fate selection during asymmetric division of the zygote (Bergmann et al. 2004; Lukowitz et al. 2004). Transcriptome analysis identified over 200 oppositely expressed transcripts between Δ*N-YDA* and *yda*. Genes establishing stomata identity or involved in regulating the asymmetric division orientation were predicted to be up regulated in *yda* and down regulated in ΔN-YDA. This was confirmed by identifying *TMM* and *SDD1* in this gene set and further tested by selecting the *FAMA* transcription factor (Bergmann et al. 2004). Further analysis of the data set will reveal if additional candidate extrinsic and intrinsic factors regulating the asymmetric division are present. However, genes with additional roles in development or genes functioning in other asymmetric divisions and therefore not only expressed in the stomata pathway may be missed as demonstrated by the absence of the *ER* family receptor kinase genes, that promote above-ground organ growth (Shpak et al. 2003, 2004), from the differential gene set.

5.5 Pollen Development

In flowering plants, male reproductive development begins with the initiation and formation of the stamens, the male reproductive organs, in the flower (Fig. 1B). Stamens consist of an anther, where pollen development takes place and a filament that supports the anther. Inside the anther the pollen mother cells undergo meiosis to form a tetrad of haploid microspores. As the microspores are freed from the tetrads by degradation of the surrounding tetrad callose wall they enlarge and undergo cytoplasmic reorganization. The result is a polarized cell with a large single vacuole and most of the cytoplasm at one side and the nucleus at the other side. Next the microspore performs a mitotic asymmetric division (pollen mitosis I, PMI) forming a large vegetative cell and a smaller generative cell. The generative cell is completely engulfed in the cytoplasm of the vegetative cell. Each cell of this bicellular pollen grain has a markedly different fate. In Arabidopsis, the generative cell undergoes another mitotic division (pollen mitosis II, PMII) giving rise to two sperm cells (Fig. 1B). The large vegetative cell does not divide again but, upon pollination, forms the pollen tube to deliver both sperm cell nuclei to the embryo sac in the ovule, where they participate in double fertilization (reviewed in Twell et al. 1998; McCormick 2004).

Experiments using in vitro cultured tobacco microspores provide evidence for separation of intrinsic fate determinants during asymmetric division.

Isolated uninucleate microspores undergo normal asymmetric division in vitro and are capable of fertilization (Tupy et al. 1991; Eady et al. 1995). Induction of symmetric PMI division in vitro using cold, caffeine, centrifugation or low concentrations of the microtubule inhibitor colchicine can result in the formation of two equally sized cells that do not have nuclear chromatin characteristic of the vegetative cell and that express the vegetative cell fate specific *LAT52* marker (Terasaka and Niitsu 1987; Eady et al. 1995). High colchicine levels block PMI, resulting in uninucleate pollen still expressing lat52 (Eady et al. 1995). Similarly, in the *solo pollen* mutant mature pollen grains may contain only a single nucleus and display vegetative cell fate (Howden et al. 1998). Together, these data demonstrate that microspores develop into a vegetative cell by default and that microtubule assisted asymmetric division is essential for generative cell differentiation.

The specific microspore nuclear migration pattern indicates polarity is decided early and possibly during tetrad formation (Twell et al. 1998). This is confirmed by mutations in the *TETRASPORE/STUD (TES/STD)* gene that encodes a kinesin functioning as a microtubule-associated motor directing cytokinesis after meiosis of the pollen mother cell. Consequently, the *tes/std* mutants form large microspores composed of four nuclei within a common cytoplasm surrounded by a callose wall. Nevertheless, all four nuclei undergo nuclear migration and normal asymmetric division within the common cytoplasm and up to four sperm-cell pairs were observed in mature *tes/std* pollen grains (Hulskamp et al. 1997; Spielman et al. 1997; Yang CY et al. 2003). This suggests that polarity determinants are already laid down towards the radial wall before separation of the microspores. Although the cell wall and its composition are important for pollen development, germination and growth (summarized in Boavida et al. 2005), nothing is known about the nature and involvement of components in the asymmetry of microspore division.

Molecular genetic studies have identified a couple of genes that affect the asymmetric microspore division. The *sidecar pollen* (*scp*) mutation causes some of the developing pollen grains to undergo a premature and symmetric cell division that produces two vegetative cells. One of the vegetative cells then continues to perform the normal asymmetric division forming the generative cell (Chen and McCormick 1996) supporting the pre-existence of a polarity cue. *SCP* might then be involved in preventing premature division until polarity is fully established or, alternatively, direct the orientation of division such that an asymmetrically localized gene product is properly partitioned. Failure to do so would result in lethality or initial symmetric fates in some cases followed by an asymmetric division still occurring in the sister cell with the correct polar determinants.

The *gemini pollen 1 (gem1)* mutants arrest after PMI produce a substantial proportion of microspores that either fail to establish a cell plate or produce partial or irregular branching cell walls that alter division symmetry. In symmetrically divided cells, both daughter cells display vegetative cell fate. In contrast to *scp*, symmetrical divisions in *gem1*mutants do not

occur premature. Typically, the *gem1* mutant exhibits uncoupled nuclear division from cytokinesis resulting in either binucleate or bicellular pollen (Park et al. 1998). *GEM1* encodes a microtubule-associated protein with a quite general role in microtubule stability and is also required for correct positioning of the phragmoplast during cytokinesis at PMI (Whittington et al. 2001; Twell et al. 2002).

In *two-in-one pollen* (*tio*) mutants the mature pollen grains contain two nuclei and displays vegetative cell fate characteristics. Polar nuclear migration and asymmetric nuclear division at PMI occur normally in *tio* mutants. However, dividing microspores have incomplete callose walls that are correctly positioned at the generative cell pole but are degraded before mid-bicellular pollen stage (Oh et al. 2005). *TIO* encodes the *Arabidopsis* member of the FUSED(FU) Ser/Thr protein kinase family which has evolved to a very different and general role as an essential phragmoplast-associated protein when compared to its counterparts that function in the animal hedgehog signaling pathway (Lum and Beachy 2004; Oh et al. 2005). Nevertheless, the conserved domains between TIO and FU and extrapolating from FU binding partners suggest the kinesin TES as a possible binding partner in regulating phragmoplast expansion (Oh et al. 2005).

Recently, a candidate segregated fate determinant encoding a germline-restrictive silencing factor (GRSF) was identified from lily (Haerizadeh et al. 2006). GRSF is present in nongerm cells, in uninucleate microspores and in the vegetative cell nucleus but absent from the generative cell nucleus. GRSF was shown to bind silencer sequences in promoters of genes specific to the male germline thereby stably repressing these genes in cells other than the germ cells. The presence of *GRSF* orthologs and similar cis-acting silencers in male germline specific genes in other plant species suggests the described silencing may be a general mechanism to regulate male germline specific gene expression (Haerizadeh et al. 2006)

Genomic studies in recent years have uncovered thousands of genes being expressed during the different stages of pollen development (Becker et al. 2003; Honys and Twell 2004; Boavida et al. 2005). While providing a wealth of information, further analysis is needed to select targets for functional studies among these. Meanwhile, clever genetic screens like marker assisted screening for gametophytic mutants (Johnson et al. 2004) continue to identify new genes with roles in asymmetric microspore division.

6 Summary

In animals, subcellular localization of specific intrinsic RNAs and/or protein is a key mechanism through which cells become polarized and the unequal inheritance of these factors can direct daughter cell fate (Roegiers and Jan 2004). PAR proteins polarize the cell by connecting to the

cytoskeleton and steer the orientation of division. Even so, the asymmetric distribution of these factors may be guided by positional cues from the surrounding tissues. Alternatively, mRNAs/proteins can be equally divided over the two daughters following zygotic division and external cues can direct their asymmetric expression or degradation.

The best studies cases of asymmetric division in plants appear diverse in that very different genes have been implicated in each of these (Table 1). However, there are important common factors. *WOX* genes are implicated intrinsic factors in asymmetric zygote division, in generating the progenitors of the root stem cell organizing cells during embryonic root formation, and in lateral root founder cell asymmetric divisions. The YDA MAPKK kinase is another common factor and suggests MAP kinase signaling in asymmetric division may be specific for plants. Finally, a persuasive association of auxin signaling with a variety of asymmetric cell divisions is observed. Auxin distribution requires polarized cells and its accumulation and signaling appears to be a common extrinsic factor to specify or enforce cell fate prior (hypophysis, lateral root founder cells) or immediately after (zygote) asymmetric division. But whether auxin is directly involved in determining the asymmetry of division and through what mechanisms is unknown.

Polarization clearly occurs in plant asymmetric cell division as evidenced by the zygote, lateral root founder cells, stomata progenitors and microspores before division. Yet, how cells acquire polarity is not completely understood and a framework for cell polarization leading to division orientation and fate segregation is lacking. The PIN proteins involved in polar auxin transport as well as ROPs display characteristic polar expression and hence can be used as valuable markers to study the acquisition of cell polarity. But proteins connecting the polar distribution of the PINs and other factors to the polarization machinery are unknown.

Plant asymmetric divisions do involve intrinsic and extrinsic mechanisms but the causal link with asymmetric localization of intrinsic factors within a cell and the nature of the external factors remain elusive. The asymmetric pollen mitosis I division producing a vegetative and a generative cell presents a good example for the segregation of intrinsic factors that determine daughter fate. The mutants involved are disturbed in proteins connected to the cytoskeleton and orientation of cell division plane. However, the polarization cue is laid down early in microspore development and the partitioned intrinsic factors remain elusive. Similarly, in the zygote, asymmetric cues may be laid down in the egg cell before fertilization making asymmetric zygotic division an intrinsic process. The *WOX* genes represent a class of putative intrinsic factors whose mRNA or protein segregation may direct the fate of daughter cells upon division. In the ground tissue, the moving SHR protein determines endodermis fate but its function is dependent on other intrinsic or extrinsic factors (possibly involving SCR) to confine the protein after asymmetric division. *SCR* presents an entry

Table 1. Genes implicated in plant asymmetric cell divisions

Gene	Identity	Asymm. Div.	References
WOX2	Homeodomain transcription factor	Zygote	Haecker et al. 2004
WOX5	Homeodomain transcription factor	Hypophysis	Haecker et al. 2004
WOX8	Homeodomain transcription factor	Zygote	Haecker et al. 2004
		Hypophysis	
WOX9	Homeodomain transcription factor	Zygote	Ning et al. 2006
			Haecker et al. 2004
WOX13	Homeodomain transcription factor	Lateral root	Haecker et al. 2004
			Vanneste et al. 2005
YODA (YDA)	MAPKK kinase	Zygote	Lukowitz et al. 2004
		Stomata	Bergmann et al. 2004
GNOM	ARF-GEF for polar vesicle transport	Zygote	Steinmann et al. 1999
			Geldner et al. 2003
PIN7	Membrane localized auxin efflux facilitator	Zygote	Friml et al. 2003
PIN1	Membrane localized auxin efflux facilitator	Hypophysis	Friml et al. 2004
PINOID (PID)	Ser/Thr kinase controls PIN polarity	Hypophysis	Friml et al. 2004
MONOPTEROS (MP)	ARF5 transcription factor	Hypophysis	Berleth and Jurgens 1993
			Wijers et al. 2006
BODENLOS (BDL)	AUX/IAA12 binds and inhibits MP	Hypophysis	Hamann et al. 1999
			Weijers et al. 2006

(*Continued*)

Table 1. Genes implicated in plant asymmetric cell divisions—(*Cont'd*)

Gene	Identity	Asymm. Div.	References
SHORT ROOT (SHR)	GRAS transcription factor	Radial, endo./cortex	Benfey et al. 1993 Helariutta et al. 2000 Nakajima et al. 2001
SCARECROW (SCR)	GRAS transcription factor	Radial, endo./cortex	Di Laurenzio et al. 1996 Heidstra et al. 2004
SOLITARY ROOT 1 (SLR1)	AUX/IAA14 inhibits ARF7/ARF19	Lateral root	Fukaki et al. 2002 Fukaki et al. 2005 Vanneste et al. 2005
PICKLE (PKL)	Chromatin remodeling factor	Lateral root	Fukaki et al. 2006
NONPHOTOTROPIC HYPOCOTYL 4 (NPH4) /ARF7 and ARF19	ARF transcription factor	Lateral root	Fukaki et al. 2005 Okushima et al. 2005 Wilmoth et al. 2005
STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1)	Subtilicin-like extra cytoplasmic protease	Stomata	Von Groll et al. 2002
TOO MANY MOUTHS (TMM)	Transmembrane LRR receptor	Stomata	Nadeau and Sack 2002a

ERECTA (ER), ER-like1 (ERL1) and ERL2	Transmembrane LRR receptor kinases	Stomata	Shpak et al. 2005
FOUR LIPS (FLP) and MYB88	R2R3 MYB transcription factor	Stomata	Yang M and Sack 1995 Lai et al. 2005
FAMA	bHLH transcription factor	Stomata	Bergmann et al. 2004 Ohashi-Ito and Bergmann 2006
SPEECHLESS (SPCH) and MUTE	bHLH transcription factor	Stomata	MacAlister et al. 2007 Pillitteri et al. 2007
SOLO POLLEN	-	Pollen	Howden et al. 1998
TETRASPORE/STUD (TES/STD)	Kinesin	Pollen	Yang CY et al. 2003
SIDECAR POLLEN (SCP)	-	Pollen	Chen and McCormick 1996
GEMINI POLLEN 1 (GEM1)	Microtubule associated protein	Pollen	Twell et al. 2002

into the signaling cascade to direct the orientation of division. An elaborate extrinsic signaling cascade has been exposed in orienting asymmetric division planes during stomata development. However, the actual signal originating from the stomata precursors is unknown. Signaling mutants form clusters of stomata suggesting segregation of intrinsic fate determinants once the stomata lineage is determined may also be controlled by this cascade. Identifying the ligand and determining how the signaling cascade translates into control of cell division plane are major challenges for the future.

In conclusion, recent studies have provided candidate intrinsic and extrinsic factors for several asymmetric divisions in plants. Nevertheless, the main questions mostly remain: What are the intrinsic cues and what is the mechanism by which they polarize the cell prior to asymmetric division? What are the extrinsic cues that determine daughter fates during asymmetric cell division, what are the signaling partners involved? Which proteins connect polarity to the cytoskeleton and how do they direct the orientation of division? Finally, are all the components involved in the asymmetric cell divisions going to be linked to one or more "ground themes" for plant asymmetric division? When the research on plant models continues to progress with the current pace, we should soon learn the answer to these remaining questions.

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References

Abe M, Katsumata H, Komeda Y, Takahashi T (2003) Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in Arabidopsis. Development 130:635–643

Aida M, Beis D, Heidstra R, Willemsen V, Blilou I, Galinha C, Nussaume L, Noh YS, Amasino R, Scheres B (2004) The PLETHORA genes mediate patterning of the Arabidopsis root stem cell niche. Cell 119:109–120

Aloni R, Aloni E, Langhans M, Ullrich CI (2006) Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann Bot (Lond) 97:883–893

Bagnat M, Simons K (2002) Cell surface polarization during yeast mating. PNAS 99:14183–14188

Baum SF, Karanastasis L, Rost TL (1998) Morphogenetic effect of the herbicide Cinch on Arabidopsis thaliana root development. J Plant Growth Regul 17:107–114

Becker JD, Boavida LC, Carneiro J, Haury M, Feijo JA (2003) Transcriptional profiling of Arabidopsis tissues reveals the unique characteristics of the pollen transcriptome. Plant Physiol 133:713–725

Beeckman T, Burssens S, Inze D (2001) The peri-cell-cycle in Arabidopsis. J Exp Bot 52:403–411
Benfey PN, Linstead PJ, Roberts K, Schiefelbein JW, Hauser MT, Aeschbacher RA (1993) Root development in Arabidopsis: four mutants with dramatically altered root morphogenesis 47. Development 119:57–70
Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115:591–602
Bennett SRM, Alvarez J, Bossinger G, Smyth DR (1995) Morphogenesis in pinoid mutants of Arabidopsis thaliana. Plant J 8:505–520
Berger D, Altmann T (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in Arabidopsis thaliana. Genes Dev 14:1119–1131
Bergmann DC, Lukowitz W, Somerville CR (2004) Stomatal development and pattern controlled by a MAPKK kinase. Science 304:1494–1497
Berleth T, Jurgens G (1993) The role of the monopteros gene in organising the basal body region of the Arabidopsis embryo. Development 118:575–587
Betschinger J, Knoblich JA (2004) Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol 14:R674–R685
Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B (2005) The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature 433:39–44
Boavida LC, Becker JD, Feijo JA (2005) The making of gametes in higher plants. Int J Dev Biol 49:595–614
Bonke M, Thitamadee S, Mahonen AP, Hauser MT, Helariutta Y (2003) APL regulates vascular tissue identity in Arabidopsis. Nature 426:181–186
Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R (2000) Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science 289:617–619
Burridge K, Wennerberg K (2004) Rho and Rac take center stage. Cell 116:167–179
Carlsbecker A, Helariutta Y (2005) Phloem and xylem specification: pieces of the puzzle emerge. Curr Opin Plant Biol 8:512–517
Casamitjana-Martinez E, Hofhuis HF, Xu J, Liu CM, Heidstra R, Scheres B (2003) Root-specific CLE19 overexpression and the sol1/2 suppressors implicate a CLV-like pathway in the control of Arabidopsis root meristem maintenance. Curr Biol 13:1435–1441
Casero PJ, Casimiro I, Rodríguez-Gallardo L, Martín-Partido G, Lloret PG (1993) Lateral root initiation by asymmetrical transverse divisions of pericycle cells in adventitious roots of Allium cepa. Protoplasma 176:138–144
Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inze D, Sandberg G, Casero PJ, Bennett M (2001) Auxin transport promotes Arabidopsis lateral root initiation. Plant Cell 13:843–852
Celenza JL Jr, Grisafi PL, Fink GR (1995) A pathway for lateral root formation in Arabidopsis thaliana. Genes Dev 9:2131–2142
Chen YC, McCormick S (1996) Sidecar pollen, an Arabidopsis thaliana male gametophytic mutant with aberrant cell divisions during pollen development. Development 122:3243–3253
Croxdale JL (2000) Stomatal patterning in angiosperms. Am J Bot 87:1069–1080
De Smet I, Vanneste S, Inze D, Beeckman T (2006) Lateral root initiation or the birth of a new meristem. Plant Mol Biol 60:871–887

Dhonukshe P, Kleine-Vehn J, Friml J (2005) Cell polarity, auxin transport, and cytoskeleton-mediated division planes: who comes first? Protoplasma 226:67–73

Di Laurenzio L, Wysocka-Diller J, Malamy JE, Pysh L, Helariutta Y, Freshour G, Hahn MG, Feldmann KA, Benfey PN (1996) The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell 86:423–433

DiDonato RJ, Arbuckle E, Buker S, Sheets J, Tobar J, Totong R, Grisafi P, Fink GR, Celenza JL (2004) Arabidopsis ALF4 encodes a nuclear-localized protein required for lateral root formation. Plant J 37:340–353

Dubrovsky JG, Doerner PW, Colon-Carmona A, Rost TL (2000) Pericycle cell proliferation and lateral root initiation in Arabidopsis. Plant Physiol 124:1648–1657

Dubrovsky JG, Rost TL, Colon-Carmona A, Doerner P (2001) Early primordium morphogenesis during lateral root initiation in Arabidopsis thaliana. Planta 214:30–36

Eady C, Lindsey K, Twell D (1995) The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. Plant Cell 7:65–74

Fiers M, Hause G, Boutilier K, Casamitjana-Martinez E, Weijers D, Offringa R, van der Geest L, van Lookeren Campagne M, Liu CM (2004) Mis-expression of the CLV3/ESR-like gene CLE19 in Arabidopsis leads to a consumption of root meristem. Gene 327:37–49

Fowler JE, Quatrano RS (1997) Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall. Annu Rev Cell Dev Biol 13:697–743

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jurgens G (2003) Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature 426:147–153

Friml J, Yang X, Michniewicz M, Weijers D, Quint A, Tietz O, Benjamins R, Ouwerkerk PB, Ljung K, Sandberg G, Hooykaas PJ, Palme K, Offringa R (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science 306:862–865

Fukaki H, Tameda S, Masuda H, Tasaka M (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. Plant J 29:153–168

Fukaki H, Nakao Y, Okushima Y, Theologis A, Tasaka M (2005) Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in Arabidopsis. Plant J 44:382–395

Fukaki H, Taniguchi N, Tasaka M (2006) PICKLE is required for SOLITARY-ROOT/IAA14-mediated repression of ARF7 and ARF19 activity during Arabidopsis lateral root initiation. Plant J 48:380–389

Furner I, Pumfrey JE (1992) Cell fate in the shoot apical meristem of Arabidopsis thaliana. Development 115:755–764

Gallagher KL, Paquette AJ, Nakajima K, Benfey PN (2004) Mechanisms regulating SHORT-ROOT intercellular movement. Curr Biol 14:1847–1851

Geisler M, Nadeau J, Sack FD (2000a) Oriented asymmetric divisions that generate the stomatal spacing pattern in arabidopsis are disrupted by the too many mouths mutation. Plant Cell 12:2075–2086

Geisler M, Nadeau J, Sack FD (2000b) Oriented asymmetric divisions that generate the stomatal spacing pattern in Arabidopsis are disrupted by the too many mouths mutation. Plant Cell 12:2075–2086

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jurgens G (2003) The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112:219–230

Geldner N, Richter S, Vieten A, Marquardt S, Torres-Ruiz RA, Mayer U, Jurgens G (2004) Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of Arabidopsis. Development 131:389–400

Gladish DK, Rost TL (1993) The effects of temperature on primary root growth dynamics and lateral root distribution in garden pea (Pisum sativum L., cv. "Alaska"). Environ Exp Bot 33:243–258

Gray JE, Hetherington AM (2004) Plant development: YODA the stomatal switch. Curr Biol 14:R488–R490

Gu Y, Wang Z, Yang Z (2004) ROP/RAC GTPase: an old new master regulator for plant signaling. Curr Opin Plant Biol 7:527–536

Haecker A, Gross-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T (2004) Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in Arabidopsis thaliana. Development 131:657–668

Haerizadeh F, Singh MB, Bhalla PL (2006) Transcriptional repression distinguishes somatic from germ cell lineages in a plant. Science 313:496–499

Hamann T, Mayer U, Jurgens G (1999) The auxin-insensitive bodenlos mutation affects primary root formation and apical-basal patterning in the Arabidopsis embryo. Development 126:1387–1395

Hamann T, Benkova E, Baurle I, Kientz M, Jurgens G (2002) The Arabidopsis BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. Genes Dev 16:1610–1615

Hardtke CS, Ckurshumova W, Vidaurre DP, Singh SA, Stamatiou G, Tiwari SB, Hagen G, Guilfoyle TJ, Berleth T (2004) Overlapping and non-redundant functions of the Arabidopsis auxin response factors MONOPTEROS and NON-PHOTOTROPIC HYPOCOTYL 4. Development 131:1089–1100

Heidstra R, Welch D, Scheres B (2004) Mosaic analyses using marked activation and deletion clones dissect Arabidopsis SCARECROW action in asymmetric cell division. Genes Dev 18:1964–1969

Helariutta Y, Fukaki H, Wysocka-Diller J, Nakajima K, Jung J, Sena G, Hauser MT, Benfey PN (2000) The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. Cell 101:555–567

Himanen K, Boucheron E, Vanneste S, de Almeida Engler J, Inze D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell 14:2339–2351

Himanen K, Vuylsteke M, Vanneste S, Vercruysse S, Boucheron E, Alard P, Chriqui D, Van MM, Inze D, Beeckman T (2004) Transcript profiling of early lateral root initiation. Proc Natl Acad Sci USA 101:5146–5151

Hobe M, Müller R, Grünewald M, Brand U, Simon R (2003) Loss of CLE40, a protein functionally equivalent to the stem cell restricting signal CLV3, enhances root waving in Arabidopsis. Devel Genes Evol 213:371–381

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol 5:R85

Horvitz HR, Herskowitz I (1992) Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. Cell 68:237–255

Howden R, Park SK, Moore JM, Orme J, Grossniklaus U, Twell D (1998) Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in Arabidopsis. Genetics 149:621–631

Hulskamp M, Parekh NS, Grini P, Schneitz K, Zimmermann I, Lolle SJ, Pruitt RE (1997) The STUD gene is required for male-specific cytokinesis after telophase II of meiosis in Arabidopsis thaliana. Devel Biol 187:114–124

Irish VF, Sussex IM (1992) A fate map of the Arabidopsis embryonic shoot apical meristem. Development 115:745–753

Itoh J, Nonomura K, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, Kitano H, Nagato Y (2005) Rice plant development: from zygote to spikelet. Plant Cell Physiol 46:23–47

Jeff A, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature 379:66–69

Jenik PD, Barton MK (2005) Surge and destroy: the role of auxin in plant embryogenesis. Development 132:3577–3585

Johnson MA, von Besser K, Zhou Q, Smith E, Aux G, Patton D, Levin JZ, Preuss D (2004) Arabidopsis hapless mutations define essential gametophytic functions. Genetics 168:971–982

Jurgens G (2005) Cytokinesis in higher plants. Annu Rev Plant Biol 56:281–299

Kemphues KJ, Priess JR, Morton DG, Cheng NS (1988) Identification of genes required for cytoplasmic localization in early C. elegans embryos. Cell 52:311–320

Knox JP (1997) The use of antibodies to study the architecture and developmental regulation of plant cell walls. Int Rev Cytol 171:79–120

Konopka CA, Schleede JB, Skop AR, Bednarek SY (2006) Dynamin and cytokinesis. Traffic 7:239–247

Kranz E, Lorz H (1993) In vitro fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. Plant Cell 5:739–746

Kranz E, Wiegen P, Lorz H (1995) Early cytological events after induction of cell division in egg cells and zygote development following in vitro fertilization with angiosperm gametes. Plant J 8:9–23

Krumlauf R (1994) Hox genes in vertebrate development. Cell 78:191–201

Lai LB, Nadeau JA, Lucas J, Lee EK, Nakagawa T, Zhao., Geisler M, Sack FD (2005) The Arabidopsis R2R3 MYB proteins FOUR LIPS and MYB88 restrict divisions late in the stomatal cell lineage. Plant Cell 17:2754–2767

Larkin JC, Marks MD, Nadeau J, Sack F (1997) Epidermal cell fate and patterning in leaves. Plant Cell 9:1109–1120

Laskowski MJ, Williams ME, Nusbaum HC, Sussex IM (1995) Formation of lateral root meristems is a two-stage process. Development 121:3303–3310

Levesque MP, Vernoux T, Busch W, Cui H, Wang JY, Blilou I, Hassan H, Nakajima K, Matsumoto N, Lohmann JU, Scheres B, Benfey PN (2006) Whole-genome analysis of the SHORT-ROOT developmental pathway in Arabidopsis. PLoS Biol 4:e143

Li X, Mo X, Shou H, Wu P (2006) Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of Arabidopsis. Plant Cell Physiol 47:1112–1123

Lindsey K, Topping JF (1993) Embryogenesis: a question of pattern. J Exp Bot 44:359–374

Lloyd C, Chan J (2006) Not so divided: the common basis of plant and animal cell division. Nat Rev Mol Cell Biol 7:147–152
Lu P, Porat R, Nadeau JA, O'Neill SD (1996) Identification of a meristem L1 layer-specific gene in Arabidopsis that is expressed during embryonic pattern formation and defines a new class of homeobox genes. Plant Cell 8:2155–2168
Lucas JR, Nadeau JA, Sack FD (2006) Microtubule arrays and Arabidopsis stomatal development. J Exp Bot 57:71–79
Lukowitz W, Roeder A, Parmenter D, Somerville C (2004) A MAPKK kinase gene regulates extra-embryonic cell fate in Arabidopsis. Cell 116:109–119
Lum L, Beachy PA (2004) The hedgehog response network: sensors, switches, and routers. Science 304:1755–1759
MacAlister CA, Ohashi-Ito K, Bergmann DC (2007) Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature epub ahead of print
Malamy JE (2005) Intrinsic and environmental response pathways that regulate root system architecture. Plant Cell Environ 28:67–77
Malamy JE, Benfey PN (1997) Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development 124:33–44
Mallory TE, Chiang S-H, Cutter EG, Gifford EM Jr (1970) Sequence and pattern of lateral root formation in five selected species. Am J B 57:800–809
Mansfield SG, Briarty LG (1991) Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. Can J Bot 69:461–476
Mayer KFX, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T (1998) Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell 95:805–815
Mayer U, Buttner G, Jurgens G (1993) Apical-basal pattern formation in the Arabidopsis embryo: studies on the role of the gnom gene. Development 117:149–162
McCabe PF, Valentine TA, Forsberg LS, Pennell RI (1997) Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. Plant Cell 9:2225–2241
McCarthy EK, Goldstein B (2006) Asymmetric spindle positioning. Curr Opin Cell Biol 18:79–85
McCormick S (2004) Control of male gametophyte development. Plant Cell 16:S142–S153
Nadeau JA, Sack FD (2002a) Control of stomatal distribution on the Arabidopsis leaf surface. Science 296:1697–1700
Nadeau JA, Sack FD (2002b) Stomatal development in Arabidopsis. The Arabidopsis book. American Society of Plant Biologists, Rockville, MD, pp 1–28
Nadeau JA, Sack FD (2003) Stomatal development: cross talk puts mouths in place. Trends Plant Sci 8:294–299
Nakajima K, Sena G, Nawy T, Benfey PN (2001) Intercellular movement of the putative transcription factor SHR in root patterning. Nature 413:307–311
Nawy T, Lee JY, Colinas J, Wang JY, Thongrod SC, Malamy JE, Birnbaum K, Benfey PN (2005) Transcriptional profile of the Arabidopsis root quiescent center. Plant Cell 17:1908–1925
Ning J, Peng XB, Qu LH, Xin HP, Yan TT, Sun M (2006) Differential gene expression in egg cells and zygotes suggests that the transcriptome is restructed before the first zygotic division in tobacco. FEBS Lett 580:1747–1752

Nothnagel EA (1997) Proteoglycans and related components in plant cells. Int Rev Cytol 174:195–291

Oh SA, Johnson A, Smertenko A, Rahman D, Park SK, Hussey PJ, Twell D (2005) A divergent cellular role for the FUSED kinase family in the plant-specific cytokinetic phragmoplast. Curr Biol 15:2107–2111

Ohashi-Ito K, Bergmann DC (2006) Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development. Plant Cell 18: 2493–2505

Okamoto T, Scholten S, Lorz H, Kranz E (2005) Identification of genes that are up- or down-regulated in the apical or basal cell of maize two-celled embryos and monitoring their expression during zygote development by a cell manipulation- and PCR-based approach. Plant Cell Physiol 46:332–338

Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologis A (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. Plant Cell 17:444–463

Park SK, Howden R, Twell D (1998) The Arabidopsis thaliana gametophytic mutation gemini pollen1 disrupts microspore polarity, division asymmetry and pollen cell fate. Development 125:3789–3799

Pennell RI, Janniche L, Kjellbom P, Scofield GN, Peart JM, Roberts K (1991) Developmental regulation of a plasma membrane Arabinogalactan protein epitope in oilseed rape flowers. Plant Cell 3:1317–1326

Pillitteri LJ, Sloan DB, Bogenschutz NL, Torii KU (2007) Termination of asymmetric cell division and differentiation of stomata. Nature epub ahead of print

Poethig RS (1987) Clonal analysis of cell lineage patterns in plant development. Am J Bot 74:581–594

Pollock EG, Jensen WA (1964) Cell development during early embryogenesis in Capsella and Gossypium. Am J Bot 51:915–921

Qin Y, Zhao J (2006) Localization of arabinogalactan proteins in egg cells, zygotes, and two-celled proembryos and effects of {beta}-D-glucosyl Yariv reagent on egg cell fertilization and zygote division in Nicotiana tabacum L. J Exp Bot 57(9):2061–2074

Reddy GV, Meyerowitz EM (2005) Stem-cell homeostasis and growth dynamics can be uncoupled in the Arabidopsis shoot apex. Science 310:663–667

Roegiers F, Jan YN (2004) Asymmetric cell division. Curr Opin Cell Biol 16:195–205

Sabatini S, Beis D, Wolkenfelt H, Murfett J, Guilfoyle T, Malamy J, Benfey P, Leyser O, Bechtold N, Weisbeek P, Scheres B (1999) An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell 99:463–472

Sabatini S, Heidstra R, Wildwater M, Scheres B (2003) SCARECROW is involved in positioning the stem cell niche in the Arabidopsis root meristem. Genes Dev 17:354–358

Sachs T (1991) Pattern formation in plant tissues. Cambridge University Press, New York.

Sack FD (2004) Plant sciences. Yoda would be proud: valves for land plants. Science 304:1461–1462

Sauer M, Friml J (2004) In vitro culture of Arabidopsis embryos within their ovules. Plant J 40:835–843

Scarpella E, Meijer AH (2004) Pattern formation in the vascular system of monocot and dicot plant species. New Phytologist 164:209–242

Schel JHN, Kieft H, van Lammeren AAM (1984) Interactions between embryo and endosperm during early developmental stage of maize caryopses (*Zea mays*). Can J Bot 62:2842–2853

Scheres B, Benfey PN (1999) ASYMMETRIC CELL DIVISION IN PLANTS. Annu Rev Plant Physiol Plant Mol Biol 50:505–537

Scheres B, Wolkenfelt H, Willemsen V, Terlouw M, Lawson E, Dean C, Weisbeek P (1994) Embryonic origin of the Arabidopsis primary root and root meristem initials. Development 120:2475–2487

Scheres B, Di Laurenzio L, Willemsen V, Hauser MT, Janmaat K, Weisbeek P, Benfey PN (1995) Mutations affecting the radial organisation of the Arabidopsis root display specific defects throughout the embryonic axis. Development 121:53–62

Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T (2000) The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100:635–644

Schuck S, Simons K (2004) Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. J Cell Sci 117:5955–5964

Sena G, Jung JW, Benfey PN (2004) A broad competence to respond to SHORT ROOT revealed by tissue-specific ectopic expression. Development 131:2817–2826

Serna L (2004) Plant biology good neighbours. Nature 430:302–304

Serna L (2005) Epidermal cell patterning and differentiation throughout the apical-basal axis of the seedling. J Exp Bot 56:1983–1989

Shevell DE, Leu WM, Gillmor CS, Xia G, Feldmann KA, Chua NH (1994) EMB30 is essential for normal cell division, cell expansion, and cell adhesion in Arabidopsis and encodes a protein that has similarity to Sec7. Cell 77:1051–1062

Shi H, Kim Y, Guo Y, Stevenson B, Zhu JK (2003) The Arabidopsis SOS5 locus encodes a putative cell surface adhesion protein and is required for normal cell expansion. Plant Cell 15:19–32

Shpak ED, Lakeman MB, Torii KU (2003) Dominant-negative receptor uncovers redundancy in the Arabidopsis ERECTA leucine-rich repeat receptor-like kinase signaling pathway that regulates organ shape. Plant Cell 15:1095–1110

Shpak ED, Berthiaume CT, Hill EJ, Torii KU (2004) Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and flower development by promoting cell proliferation. Development 131:1491–1501

Shpak ED, McAbee JM, Pillitteri LJ, Torii KU (2005) Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science 309:290–293

Smith LG, Hake S, Sylvester AW (1996) The tangled-1 mutation alters cell division orientations throughout maize leaf development without altering leaf shape. Development 122:481–489

Spielman M, Preuss D, Li FL, Browne WE, Scott RJ, Dickinson HG (1997) TETRASPORE is required for male meiotic cytokinesis in Arabidopsis thaliana. Development 124:2645–2657

Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414:98–104

Sprunck S, Baumann U, Edwards K, Langridge P, Dresselhaus T (2005) The transcript composition of egg cells changes significantly following fertilization in wheat (Triticum aestivum L.). Plant J 41:660–672

Steeves TA, Sussex IM (1989) Patterns in plant development. Cambridge University Press, Cambridge

Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, lweiler L, Palme K, Jurgens G (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science 286:316–318

Stewart RN, Dermen H (1970) Determination of number and mitotic activity of shoot apical initial cells by analysis of mericlinal chimeras. Am J Bot 57:816–826

Terasaka O, Niitsu T (1987) Unequal cell division and chromatin differentiation in pollen grain cells. I. Centrifugal, cold and caffeine treatments. Bot Mag Tokyo 100:205–216

Torres-Ruiz RA, Jurgens G (1994) Mutations in the FASS gene uncouple pattern formation and morphogenesis in Arabidopsis development. Development 120:2967–2978

Traas J, Bellini C, Nacry P, Kronenberger J, Bouchez D, Caboche M (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature 375:676–677

Tupy J, Rihova L, Zarsky V (1991) Production of fertile tobacco pollen from microspores in suspension culture and its storage for in situ pollination. Sex Plant Reprod 4:284–287

Twell D, Park SK, Lalanne E (1998) Asymmetric division and cell-fate determination in developing pollen. Trends Plant Sci 3:305–310

Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A, Hussey PJ (2002) MOR1/GEM1 has an essential role in the plant-specific cytokinetic phragmoplast. Nat Cell Biol 4:711–714

van den Berg C, Willemsen V, Hage W, Weisbeek P, Scheres B (1995) Cell fate in the Arabidopsis root meristem determined by directional signalling. Nature 378:62–65

van den Berg C, Willemsen V, Hendriks G, Weisbeek P, Scheres B (1997) Short-range control of cell differentiation in the Arabidopsis root meristem. Nature 390:287–289

Vanneste S, De RB, Beemster GT, Ljung K, De SI, Van IG, Naudts M, Iida R, Gruissem W, Tasaka M, Inze D, Fukaki H, Beeckman T (2005) Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. Plant Cell 17:3035–3050

Von Groll U, Berger D, Altmann T (2002) The subtilisin-like serine protease SDD1 mediates cell-to-cell signaling during Arabidopsis stomatal development. Plant Cell 14:1527–1539

Weijers D, Schlereth A, Ehrismann JS, Schwank G, Kientz M, Jurgens G (2006) Auxin triggers transient local signaling for cell specification in Arabidopsis embryogenesis. Dev Cell 10:265–270

Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K, Rashbrooke MC, Wasteneys GO (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411:610–613

Wildwater M, Campilho A, Perez-Perez JM, Heidstra R, Blilou I, Korthout H, Chatterjee J, Mariconti L, Gruissem W, Scheres B (2005) The RETINOBLASTOMA-RELATED gene regulates stem cell maintenance in Arabidopsis roots. Cell 123:1337–1349

Willats WGT, Knox JP (1996) A role for arabinogalactan-proteins in plant cell expansion: evidence from studies on the interaction of beta-glucosyl Yariv reagent with seedlings of Arabidopsis thaliana. Plant J 9:919–925

Willemsen V, Friml J, Grebe M, van den Toorn A, Palme K, Scheres B (2003) Cell polarity and PIN protein positioning in Arabidopsis require STEROL METHYLTRANSFERASE1 function. Plant Cell 15:612–625

Wilmoth JC, Wang S, Tiwari SB, Joshi AD, Hagen G, Guilfoyle TJ, Alonso JM, Ecker JR, Reed JW (2005) NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. Plant J 43:118–130

Wodarz A (2002) Establishing cell polarity in development. Nat Cell Biol 4:E39–E44

Xu J, Scheres B (2005) Cell polarity: ROPing the ends together. Curr Opin Plant Biol 8:613–618

Yang CY, Spielman M, Coles JP, Li Y, Ghelani S, Bourdon V, Brown RC, Lemmon BE, Scott RJ, Dickinson HG (2003) TETRASPORE encodes a kinesin required for male meiotic cytokinesis in Arabidopsis. Plant J 34:229–240

Yang M, Sack FD (1995) The too many mouths and four lips mutations affect stomatal production in Arabidopsis. Plant Cell 7:2227–2239

Zhao L, Sack FD (1999) Ultrastructure of stomatal development in Arabidopsis (Brassicaceae) leaves. Am J Bot 86:929

Asymmetric Cell Division – How Flowering Plant Cells Get Their Unique Identity

R.M. Ranganath

Abstract

A central question in biology is how cell fate is specified during development of a multicellular organism. Flowering plants use two major pathways of asymmetric cell divisions in a spatio-temporal manner to achieve required cellular differentiation. In the 'one mother – two different daughters' pathway, a mother cell mitotically divides to produce two daughter cells of different size and fate. By contrast, the 'coenocyte-cellularization' pathway involves formation of a coenocyte, nuclear migration to specific locations of the coenocyte and cellularization of these nuclei by unique wall forming processes. Given that cell fate determinants play a key role in establishing cell identity, their allocation to daughter cells in the two pathways needs to be understood in terms of the unique cell cycle regulatory mechanisms involved. Most of the information available on cell fate determination in flowering plants is in the form of genes identified from mutant analysis. Novel techniques of interrogating individual plant cells *in vivo* are necessary to advance the extant knowledge from genetics to functional genomics data bases.

1 Introduction

Growth, cell division and cellular differentiation are integral parts of eukaryotic development. The derivatives of a zygote should proliferate and also differentiate in order to produce a fully mature multicellular organism. Asymmetric cell divisions (ACDs) play a significant role in the process of cellular differentiation.

A cell division that generates distinct fates in the daughter cells is termed an ACD (Horvitz and Herskowitz 1992). There are two major pathways by which flowering plants execute ACDs. In the widely known 'one mother – two different daughters (1M-2DD)' pathway, a mother cell divides to produce two daughter cells which may be *ab initio* different in size and fate or the daughter cells may be initially similar in size but subsequently acquire different fates. For example, a1M-2DD pathway is used during the zygotic division to differentiate suspensor and embryo precursor cells, during the

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microspore division to differentiate a vegetative cell and a generative cell and to differentiate a stomatal complex. A mother cell committed to ACD is polarized and the spindle formation and its positioning are synchronized with cell polarity. Either the nucleus or the spindle itself migrates to an asymmetric cellular location so that the cell plate formed at the spindle mid-zone partitions the polarized mother cell into two daughter cells of different sizes. The entire process is coordinated in such a way that the cell fate determinants (CFDs) are differentially segregated to the daughter cells to give them unique identities. Alternatively, differential positional cues may play key roles in cell fate specification (see Laux et al. 2004).

The coenocyte-cellularization (C-C) pathway is used during the development of female gametophyte, cereal endosperm and pseudomonad in sedges. The mother cell first forms a coenocyte by a predetermined number of nuclear division cycles. The nuclei migrate to specific locations in the coenocyte and are then cellularized by unique cell wall formation processes so that each cell is allocated a single nucleus, as a rule. The mechanisms of CFD allocation, generally associated with the spindle as in 1M-2DD ACDs, are irrelevant in the C-C pathways since cell specification occurs only after the nuclear division cycles are completed. Thus, CFD allocation and cell specification have to be understood in a totally altered cell cycle and signaling environment within a coenocyte. Flowering plants use both these pathways in precise time windows of development. Interestingly, the C-C pathways are associated with both mitotic and meiotic cell cycles and the number and types of specialized cells formed depends on the developmental context.

In this review, I have tried to provide a concise overview of plant development driven by these two types of ACDs. For general information on polarity, signals, CFD allocation, cytokinesis/cellularization and cell cycle regulation, see Ranganath (2005) and Hiedstra R (2007), chapter 1 in this publication. The reader is also directed to other reviews for wider information, wherever necessary.

2
Embryo Development

In flowering plants, embryogenesis generates the primary body organization as represented in the seedling: shoot meristem, cotyledons, hypocotyls, root and root meristem along the apical axis and a concentric arrangement of epidermis, subepidermal ground tissue and central vascular cylinder along the radial axis. The meristems arise as the terminal elements of the apical-basal pattern along the axis of polarity. New structures are added from the meristems of the shoot and the root during post-embryonic development (reviewed by Laux et al. 2004). The cotyledons, hypocotyls and radical (embryonic root) do not contribute to the post-embryonic development (Mayer et al. 1993). Embryonic lethal mutants are isolated from both

Arabidopsis and maize (reviewed by Meinke 1991) and have helped to delineate three regions in the apical-basal axis: epicotyl and cotyledons, hypocotyl and root (Mayer et al. 1993). Apical and basal pattern formation during *Arabidopsis* embryogenesis has been reviewed by Jurgens (2001).

Most dicots go through similar developmental stages – globular shape, heart shape and torpedo shape, and are patterned similarly (Jenik et al. 2005). In *Arabidopsis*, the zygote elongates and then executes a 1M-2DD type of ACD. The apical daughter cell, after two rounds of longitudinal and a single round of transverse divisions, gives raise to an eight-celled embryo proper. The basal cell divides transversely to produce a suspensor and its uppermost cell forming the hypophysis. At the eight-celled stage of the embryo, four regions with different developmental fates could be identified: 1) four most apical cells forming the apical embryo domain which generates the shoot apical meristem and most of the cotyledons, 2) lower cells forming the central embryo domain which generates hypocotyls and root, and contribute to cotyledons and root meristem, 3) the basal embryo domain (hypophysis) which gives raise to distal part of the root meristem, the Quiescent Centre (QC) and the stem cells of the central root cap and 4) the extra-embryonic suspensor (reviewed by Laux et al. 2004). Organ and tissue types are distributed along the perpendicular axis whereas the vasculature, endodermis, cortex and epidermis are arranged along the radial (inside-outside) axis. The shoot meristem, hypocotyls, root and root meristem lie along the shoot-root (apical-basal) axis (Natesh and Rau 1984). The first manifestation of the apical-basal axis in plants, ACD of the zygote, produces a basal cell that transports and an apical cell that responds to the signaling molecule auxin. PIN7 protein, a component of auxin efflux, is localized to the apical region of the basal cell and regulates the auxin efflux from basal cell to the apical cell (see Friml et al. 2003). The serine/threonine kinase PINOID (PID) plays a critical role in the localization of the PIN protein – high levels of PID localize PIN to the apical membrane whereas low levels to the basal membrane (Xu and Scheres 2005). The apical cell of the two-celled embryo accumulates *ARABIDOPSIS THALIANA MERISTEM LAYER1* (*AtML1*) gene transcript but not the basal cell (suspensor precursor cell) (see Lu et al. 1996).

Several mutations are known to affect the suspensor and the embryo (Mayer et al. 1993; Willemsen et al. 1998 and the references therein). In the *twin* (*twn*) mutants, suspensor cells form secondary embryos (Vernon and Meinke 1994). A MAPKK kinase – YODA (YDA) acts as a molecular switch promoting proper development of the suspensor (see Lukowitz et al. 2004). However, Bergmann et al. (2004) show that in *Arabidopsis,* YDA is also involved in cell fate specification of epidermis.

In flowering plants, many somatic cells are also totipotent. Activation of embryo-specific genes is necessary in the zygote for embryonic development (Russinova and de Vries 2000). Although a catalogue of such genes is not yet available, several genes which can induce embryonic development in somatic cells have been reported. Dodeman et al. (1997) have provided a comparative

account of zygotic and somatic embryogenesis in plants. The genes *BABY BOOM* (*BBM*) (Boutilier et al. 2002), *LEAFY COTYLEDON1* (*LEC1*) and *LEC2* (Lotan et al. 1998; Stone et al. 2001) and *WUSCHEL* (Zuo et al. 2002) can induce embryo-like structures in somatic cells. Cultured cells of carrot can either be maintained in an undifferentiated condition or induced to form embryos by transferring to an auxin-free environment (see Krikorian and Smith 1992). In carrot cultures, the *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) gene plays a crucial role in somatic embryogenesis (Schimdt et al. 1997). During adventitious embryony, a nucellar cell transdifferentiates directly into an embryo (see Asker and Jerling 1992). Given that certain basic requirements of *de novo* gene expression have to be met for a cell to get a particular fate, the activities of embryo-inducing genes in the transdifferentiation of a nucellar cell into an adventitious embryo have to be examined in both genetic and epigenetic contexts (Ranganath 2004). Xiao et al. (2006) have shown that in *Arabidopsis*, mutations in the DNA methylation genes *METHYLTRANSFERASE1* (*MET1*) and *CHROMOMETHYLASE3* (*CMT3*) misexpress embryo cell identity genes and result in improper auxin gradients. By contrast, plant chromatin regulators such as PK1 and EMF2 control the transition from embryonic to vegetative phase (Gaudin et al. 2001; Moon et al. 2002).

3
Stem Cells in Flowering Plants

Stem cells make a facultative use of symmetric and asymmetric divisions to expand their number or generate differentiated progeny cells during development, respectively (Morrison and Kimble 2006). In both plants and animals, maintenance of stem cells is by reciprocal signaling between stem cells and their surrounding tissue microenvironment called niche. Distribution of auxin and the establishment of auxin maxima are early formative steps in niche specification which depends on the expression and distribution of auxin carriers (Jiang and Feldman 2005). Plant stem cell niches are located within the meristems (reviewed by Singh and Bhalla 2006). It is also argued that 'stemness' is a transient trait or a state and cannot be predicted on the basis of momentary gene expression patterns. An overall genomic and proteomic analysis coupled with mathematic modeling is necessary to determine a stem cell state (see Zipori 2004). Between the stem cell and its terminally differentiated population of cells, an intermediate population of committed progenitors, called transit amplifying (TA) cells with a restricted differentiation potential, is formed (see Singh and Bhalla 2006). Laux (2003) has discussed the concept of stem cells in plants. For a list of putative chromatin factors controlling meristem phase transition, see Reyes (2006).

3.1
Shoot Apical Meristem (SAM)

In flowering plants, SAM gives rise to aerial parts by continuously initiating new organs. Stem cells in the SAM divide slowly and produce daughter cells of two types: ones that remain in the centre as stem cells whereas others are continuously displaced outwards (Reddy et al. 2004) and form the founder cells for the formation of either lateral organs or the main stem. The number of stem cells remains constant in spite of the continuous departure of their daughters into differentiation pathways (Williams and Fletcher 2005). In *Arabidopsis*, SAM is about 100 μm in diameter and about 100 cells in number (Weigel and Jurgens 2002).

There are two main pathways known to maintain stem cell fate in the SAM: the *WUSCHEL* (*WUS*) – *CLAVATA* (*CLV*) gene expression pathway and the *KNOX* and *BELL* class gene expression pathway. *WUS* is a homeobox gene expressed very early during embryogenesis and confined to a few cells in the inner layers of the central zone, called organizing centre-OC (Haecker et al. 2004) (see Fig. 1A). *WUS* gene product acts in concert with a signaling complex comprising of CLV genes products. This feedback loop regulates a small population of stem cells in the central zone. The microRNA – *miR166g* might be the first of many small regulatory RNAs involved in SAM activities (see Williams and Fletcher 2005).

Products of both *KNOX* and *BELL* class genes belong to TALE class of homeodomain TFs. Class I *KNOX* genes are expressed in SAM and are down regulated in differentiating cells (Jackson et al. 1994). In *Arabidopsis*, mutations in the *KNOX1* gene *SHOOT MERISTEMLESS* (*STM*) results in loss of meristem functions (Long et al. 1996) through repression of *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* genes. *KNOX*

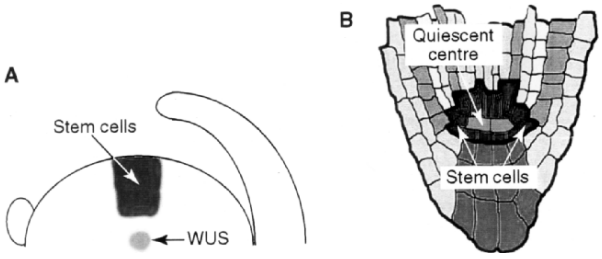

Fig. 1. A,B Schematic organization of plant meristems: **A** shoot apical meristem: the expression domain of WUS, which maintains the stem cell niche, subtends that of the stem cells at the centre of the meristem; **B** root apical meristem: the quiescent centre maintains the stem cell niche for the surrounding stem cells. Reproduced from Doerner P, Curr Biol Vol 16(6), (2006), with permission from Elsevier Science

genes therefore function redundantly to maintain shoot stem cell fate. Expression of *STM* is evident only at the globular embryo stage and very little is known about the origin and development of the SAM before this stage (see Mayer et al. 1998). Mis-expression of *KNOX* genes alone can induce ectopic meristems indicating that these genes can establish stem cell fate in differentiated cells. Genes such as *AS1*, *AS2*, *BLADE ON PETIOLE* (*BOP*) are known to represses *KNOX* in lateral organs whereas members of the *YABBY* gene family such as *FILAMENTOUS FLOWER* and *YABBY3* are required for correct patterning of the lateral organs, and to maintain differentiated cell fate by repression *KNOX* gene functions (reviewed by Byrne et al. 2003). Tsiantis (2001) has discussed the importance of homeobox-defined developmental pathways in the control of shoot cell fate. Plant hormones gibberellins and cytokinins together are involved in the regulation of *KNOX1* control of SAM cell identity (Jasinski et al. 2005; Yanai et al. 2005). Shani et al. (2006) have also pointed out that both KNOX and WUS TFs facilitate high cytokinin activity in the SAM whereas auxin activities promote initiation of lateral organs at specific sites in the SAM flanks.

3.2 Root Apical Meristem (RAM)

A typical dicotyledonous root, as in *Arabidopsis*, is formed by the primary root during embryogenesis. The root tissues are generated by the RAM (Fig. 1B) and comprise concentric single cell layers of epidermis, cortex, endodermis and pericycle which surround the vascular tissues (reviewed by Hardtke 2006). RAM is derived from the upper most cell of the suspensor. As in SAM, the RAM has a group of slowly dividing cells – the quiescent centre (QC), which is established early during embryogenesis. ACDs from QC produce daughter cells which retain QC function or replace an adjacent cell of a specific cell type of the root (Kidner et al. 2000). Laser ablation experiments in *Arabidopsis* have shown that a new QC is developed from proximal cells, emphasizing the importance of positional information. (van den Berg et al. 1995). Nawy et al. (2005) have provided a transcriptional profile of QC in *Arabidopsis* root.

SCARECROW (SCR), a GRAS family TF (Di Laurenzio et al. 1996) is required cell autonomously for distal specification of QC, which in turn regulates the stem cell fate of surrounding cells (Sabatini et al. 2003). Loss of *SCR* gene function disrupts QC and stem cell identity which can be restored by cell autonomous expression of *SCR* in the initial cells. *SCR* is first expressed in the QC precursor cells during embryogenesis, after which it extends to the initial cells of the ground tissue (cortex and endodermis) (Wysoka-Diller et al. 2000). This expression pattern persists in the post-embryonic root. In *scr1* mutant, the ACD of the daughter of

the cortex/endodermis does not occur resulting in a mixed cell identity (Sabatini et al. 2003). *SHORTROOT* (*SHR*), a related GRAS family gene, is also required for the ACD responsible for formation of ground tissue (cortex and endodermis) as well as specification of endodermis in *Arabidopsis* root (Helariutta et al. 2000). SHR protein, required for endoderm specification and up regulation of *SCR* expression in the ground tissue, is expressed in vascular cells and may move as a protein to the surrounding cell layer, including the QC (Helariutta et al. 2000; Nakajima et al. 2001). The coordinated actions of SCR and SHR proteins pattern the entire tissue surrounding the vascular tissue (ground tissue patterning) in *Arabidopsis* (Scheres et al. 1995). In the *Arabidopsis* root meristem, ground tissue stem cell daughters perform an ACD to form endodermis and cortex. SCR limits SHR movement, and transient SCR action is sufficient to separate endodermis and cortex fates by ACD (Heidstra et al. 2004)

Some of the proteins seem to be required by both SAM and RAM. For example, SCR and SHR proteins are required for ground tissue organization in both root and shoot (Fukaki et al. 1998). In *Oriza sativa*, *SCR* (*osSCR*) gene is involved not only in the ACD of the cortex/endodermis progenitor cell but also during stomata and ligule formation by polarization of cytoplasm (Kamiya et al. 2003).

4
Formation of Lateral Organs

During post-embryonic development, all the aerial organs of a plant are ultimately derived from a few pluripotent stem cells in the SAM (Baurie and Laux 2003). The main tissue types such as epidermis, ground tissue and vascular tissue form a second patterning in the seedling which is organized perpendicular to the axis of polarity.

Above ground lateral organs are produced from the flanks of the SAMs. The shoot system consists of a leaf, a stem and a lateral bud that differentiates into a lateral shoot. Floral organs are considered as modified leaves. For lateral organ formation, it is essential that the genes responsible for establishment/maintenance of SAM should be down regulated (see Hake et al. 1995). Simultaneously, genes required for lateral organ primordia are expressed. A genetic system comprising of .class III *HD-ZIP* and *KANADI* genes is involved in adaxial–abaxial polarity in lateral organs produced from the apical meristems in *Arabidopsis* (Emery et al. 2003). A comprehensive account of leaf development is provided by Tsukaya (2002) and Byrne (2005). Termination of stem cell activity during flower development is regulated by a temporal feedback loop involving both stem cell maintenance genes and flower patterning genes (Fletcher 2002). See Coen and Mayerowitz (1991, the ABC model), Levy and Dean (1998) for floral meristem and organ identity genes and Jack (2004) for specification of floral organs. The Floral Genome

Project has identified 15 representative species from gymnosperms, basal angiosperms, monocots and core eudicots to examine the spatio-temporal patterns of gene expression during early flower development based on EST and cDNA analyses (see Eckardt 2002 and the references therein).

4.1 Epidermis

The epidermis of the shoot is derived from the outermost layer of cells covering the SAM through repeated anticlinal divisions. By contrast, the epidermis of the root is derived from an internal ring of cells, located at the centre of the RAM, by a precise series of both anticlinal and periclinal divisions. In *Arabidopsis*, the shoot epidermis comprises pavement cells, hair cells, (trichomes), stomatal guard cells and other epidermal cells (see Dolan and Roberts 1995; Dolan and Costa 2001). The root epidermis comprises two cell types: trichoblasts that form root hair cells and atrichobalsts that form non-hair cells. Shoot epidermal hairs are called trichomes – which can be secretary, glandular or nonglandular. Most of the information on trichomes has come from leaf trichomes. Hulskamp (2004) has reviewed the importance of trichome as a model for studying cellular differentiation. See Box 1 in Dolan and Roberts (1995) for mutations affecting patterning and morphogenesis in shoot and root epidermis of *Arabidopsis*. Similarly, for details of stomatal development and patterning see Geisler et al. (2003), Nadeau and Sack (2003) and Shpak et al. (2005). Although, a series of 1M-2DD type of ACDs is required to produce a guard mother cell (GMC), it is a symmetric division of the GMC that eventually produces two daughter (guard) cells. The cell plate involved in the formation of guard cells is also unique since it is curved to form a hole in the center (reviewed by Verma 2001), giving rise to daughters of same fate but with an asymmetric cell plate. In the *discordia* mutant, cell plate of the guard cells do not curve while those during the ACDs are not affected, showing that the two types of cytokinesis are differently regulated (see Gallagher and Smith 1999).

4.2 Vascular Differentiation

The vascular system consists of a continuous cellular network with interconnected vascular strands. Procambial cells or vascular stem cells are derived from apical meristem and give rise to xylem and phloem precursor cells: the former differentiate xylem, comprising tracheary elements (TEs), xylem parenchyma cells and xylem fibers. The latter differentiate into phloem which includes sieve elements, companion cells, phloem parenchyma cells and phloem fibers. Most of vascular cell differentiation is known with regard to information on *Arabidopsis* mutant analysis and *Zinnea elegans* single cell xylogenic cultures (Fukuda 2004). Microarray analysis has

shown that about 9000 genes change their expression pattern during xylem cell transdifferentiation (Demura et al. 2002). The *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) and *VND7* gene products may be the transcription switches to induce transdifferentiation of various cells into metaxylem and protoxylem-like elements in *Arabidopsis* and *Populus* (Kubo et al. 2005). Auxin plays an important role in the formation of vascular tissue. Mutations in the *Arabidopsis MONOPTEROS* [*MP*/*AUXIN RESPONSE FACTOR 5* (*ARF5*)], *AUXIN RESISTANT6* (*ARX6*) and *BODENLOS* (*BDL*) genes show disruption in vascular tissue patterning (see Mattsson et al. 2004 and the references therein). Phloem is established through ACDs and subsequent differentiation. The *ALTERED PHLOEM DEVELOPMENT* (*APL*) gene encodes a MYB coiled-coil type of TF which is required for phloem identity in *Arabidopsis* (Bonke et al. 2003). Thompson (2006) has reviewed the conceptual integration of structure and duality of function aspects of phloem sieve element-companion cell complexes. Intercellular signaling molecules such as auxin, cytokinin and plant steroid hormones – Brassinosteroids (BRs) – are involved in differentiation and/or maintenance of procambial cells through distinct gene expression machineries including *HD-ZIP-III* gene family and microRNAs (reviewed by Dinneny and Yanofsky 2004; Fukuda 2004; Sieburth and Deyholos 2005). HD ZIP and KANADI TFs are important regulators of radial patterning during secondary growth and TFs belonging to *G2-like*, *NAC*, *AP2*, *MADS* and *MYB* gene families regulate xylem and phloem differentiation activity (Zhao et al. 2005). See Fig. 2 for an overview of vascular development.

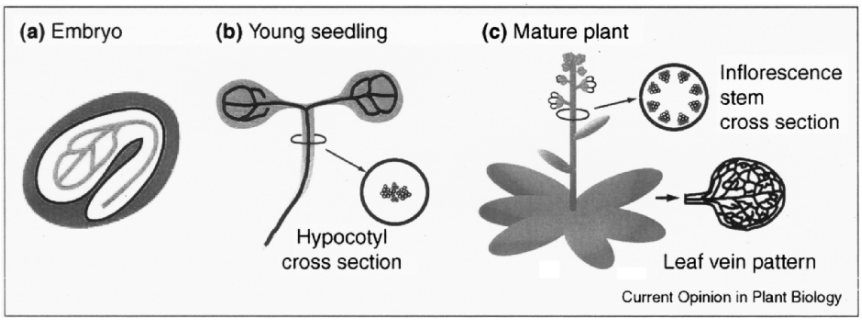

Fig. 2. a–c Overview of vascular development: **a** in the dry seed embryo already has procambium along the apical-basal axis and in patterns in the cotyledons; **b** in young seedling, the procambium has differentiated (*black lines*), and in the hypocotyls, the vascular tissues are organized in a central core; **c** in the mature plant, the stem has a new vascular pattern that includes dispersed vascular bundles, and the leaf has a dispersed network of veins. Reproduced from Sieburth LE, Deyholos MK Curr Opin Plant Biol Vol 9 (2006), with permission from Elsevier Science

5
Gametogenesis

It is noteworthy that, in flowering plants, male gamete formation involves a 1M-2DD type of ACD (see Fig. 3A) whereas female gamete formation involves a C-C pathway (see Fig. 4A). At least 200 genes are estimated to be required for gametophyte development (see Bonhomme et al. (1998); Yang and Sundaresan (2000); Wilson and Yang (2004)).

5.1
Microsporogenesis and Male Gametophyte Development

The microspore undergoes an ACD, giving rise to two daughter cells of different fates – the larger vegetative cell (VC) and the smaller generative cell (GC). The VC is arrested in G1 phase of cell cycle while the GC divides once mitotically to produce two sperm cells (Fig. 3A: a-b-c-d-e; Fig. 3A: a-f-g-c-d-e). Several unique cellular events occur before the microspore division. Polarity is established by nuclear migration and an asymmetric spindle is formed. Control of gametophytic cytokinesis is a critical process in male germ line cell fate determination and is required for asymmetric distribution of cellular components that presumably include CFDs (Twell et al. 1998).

Random mutagenesis experiments in *Arabidopsis* have shown that many genes are required for development of functional pollen (Lalanne et al. 2004; Johnson-Brousseau and McCormick 2004; Ranganath 2005). Rotman et al. (2005) have reported a novel, pollen specific *R2R3 MYB* gene, *DUO1*, involved in the process of normal sperm production in *Arabidopsis*. DUO1 protein accumulates in the sperm cell nuclei and in *duo1* mutant, a single large diploid sperm cell, unable to perform fertilization is formed. By contrast, in the *cdc*2 mutant of *Arabidopsis*, only a single sperm cell instead of two is formed with the pollen being viable. The single sperm fertilizes the egg (double fertilization Vs single fertilization) while the endosperm (diploid) develops autonomously indicating that a positive signal from the fertilized egg is required for its development (see Nowack et al. 2006; Iwakawa et al. 2006). Interestingly *SERK1* and *SERK2* gene products which are involved in embryogenesis are also required for tapetum development and microspore maturation in *Arabidopsis* (see Colcombet et al. 2005). For recent information on molecular and genetic controls over microspororgenesis and male gametophyte development, see McCormick (2004), Johnson et al. (2004) and Ma (2005). A transcriptome analysis of *Arabidopsis* male gametophyte is provided by Honys and Twell (2004).

Microspore (pseudomonad) production in sedges and the members of Styphelieae of Epacridaceae (Smith-White 1959) is uniquely different from

other angiosperms (Fig. 3A: a-f-g-h-i). As the pathway manifests both positive and negative developmental outputs (one large, functional and three, smaller nonfunctional microspores) from an ACD associated with meiosis, it is termed programmed cell elimination (PCE), as compared to

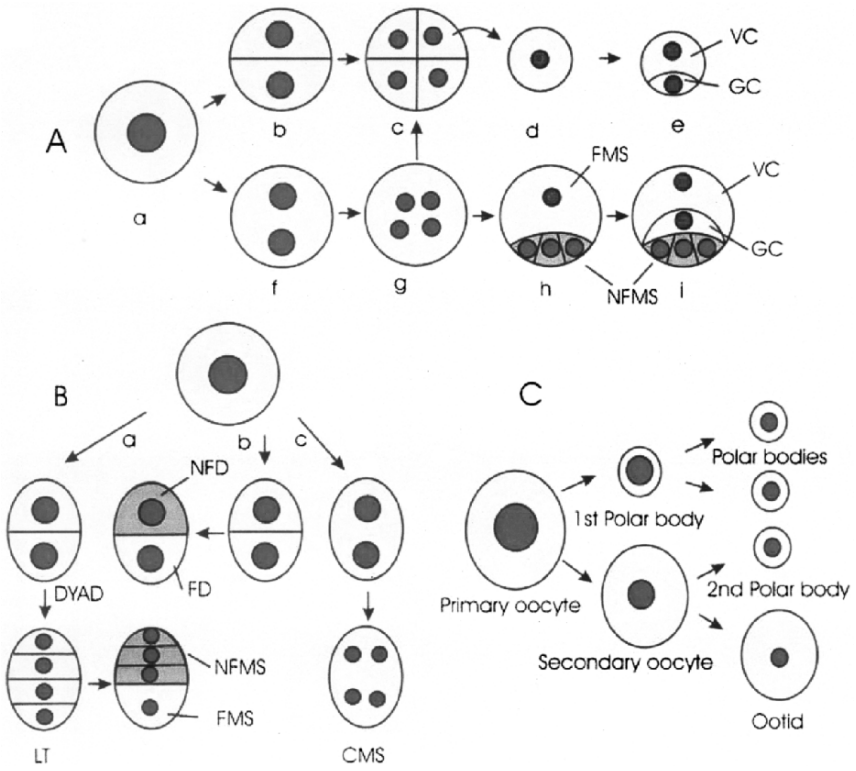

Fig. 3. A–C Asymmetric cell divisions during gametogenesis: **A** pollen development in angiosperms – a microspore mother cell (MMC) produces four haploid microspores either through pathway a-b-c (successive cytokinesis) or pathway a-f-g-c (simultaneous cytokinesis). In the sedges/Stypheliеae (pathway a-f-g-h-i) asymmetric partitioning of the meiocyte produces one large, functional microspore (FMS) and three smaller nonfunctional microspores (NFMSs); **B** megasporogenesis in angiosperms – the cell cycle regulators as well as the differentiation related molecules have to be some how segregated to the FMS in the monosporic type (pathway -a) and functional dyad (FD) (pathway -b) respectively, whereas in the tetrasporic type (pathway -c), they remain in the same coenocytic megaspore (CMS) due to lack of cytokineses during meiosis. NFMSs/dyad in pathways (a) and (b) are shown in *gray*; **C** female meiosis in animals – successive cytokineses that asymmetrically partition the meiocyte are part of the meiotic cell cycle as compared to the post-meiotic and simultaneous cytokinesis seen in the sedges/Stypheliеae pathway (A-a,f,g,h,i). Reproduced from Ranganath RM, Plant Biology Vol 7 (2005), with permission from Thieme-Verlag

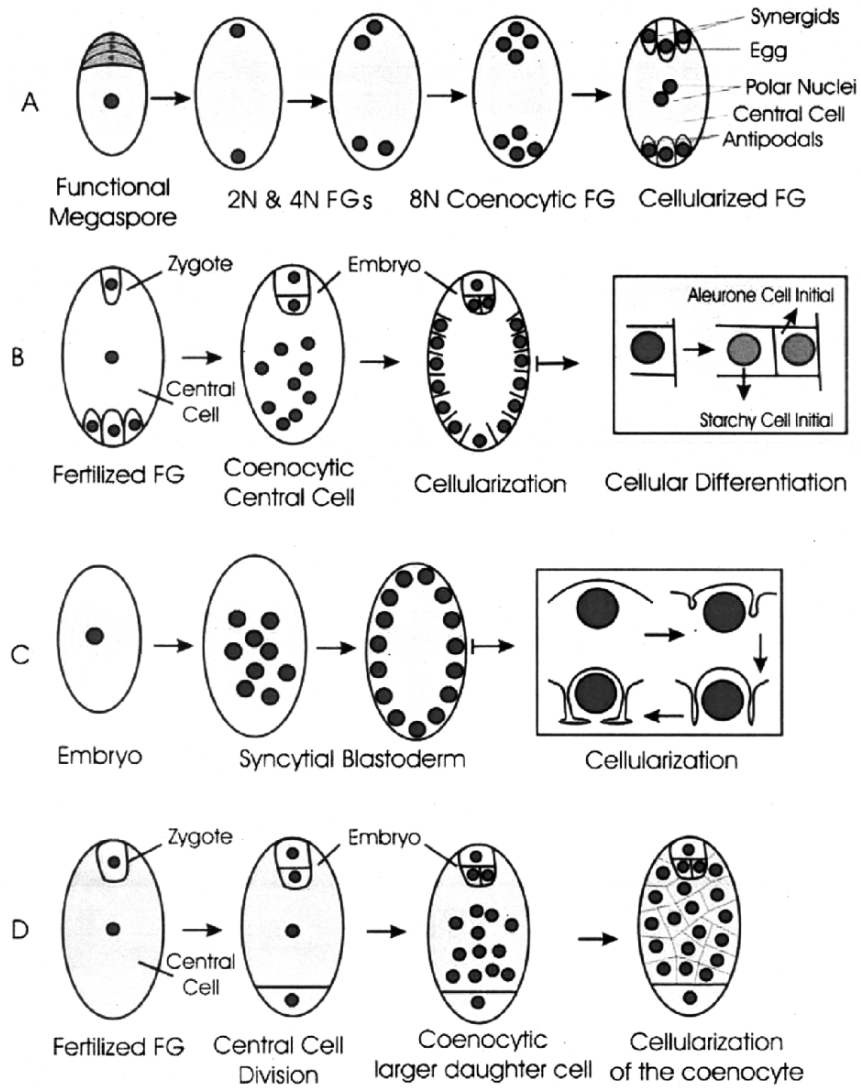

Fig. 4. Coenocyte-cellularization pathways: **A** FG development (Polygonum type) in flowering plants – the functional megaspore undergoes three mitotic division cycles to produce a eight-nucleate-coenocyte which is subsequently cellularized to form a seven celled FG. Note the differences in the size and fate of the cells, particularly the egg and the central cell in the mature FG; **B** cereal endosperm development – cellularization of the cocenocytic central cell initially involves anticlinal divisions resulting in open-ended alveoli. The first of the periclinal divisions produces the aleurone cell initials (ACIs) towards the periphery and starchy cell initials (SCIs) with open ended alveoli towards the center of the central cell. Repeated clonal divisions of the ACIs and SCIs eventually increase their cell number. Since the CFDs involved have to be at the right place at the right time, the key issue to be resolved in this and the other coenocyte-cellularization pathways is the manner in which cellularization

programmed cell death (PCD) where somatic cells die by a genetically predetermined programme before their elimination from a developmental pathway (see Ranganath and Nagashree 2000, 2001 and the references therein). Microspore (cell) death in sedges (Fig. 3A: h-i) is a consequence of development rather than its cause as in PCD.

5.2 Megasporogenesis and Female Gametophyte Development

More than 70% of the angiosperm species examined have shown monosporic type of female gametophyte (FG) development (Huang and Russell 1992), where only one of the four megaspores produced by meiosis is functional and produces the typical 8-nucleate, 7 celled FG (Fig. 4A). The other three megaspores degenerate by a distinct form of PCD (Yadegari and Drews 2004). In the bisporic type, one of the dyads is eliminated and the surviving dyad functions as the 'functional megaspore' due to absence of cytikinesis during MII, whereas in the tetrasporic type, all the haploid nuclei produced by meiosis lie in the same cell due to absence of cytokinesis during both MI and MII. Consequently, the traditional cellular barrier between meiosis and mitosis does not exist, creating a unique situation seen in some of the ascomycetes and zygomycetes which execute sequential meiosis and mitosis in the same cellular space (reviewed by Ranganath 2003) (Fig. 3B: b, c). Given that the subsequent developments of FG formation, double fertilization, endosperm and embryo development occur in the 'functional megaspore cell space', it should be explained as to how the necessary cell cycle regulators and the CFDs are segregated into the functional megaspore, particularly into one of the four megaspores in the monosporic and one of the dyads in the bisporic types. In both, segregation of the determinants has to be understood in the context of the meiotic cell cycle where there are no drastic differences in the size of the cells involved, as compared to the classical 1M-2DD type of ACDs. Due to the coenocytic nature of the functional megaspore in the tetrasporic type, the problem of segregation of the determinants does not arise. Although, a direct consequence of the cellularization process is the formation of different cell types of the FG, the molecular mechanisms that underlie their specification in terms of CFD allocation are yet to be understood. Kim et al. (2005) have shown that Lysophosphotidyl Acetyltransferease (LAPT2), located in the

Fig. 4. (*Cont'd*) process is coordinated with cell-specific CFD allocation; **C** early embryo development in *Drosophila* – note the mechanistic similarities with the cereal endosperm development; **D** helobial endosperm combines the characteristics of both cellular and nuclear (coenocyte-cellularization) endosperm development pathways. Reproduced from Ranganath RM, Plant Biology Vol 7 (2005), with permission from Thieme-Verlag

endosplasmic reticulum, is required for development of FG but not male gametophyte.

A number of genes with a function during FG development are known (Ranganath 2003; Pagnussat et al. 2005; Acosta-Garcia and Vielle-Calzada 2004; Ebel et al. 2004; Shi et al. 2005; Dresselhaus 2006 and the references therein). Among thousands of genes expressed in the FG, only about 600 could be identified by genetic screens (Drews and Yadegari 2002). By using ATH1 *Arabidopsis* whole-genome oligonucleotide array for transcriptome profile analyses and the *Arabidopsis thaliana* mutant *sporocyteless*, which produces ovules without FGs, 225 FG-specific genes have been identified (see Yu et al. 2005). cDNA libraries constructed from eggs and two-celled embryos isolated from wheat FGs showed unique transcriptional profiles (Sprunck et al. 2005). Genes required for FG development include those involved in protein degradation, cell death, signal transduction and transcription regulation (Pagnussat et al. 2005).

6
Endosperm Development

The endosperm in angiosperms develops from a fertilized central cell through *ab initio* cellular or *ab initio* nuclear or helobial pathways. Helobial endosperm combines the characteristics of both cellular and C-C pathways. In spite of considerable size differences, there are no qualitative differences between the daughter cells of the central cell division as well as the cells formed after cellularization of the coenocytic upper cell (Fig. 4D). Olsen (2004b) has provided a comparative account of endosperm developments in *Arabidopsis* and cereals.

6.1
Cereal Endosperm

Cereal endosperm has been a good model for studying cell specification. The central cell follows a typical C-C pathway. In maize, the coenocyte may have 256 to 512 free nuclei before cellularization begins (Walbot 1994). The large number of free nuclei is arranged peripherally prior to cellularization (Fig. 4B). The coenocytic central cell in cereals shows mechanistic similarities to the early embryo development in *Drosophila* (see Gilbert 2000) (Fig. 4B,C). The mature cereal endosperm shows five different types of cellular zones: aleurone, subaleurone, starchy, embryo-surrounding and transfer cell zones. Olsen (2001) suggests that cell fate specification in cereals occurs by positional signaling.

The embryo surrounding region (ESR) cells are densely cytoplasmic and express *ESR1, ESR2 and ESR3* genes, *ZmAE1* (*Zea mays* androgenic

embryo1) and *ZmAE3* genes and other gene products preferentially found in the transfer cells (reviewed by Berger 1999, Olsen 2004a). Starchy cells represent bulk of the endosperm. These cells accumulate starch and prolamine storage proteins encoded by genes expressed exclusively in these cells. Interestingly, in the maize mutants that lack aleurone cells, *crinkly4* (*cr4*) (Becraft and Asuncion-Crabb 2000), starchy endosperm cells are formed in place of aleurone cells.

The aleurone cells cover the endosperm excepting the region of transfer cells. Molecular markers of aleurone include *Ltp2*, *B22E*, *pZE40*, *ole1*, *ole2*, *per1hi33* (Olsen 2004a, b and the references therein). Aleurone cell fate in barley endosperm is established after the first periclinal division of the alveolar nuclei, with the outer sister nucleus destined to be cellularized to form an aleurone cell (Fig. 4B). Several mutations affecting aleurone cell fate and number are known in maize (see Shen et al. 2003). The *DEK* gene codes for a membrane protein with high homology to animal calpains, and may function to maintain and restrict the aleurone cell fate imposed by *CRINKLY4* (*CR4)* gene (Lid et al. 2002). However, *DEK1* gene is not specific to aleurone alone (see Becraft et al. 2002). Interestingly, the *Arabidopsis* epidermal gene *ATM1* is expressed in aleurone cells indicating their epidermal nature.

7 Future Prospects

As compared to animals, in flowering plants differential inheritance of CFDs (generally signal molecules or TFs) by the daughter cells after an ACD is indirectly inferred from gene expression and mutant analysis. Although some of the gene products are known, their cellular interactions are yet to be delineated. For example, intercellular movement of TFs is known to regulate several cell specification and patterning events in plants. Immunological detection techniques have shown that in *Arabidopsis* root, the SHORTROOT (SHR) protein moves from the stele cells to the endodermis where it activates cell differentiation and division (see Helariutta et al. 2000; Nakajima et al. 2001). Similarly, the product of *LEAFY* (*LFY*), one of the floral identity genes, has the ability to move between cells (Sessions et al. 2000). The MADS-box type of TFs – DEFICIENS (DEF) and GLOBOSA (GLO) involved in floral development – can move from inner L2 to outer L1 layer to control petal identity (see Kurata et al. 2005).

In the genomics era, molecular identity of a cell has to be ultimately understood in a functional genomics perspective. It has already been established that DNA sequence data from model organisms can provide a catalogue of genes and their predicted proteins, but how, where and when these proteins are expressed and interact in a particular developmental context

have to be understood only in a proteomic (expressed proteins in a cell) context. Fifty years of nuclear transplantation experiments have also conclusively established that it is the cytoplasm that directs nuclear functions (Gurdon and Byrne 2003). However, the inaccessibility of plant tissues or organs has been a major impediment in exploring cellular gene and protein networks. Future challenges lie in crafting innovative techniques to illuminate the molecular identity of a differentiated plant cell.

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References

Acosta-Garcia G, Vielle-Calzada J-P (2004) A classical Arabinogalactan protein is essential for the initiation of female gametogenesis in *Arabidopsis*. Plant Cell 16:2614–2628

Asker SE, Jerling L (1992) Apomixis in plants. CRC, Boca Raton

Baurie I, Laux T (2003) Apical meristems: the plant's fountain of youth. BioEssays 25:961–970

Becraft PW, Asuncion-Crabb Y (2000) Positional cues specify and maintain aleurone cell fate in maize endosperm development. Development 127:4039–4048

Becraft, PW, Li K, Dey N, Asuncion-Crabb Y (2002) The maize dek1 gene functions in embryonic pattern formation and cell fate specification. Development 129:5217–5225

Berger F (1999) Endosperm development. Curr Opin Plant Biol 2:28–32

Bergmann DC, Lukowitz W, Somerville CR (2004) Stomatal development and pattern controlled by a MAPKK kinase. Science 304:1494–1497

Bonhomme S, Horlow C, Vezon D et al. (1998) T-DNA mediated disruption of essential genes in *Arabidopsis* is unexpectedly rare and can not be inferred from segregation distortion alone. Mol Gen Genet 260:444–452

Bonke M, Thitamadee S, Mahonen AP, Hauser M-T, Helariutta Y (2003) APL regulates vascular tissue identity in *Arabidopsis*. Nature 426:181–186

Boutilier K, Offringa R, Sharma VK et al. (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell 14:1737–1749

Byrne ME (2005) Networks in leaf development. Curr Opin Plant Biol 8:59–66

Byrne ME, Kidner CA, Martienssen RA (2003) Plant stem cells: divergent pathways and common themes in shoots and roots. Curr Opin Genet Devel 13:551–557

Coen ES, Mayerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353:31–37

Colcombet J, Bolsson-Demier A, Ros-Palau R, Vera CE, Schroeder JI (2005) *Arabidopsis* SOMATC EMBRYOGENESIS RECEPTOR KINASE1 and 2 are essential for tapetum development and microspore maturation. Plant Cell 17: 3350–3361

Demura T, Tashiro G, Horiguchi G, Kishimoto N et al. (2002) Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. Proc Natl Acad Sci USA 99:15794–15799

Di Laurenzio L, Wysoka-Diller J, Malamy JE, Pysh L, Helariutta Y, Freshour G, Hahn MG, Feldmann KA, Benfey PN (1996) The SCARECROW gene regulates an asymmetric division that is essential for generating the radial organization of the *Arabidopsis* root. Cell 86:423–433

Dinneny JR, Yanofsky MF (2004) Vascular patterning: xylem or phloem? Curr Biol 14(3):R112–R114

Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48:1493–1509

Dolan L, Costa S (2001) Evolution and genetics of root hair stripes in the root epidermis. J Exp Bot 52:413–417

Dolan L, Roberts K (1995) Two ways to skin a plant: the analysis of root and shoot epidermal development in *Arabidopsis*. BioEssays 17:865–872

Dresselhaus T (2006) Cell-cell communication during double fertilization. Curr Opin Plant Biol 9:41–47

Drews GN, Yadegari R (2002) Development and function of the angiosperm female gametophyte. Ann Rev Genet 36:99–124

Ebel C, Mariconti L, Gruissem W (2004) Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. Nature 429:776–780

Eckardt NA (2002) Plant reproduction: insights into the "Abominable Mystery". (Meeting Report). Plant Cell 14:1669–1673

Emery JF, Floyd SK, Alvarez J et al. (2003) Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes. Curr Biol 13:1768–1774

Fletcher N (2002) Shoot and floral meristem maintenance in *Arabidopsis*. Annu Rev Plant Biol 53:45–66

Friml J, Vieten A, Sauer M et al. (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature 426:147–153

Fukaki H, Wysoka-Diller J, Kato T, Fujisawa H, Benfey PN, Tasaka M (1998) Genetic evidence that the endodermis is essential for shoot gravitropism in *Arabidopsis thaliana*. Plant J 14:425–430

Fukuda H (2004) Signals that control plant vascular cell differentiation. Nature Rev Mol Cell Biol 5:379–391

Gallagher K, Smith LG (1999) *Discordia* mutations specifically misorient cell division during development of maize leaf epidermis. Development 126: 4623– 4633

Gaudin V, Libault M, Pouteau S, Juul T, Zhao G, Lefebvre D, Grandjean O (2001) Mutations in LIKE HETEROCHROMATIN PROTEIN1 affect flowering time and plant architecture in *Arabidopsis*. Development 128:4847–4858

Geisler MJ, Deppong DO, Nadeau JA, Sack FD (2003) Stomatal neighbour cell polarity and division in *Arabidopsis*. Planta 216:571–579

Gilbert SF (2000) Developmental biology, 6th edn. Sinauer Associates, Sunderland, Massachussets

Gurdon JB, Byrne JA (2003) The first half century of nuclear transplantation. Proc Natl Acad Sci USA 100:8048–8052

Haecker A, Gross-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T (2004) Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development 131:657–668

Hake S, Char BR, Chuck G, Foster T, Long J, Jackson D (1995) Homeobox genes in the functioning of plant meristems. Phil Trans R Soc London B 350:45–61

Hardtke CS (2006) Root development – branching into novel spheres. Curr Opin Plant Biol 9:66-71

Heidstra R, Welch D, Scheres B (2004) Mosaic analyses using marked activation and deletion clones dissect *Arabidopsis* SCARECROW action in asymmetric cell division. Genes Devel 18:1964–1969

Helariutta Y, Fukaki H, Wysocka-Diller J, Nakajima K, Jung J, Sena G, Hauser M-T, Benfey PN (2000) The *SHORT-ROOT* gene controls radial patterning of the *Arabidopsis* root through radial signaling. Cell 101:555–567

Honys D, Twell D (2004) Transcriptome analysis of male gametophyte development in *Arabidopsis*. Genome Biol 5:R85 (doi 10:1186/gb2004-5-11-85)

Horvitz HR, Herskowitz I (1992) Mechanisms of asymmetric division: two Bs are not two Bs, that is the question. Cell 68:237–255

Huang B-Q, Russell SD (1992) Female germ unit: organization, isolation and function. Int Rev Cytol 140:233–292

Hulskamp M (2004) Plant trichomes: a model for cell differentiation. Nature Rev Mol Cell Biol 5:471–480

Iwakawa H, Shinmyo A, Sekine M (2006) *Arabidopsis* CDKA1, a cdc2 homologue, controls proliferation of generative cells in male gametogenesis. Plant J 45: 819–831

Jack T (2004) Molecular and genetic mechanisms of floral control. Plant Cell 16:S1–S17

Jackson D, Veit B, Hake S (1994) Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development 120:405–413

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Current Biol 15:1560–1565

Jenik PD, Jurkuta REJ, Barton MK (2005) Interactions between the cell cycle and embryonic patterning in *Arabidopsis* uncovered by a mutation in DNA polymerase E [WOA]. Plant Cell 17:3362–3377

Jiang K, Feldman LJ (2005) Regulation of root apical meristem development. Ann Rev Cell dev Biol 21:485–509

Johnson MA, von Besser K, Zhou Q, Smith E, Aux G, Patton D, Levin JZ, Preuss D (2004) *Arabidopsis hapless* mutations define essential gametophytic functions. Genetics 168:971–982

Johnson-Brousseau SA, McCormick S (2004) A compendium of methods useful for characterizing *Arabidopsis* pollen mutants and gametophytically expressed genes. Plant J 39:761–775

Jurgens G (2001) Apical-basal pattern formation in *Arabidopsis* embryogenesis. EMBO J 20:3609–3616

Kamiya N, Itoh J-I, Morikami A, Nagato Y, Matsuoka M (2003) The SCARECROW gene's role in asymmetric cell divisions in rice. Plant J 36:45–54

Kidner C, Sundaresan V, Roberts K, Dolan L (2000) Clonal analysis of the *Arabidopsis* root confirms that position, not lineage, determines cell fate. Planta 211:191–199

Kim HU, Li Y, Huang AHC (2005) Ubiquitous and endosplasmic reticulum-located Lysophosphotidyl Acetyltransferase, LAPT2, is essential for female but not male gametophyte development in *Arabidopsis*. Plant Cell 17:1073–1089

Krikorian AD, Smith DL (1992) Somatic embryogenesis in carrot (*Daucus carota*). In: Lindsey K (ed) Plant tissue culture manual. Kluwer Academic Publishers, Dordrecht, A9, pp 1–32

Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T (2005) Transcriptome switches for protoxylem and metaxylem vessels. Genes Dev 19:1855–1860

Kurata T, Okada K, Wada T (2005) Intercellular movement of transcription factors. Curr Opin Plant Biol 8:600–605

Lalanne E, Michelidis C, Moore JM, Gagliano W, Johnson A, Patel R, Howden R, Vielle-Calzada J-P, Grossniklaus U, Twell D (2004) Analysis of transposon insertion mutants highlights the diversity of mechanisms underlying male progamic development in *Arabidopsis*. Genetics 167:1975–1986

Laux T (2003) The stem cell concept in plants: a matter of debate. Cell 113:281–283

Laux T, Wurschum T, Breuninger H (2004) Genetic regulation of embryonic pattern formation. Plant Cell 16:S190–S202

Levy YY, Dean C (1998) The transition to flowering. Plant Cell 10:1973–1990

Lid SE, Gruis D, Jung R et al. (2002) The defective kernel 1 (dek1) gene required for aleurone cell development in the endosperm of maize grains encodes a membrane protein of the calpain gene superfamily. Proc Natl Sci Acad USA 99:5460–5465

Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeobox proteins encoded by the STM gene of *Arabidopsis*. Nature 379:66–69

Lotan T, Ohto M-A, Yee KM et al. (1998) *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell 93:1195–1205

Lu P, Porat R, Nadeau JA, O'Neill SD (1996) Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. Plant Cell 8:2155–2168

Lukowitz W, Roeder A, Parmenter D, Somerville C (2004) A MAPKK kinase gene regulates extra-embryonic cell fate in *Arabidopsis*. Cell 116:109–119

Ma H (2005) Molecular genetic analyses of microspororgenesis and microgametogenesis in flowering plants. Ann Rev Plant Biol 56:393–434

Mattsson J, Ckurshumova W, Berleth T (2004) Auxin signaling in *Arabidopsis* leaf vascular development. Plant Physiol 131:1327–1339

Mayer U, Buttner G, Jurgens G (1993) Apical-basal pattern in the *Arabidopsis* embryo: studies on the *gnom* gene. Development 117:149–162

Mayer KFX, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T (1998) Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell 95:805–815

McCormick S (2004) Control of male gametophyte development. Plant Cell 16:S142–S153

Meinke DW (1991) Perspectives on genetic analysis of plant embryogenesis. Plant Cell 3:857–876

Moon Y-H, Chen L, Pan R-L et al. (2002) EMF repression of embryo and flower development in *Arabidopsis*. Abstract 11-22, XIII International Conference on *Arabidopsis* Research, Seville

Morison SJ, Kimble J (2006) Asymmetric and symmetric stem cell divisions in development and cancer. Nature 441

Nadeau JA, Sack FD (2003) Stomatal development: cross talk puts mouths in place. Trends Plant Sci 8:294–299

Nakajima K, Sena G, Benfey PN (2001) Intercellular movement of the putative transcription factor SHR in root patterning. Nature 413:307–311
Natesh S, Rau MA (1984) The embryo: in embryology of angiosperms. Johri BM (ed) Springer, Berlin Heidelberg New York, pp 377–443
Nawy T, Lee J-Y, Colinas J et al. (2005) Transcriptional profile of the *Arabidopsis* root Quiescent Center. Plant Cell 17:1908–1925
Nowack MK, Grini PE, Jakoby MJ, Lafos M, Koncz C, Schnittger A (2006) A positive signal from the fertilization of the egg cell sets of endosperm proliferation in angiosperm embryogenesis. Nature Genet 38:63–67
Olsen O-A (2001) Endosperm development: cellularization and cell fate specification. Ann Rev Plant Physiol 52:233–267
Olsen O-A (2004a) Dynamics of maize aleurone cell formation: the 'surface' rule. Maydica 49(1):37–40
Olsen O-A (2004b) Nuclear endosperm development in cereals and *Arabidopsis thaliana*. Plant Cell 16:S214–S227
Pagnussat GC, Yu H-J, Ngo QA, Rajani S, Mayalagu S, Johnson CS, Capron A, Xie L-F, Ye D, Sundaresan V (2005) Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. Development 132:603–614
Ranganath RM (2003) Female gametophyte development in higher plants meiosis and mitosis break the cellular barrier. Plant Biol 5:42–49
Ranganath RM (2004) Harnessing the developmental potential of nucellar cells: barriers and opportunities. Trends Biotechnol 22:504-510
Ranganath RM (2005) Asymmetric cell divisions in flowering plants: One mother, 'two-many' daughters. Plant Biol 7:425–448
Ranganath RM, Nagashree NR (2000) Selective cell elimination during microsporogenesis in sedges. Sexual Plant Reprod 13(1):53–60
Ranganath RM, Nagashree NR (2001) Role of programmed cell death during development. Int Rev Cytol 202:159–244
Reddy GV, Heisler MG, Ehrhardt DW, Mayerowitz EM (2004) Real-time lineage analysis reveals oriented cell divisions associated with morphogenesis at the shoot apex of *Arabidopsis thaliana*. Development 131:4225–4237
Reyes JC (2006) Chromatin modifiers that control plant development. Curr Opin Plant Biol 9:21–27
Rotman N, Durbarry A, Wardle A, Yang WC, Chaboud A, Faure J-E, Berger F, Twell D (2005) A novel class of MYB factors controls sperm-cell formation in plants. Cell 15:244–248
Russinova E, de Vries S (2000) Parental contribution to plant embryos. Plant Cell 12:461–464
Sabatini S, Heidstra R, Wildwater M, Scheres B (2003) SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. Genes Dev 17:354–358
Scheres B, Benfey PN (1999) Asymmetric cell division in plants. Annu Rev Plant Physiol 50:505–537
Scheres B, Di Laurenzio L, Willemsen V, Hauser MT, Janmaat K, Weisbeek P, Benfey PN (1995) Mutations affecting the radial organization of the *Arabidopsis* root display specific defects throughout the embryonic axis. Development 121:53–62

Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124:2049–2062

Sessions A, Yanofsky MF, Weigel D (2000) Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1. Science 289:779–781

Shani E, Yanai O, Ori N (2006) The role of hormones in shoot apical meristem function. Curr Opin Plant Biol 9:484–489

Shen B, Li C, Min Z, Meeley RB, Tarczynski MC, Olsen O-A (2003) *sal1* determines the number of aleurone cell layers in maize and encodes a class E vacuolar sorting protein. Proc Natl Acad Sci USA 100:6552–6557

Shi D-Q, Liu J, Xiang Y-H, Ye D, Sundaresan V, Yang W-C (2005) SLOW WALKER1, essential for gametogenesis in *Arabidopsis* encodes a WD-40 protein involved in 18S ribosomal biogenesis. Plant Cell 17:2340–2345

Shpak ED, McAbee JM, Pillitteri LJ, Torii KU (2005) Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science 309:290–293

Sieburth LE, Deyholos MK (2005) Vascular development: the long and winding road. Curr Opin Plant Biol 9:48–54

Singh MB, Bhalla PL (2006) Plant stem cells carve their own niche. Trends Plant Sci 11:241–246

Smith-White S (1959) Pollen development patterns in Epacridaceae: a problem in cytoplasmic-nuclear interaction. Proc Linnean Soc NSW 84:8–35

Sprunck S, Baumann U, Edwards K, Langridge P, Dresselhaus T (2005) The transcript composition of egg cells changes significantly following fertilization in wheat (*Triticum aestivum* L.). Plant J 41:660–672

Stone SL, Kwong LW, Yee KM et al. (2001) LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. Proc Natl Acad Sci USA 98:11806–11811

Thompson MV (2006) Phloem: the long and short of it. Trends Plant Sci 11(1):26–32

Tsiantis M (2001) Control of shoot cell fate: beyond homeoboxes. Plant Cell 13:733–738

Tsukaya H (2002) Leaf development. The *Arabidopsis* book. Doi:10.1199/tab.0072

Twell D, Park SK, Lalanne E (1998) Asymmetric division and cell fate determination in developing pollen. Trends Plant Sci 3(8):305–310

van den Berg C, Willemsen V, Hege W, Weisbeek P, Scheres B (1995) Cell fate in the *Arabidopsis* root meristem is determined by directional signalling. Nature 378:62–65

Verma DPS (2001) Cytokinesis and building of cell plate in plants. Ann Rev Plant Physiol 52:751–784

Vernon DM, Meinke DW (1994) Embryogenic transformation of the suspensor in *twin*, a polyembryonic mutant of *Arabidopsis*. Dev Biol 165:566–573

Walbot V (1994) Overview of key steps in aleurone development. In: Freeling M, Walbot V (eds) The maize handbook . Springer, Berlin Heidelberg New York, pp 78–80

Weigel D, Jurgens G (2002) Stem cells that make stems. Nature 415:751–754

Willemsen V, Friml J, de Vrieze G, Weisbeek P, Scheres B (1998) The HOBBIT gene is required for formation of the root meristem in the *Arabidopsis* embryo. Development 125:521–531

Williams L, Fletcher JC (2005) Stem cell regulation in the *Arabidopsis* shoot apical meristem. Curr Opin Plant Biol 8:582–586

Wilson ZA, Yang C (2004) Plant gametogenesis: Conservation and contrasts in development. Reproduction 128:483–492

Wysocka-Diller JW, Helariutta Y, Fukaki H, Malamy JE, Benfey PN (2000). Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. Development 127:595–603

Xiao W, Custard KD, Brown RC et al. (2006) DNA methylation is critical for *Arabidopsis* embryogenesis and seed viability. Plant Cell 18:805–814

Xu J, Scheres B (2005) Cell polarity: ROPing the ends together. Curr Opin Plant Biol 8:613–618

Yadegari R, Drews GN (2004) Female gametophyte development. Plant Cell (Suppl) 16:S133–S141

Yanai O, Shani E, Dolezal K, Tarkowski P, Sablowski R, Sandberg G, Samach A, Ori N (2005) *Arabidopsis* KNOX1 proteins activate cytokinin biosynthesis. Curr Biol 15:1566–1571

Yang W-C, Sundaresan V (2000) Genetics of gametophyte biogenesis in *Arabidopsis*. Curr Opin Plant Biol 3:63–67

Yu H-J, Hogan P, Sundaresan V (2005) Analysis of the female gametophyte transcriptome of *Arabidopsis* by comparative expression profiling. Plant Physiol 139:1853–1869

Zhao C, Craig JC, Petzold HE et al. (2005) The xylem and phloem transcriptomes from secondary tissues of *Arabidopsis* root-hypocotyl. Plant Physiol 138:803–818

Zipori D (2004) The nature of stem cells: state rather than entity. Nature Rev Genet 5:873–878

Zuo J et al. (2002) The *WUSCHEL* gene promotes vegetative to embryonic transition in *Arabidopsis*. Plant J 30:349–360

Symmetry Breaking in Stem Cells of the Basal Metazoan Hydra

Thomas C.G. Bosch

Abstract

Among the earliest diverging animal phyla are the Cnidaria. Cnidaria were not only first in evolution having a tissue layer construction and a nervous system but also have cells of remarkable plasticity in their differentiation capacity. How a cell chooses to proliferate or to differentiate is an important issue in stem cell biology and as critical to human stem cells as it is to any other stem cell. Here I revise the key properties of stem cells in the freshwater polyp *Hydra* with special emphasis on the nature of signals that control the growth and differentiation of these cells.

1 Stem Cells and the Need to Have Comparative Data from Ancestral Metazoans

Stem cells have the unique ability to undergo self-renewing mitotic divisions in which one or both progeny retain stem cell identity and the capacity to replicate almost indefinitely. The daughters of stem cell divisions also have the option to follow a differentiation pathway. The balance between self-renewal and differentiation must be strictly regulated to maintain the stem cell pool and to generate the required supply of fully differentiated cells. Understanding how stem cells are regulated is crucial in learning how tissues are formed and maintained. The self-renewing ability is regulated both by an intracellular mechanism and by intercellular signalling. Cell-autonomous mechanisms governing asymmetric cell divisions in invertebrates have been elucidated in a few stem cell models such as neuroblasts and germ-line stem cells (GSC) in *Drosophila* (Deng and Lin 1997; Lin and Schagat 1997), whereas the role of extrinsic signalling in controlling asymmetric cell divisions has been implicated in several systems (Morrison et al. 1997). External stimuli that alter self-renewal of several classes of stem cells and affect asymmetric divisions include cytokines, matrix proteins, hormones, and local interactions between stem cells and their neighboring

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cells. These extracellular signals may then influence the cell cycle machinery or the cytoskeletal organization of stem cells for their formation and/or divisional asymmetry. Asymmetric cell divisions also involve marked differences in gene expression as well as extraordinary genome modifications. For example, in *C. elegans* and *Drosophila* transcriptional quiescence in early germ cells is thought to be essential for the establishment of distinct germ-line and somatic fates (Seydoux 1996; Bashirullah et al. 1998). Genome modifications associated with asymmetric divisions are of importance in blood stem cells (Akashi et al. 2003) and also play crucial roles in switching of mating types in yeast, programmed chromosome breakage and chromatin elimination in *Ascaris*, and the development of a transcriptionally inert germline micronucleus in spirotrichous ciliates. Studies in insects and worms have shown that just a few signalling pathways generate most of the cellular and morphological diversity during the development of individual organisms (Pires-DaSilva and Sommer 2003). This similarity in signalling pathways between disparate animal phyla has provided convincing evidence for the monophyletic origin of metazoa. Insects, worms and vertebrates all derive from the "triploblast" or "Bilateria" clade of metazoans. Since several animal phyla diverged, however, before the origin of this clade, the discovery of shared signalling cascades tells us little about their origin and original roles, until we have comparative data from more basal animals. The aim of this chapter, therefore, is to review our understanding of the pathways that regulate proliferation, self-renewal and differentiation of stem cells in the basal metazoan *Hydra*.

2
At the Origin of Metazoan Evolution: Placozoa, Porifera and Cnidaria

Since the nineteenth century, *Trichoplax adhaerens* has been considered the most simple metazoan and proposed to be a model organism for the transition from single cellular protists to multicellular metazoa (e.g. Metschnikoff 1883; Collins 1998; Schierwater and Kuhn 1998). *Trichoplax* is composed of a ciliated epithelium that is differentiated dorsally and ventrally and contains just four distinct somatic cell types (Grell 1971). Its particular morphology, characterized by an extreme form of simplicity, has justified the creation of an own phylum, the Placozoa (Grell 1971). This simplicity together with molecular data from the *Trichoplax* mitochondrial genome (Dellaporta et al. 2006) provide convincing support for the phylogenetic placement of the phylum Placozoa at the root of the Metazoa. With regard to asymmetric cell divisions, with only one transcription factor of the Antp superclass gene, Trox-2, cloned so far, very little is known about the molecules involved leaving the molecular machinery controlling *Trichoplax* asymmetric cell divisions sitting in the dark.

Porifera (sponges) most likely evolved from simple unicellular flagellates, the choanoflagellates (Leys and Ereskovsky 2006). Porifera have six to ten different cell types including ciliated choanocytes that drive water through canals and chambers. There is a great deal of cell mobility and reversal of cell differentiation (Ruppert and Barnes 1994) and many sponges have remarkable powers of regeneration. Sponges have signal transduction pathways including receptor tyrosine kinases and protein kinase C, that are the basis for physiological processes in higher metazoa (Müller 2001). This and other observations indicate that sponges share a common ancestor with all other metazoa and that multicellularity is associated with the presence of an extracellular matrix, cell adhesion molecules and membrane associated receptors. Little is known, however, about the signals and interactions required for differentiation of sponge-specific features such as the choanocytes (Leys and Ereskovsky 2006). So far only a few homologues of homeobox transcription factors have been isolated from sponges (Müller 2001). Although these observations providing clear evidence supporting the monophyletic origin of the Metazoa, molecular techniques are still in their infancy in the Porifera (Leys and Ereskovsky 2006) and the function of developmental control genes remains to be elucidated. However, with a genome project currently underway to sequence the ceractinomorph demosponge *Reniera* sp. at the Joint Genome Institute, molecular analysis of poriferan development should make significant progress in the next few years and also offer insight into the molecular machinery needed for asymmetric cell divisions in this most basal metazoan phylum.

Among the basal metazoa, Cnidaria, the sister group of the Bilateria, are the first in evolution that have a defined body plan, stem cells, a nervous system, and a tissue layer construction. Cnidarians are diploblastic consisting of two epithelia, the ectoderm and the endoderm surrounding a gastric cavity. Cnidarians such as the freshwater polyp *Hydra* have a long history as model systems in developmental biology because of their remarkable capacity to regenerate missing body parts. Most spectacularly, *Hydra* polyps when dissociated into a suspension of single cells and pelleted into aggregates by centrifugation will organize themselves into complete polyps within a few days (Gierer et al. 1972). This ability for self-organization is at least partially due to the continuous presence of stem cells with high self-renewal capacity and high phenotypic plasticity in adult tissue. Multipotent interstitial stem cells have been identified in *Hydra* by in vivo cloning (Bosch and David 1987). Interestingly, totipotent stem cells have also been identified recently in the colonial hydrozoan *Hydractinia echinata* (Müller et al. 2004) by repopulating interstitial cell free colonies with allogeneic interstitial cells and demonstrating that the resulting phenotype was reverted to that of the donor colony. As I have outlined elsewhere (Bosch 2006), the stem cell-ness of the *Hydra* tissue is not only sufficient to explain Hydra's unprecedented regeneration capacity, but also allows *Hydra* its unique life cycle in which proliferation occurs mostly asexual by budding.

3
Key Properties of Epithelial and Interstitial Stem Cells in *Hydra*

In *Hydra*, there are about 20 cell types distributed among 3 cell lineages. Each of the epithelial layers is made up of a cell lineage, while the remaining cells are part of the interstitial cell lineage which reside among the epithelial cells of both layers. Both the epithelial cells as well as the interstitial cells in the body column continuously undergo self-renewing mitotic divisions (Dübel et al. 1987). To prove that the epithelial cells indeed have stem cell properties, we recently (Wittlieb et al. 2006) have made use of transgenic polyps and transplanted a single GFP-expressing endodermal epithelial cell into a nontransgenic polyp. By doing so we have generated polyps in which the entire ectodermal or endodermal epithelium contains the transgene (Wittlieb et al. 2006). Supporting earlier observations, such in vivo tracking of GFP labelled epithelial cells also showed that there is continuous tissue displacement towards the extremities. Displacement of ectodermal epithelial cells into the tentacles results in differentiation of battery cells. Displacement of epithelial cells towards the lower body regions results in budding and – at the boundary between the peduncle and the basal disk – in differentiation of epithelial cells into basal disk cells which begin to secret mucus just after passing through that boundary. This remarkable phenotypic plasticity of epithelial cells in response to positional signals allows *Hydra* to build complex structures such as the tentacles with only a limited number of different cell types.

Located primarily between the ectodermal epithelial cells throughout the gastric region there are the interstitial stem cells. The main evidence for their stem cell properties comes from in vivo cloning experiments which have shown (see Fig. 1) that these cells are multipotent and capable of somatic and germ line differentiation (David and Murphy 1977; Bosch and David 1987). Multipotent interstitial stem cells in *Hydra* give rise to neurons (see Fig. 2), secretory cells and gametes in a position dependent manner (Bosch and David 1987). These stem cells also give rise to cnidocytes, which are unique to and characteristic of all cnidarians. Interstitial stem cells in *Hydra* are found throughout the gastric region; they are absent, however, in the head and foot region (David and Plotnick 1980). In numerous cloning experiments no stem clones were found containing only one differentiated type of somatic cells. Thus, there is no evidence for extensively proliferating subpopulations of somatic intermediates in Hydra (Fig. 1).

Cnidocyte differentiation occurs exclusively in the gastric region as a highly complex, multistep process (reviewed in Tardent 1995). Cnidocytes differentiate in clusters of 8–32 cells in the body region (David and Challoner 1974). Cells within clusters remain interconnected by cytoplasmic

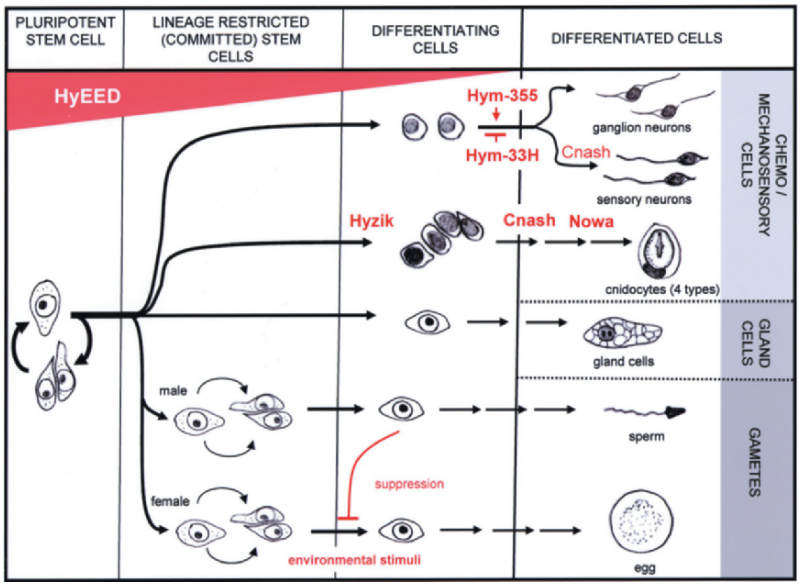

Fig. 1. A model for the differentiation of interstitial stem cells in *Hydra*. Factors affecting steps of differentiation are shown in red. For details and references see text

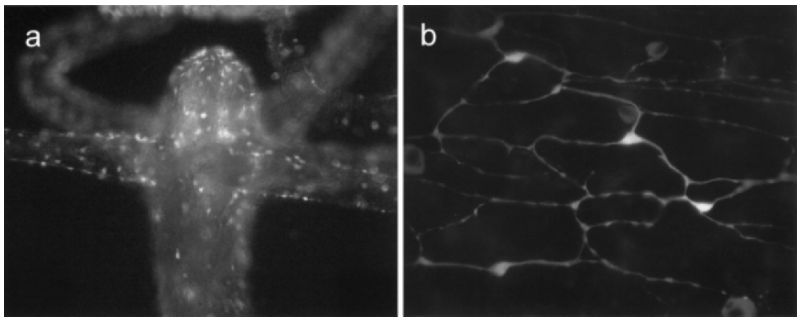

Fig. 2. **a,b** Transgenic Hydra polyps are paving the way for in vivo imaging to analyze nerve cell differentiation at the basis of metazoan evolution: **a** *Hydra vulgaris* (AEP) polyp expressing GFP exclusively in the interstitial cell lineage which includes the nerve precursors; **b** GFP expressing ganglion neurons

bridges. During differentiation (Shimizu and Bode 1995) each nematoblast produces a nematocyst capsule inside a secretory vesicle. Following capsule differentiation, the clusters of differentiating cnidocytes break up into single cells that migrate towards the tentacles and become mounted in specialized tentacle epithelial cells, termed battery cells (David and Gierer 1974).

Neuron differentiation from multipotent interstitial stem cells also occurs exclusively in the gastric region (David and Gierer 1974). After entering the neuron differentiation pathway, about a half of the neuron precursor cells migrate toward the head and foot (Heimfeld and Bode 1984; Fujisawa 1989; Teragawa and Bode 1990, 1995; Technau and Holstein 1996; Hager and David 1997). The remaining half of the neuron precursors do not migrate, but complete differentiation and are integrated into the nerve net (Fig. 2).

Little is known about gland cell differentiation from interstitial stem cells. Previous studies have described gland cells in *Hydra* as secretory cells distributed gradually along the body column and used for extracellular digestion of prey (Schmidt and David 1986; Bode et al. 1987). Recent studies based on the expression pattern of several genes (Augustin et al. 2006; Guder et al. 2006) suggest that gland cells are distributed along the whole body column down to the basal disc. The previously reported graded distribution of gland cells with a maximum density in the subhypostomal region (Schmidt and David 1986, Bode et al. 1987) appears to reflect the previously used analytical methods which were mostly histological observations.

In the gamete differentiation pathway, unipotent subpopulations of interstitial cells have been isolated (Fig. 1). They are capable of extensive proliferation but committed to either spermatogenesis (Littlefield 1985; Nishimiya-Fujisawa and Sugiyama 1993) or oogenesis (Littlefield 1991; Nishimiya-Fujisawa and Sugiyama 1995). These unipotent stem cells are present in asexually proliferating polyps in low numbers and dividing at a slower rate than their multipotent precursor cells (Holstein and David 1990). In response to environmental stimuli, these cells start to proliferate rapidly and differentiate into gametes. Interstitial cells are not only the precursors for gametes but also themselves responsible for sex determination (Littlefield 1984; Campbell 1985). Surprisingly, somatic components (e.g. epithelial cells) do not play a role in determining the sexual phenotype. In addition, male interstitial cells of *Hydra* suppress the ability of female stem cells to differentiate eggs thereby causing "masculinization" of females (Sugiyama and Sugimoto 1985; Littlefield 1994). They, however, do not interfere with the ability of female stem cells to proliferate and produce somatic cells (Bosch and David 1986). The molecular nature of this suppression is completely unkown.

Figure 1 summarizes these findings and illustrates the current view of the differentiation and proliferation potential of interstitial stem cells in *Hydra*. Transplantation experiments with genetically marked interstitial cell clones indicated that interstitial stem cells in *Hydra* display very little migratory activity and grow as contiguous patches (Bosch and David 1990). The growth of interstitial cells in clonal patches has an important implications since it affects the distribution of stem cells to daughter polyps and, for example, leads to male polyps which occasionally give rise to female polyps (Bosch and David 1987). Taken together, the differentiation

pattern of interstitial stem cells exhibits a strong dependence on position along the body axis indicating that these cells are capable to interpret positional information to differentiate according to their genetic program. Stem cells in *Hydra*, therefore, point to the importance of spatial organization and thus extrinsic influences (Wolpert 1988). Can we define these extrinsic influences in molecular terms?

4
Hydra Interstitial Stem Cells and their Niches

A specialized microenvironment, the stem cell niche, is one of the factors regulating stem cell maintenance and the crucial choice between self renewal and the initiation of differentiation (Spradling et al. 2001; Moore and Lemischka 2006). Although the niche concept was introduced already in 1978 (Schofield 1978), only recently it became clear that stem cells in vertebrates as in invertebrates require paracrine signals from the surrounding microenvironmental cells to maintain their identity and self-renewal capacity (Moore and Lemischka 2006). The best understood examples of regulation of stem-cell renewal by the niche are to be found in the female and male germ-line stem cells of *Drosophila* (Yamashita et al. 2005). In *Drosophila* female germ line, where the regulatory circuitry from the signal to the key target in the stem cell has been worked out, the main role of the signal from the niche is to block expression of genes that trigger the onset of differentiation (Chen and McKearin 2003; Xie and Spradling 2000). In *Hydra*, the interstitial cells grow and differentiate in the interstices between ectodermal epithelial cells. As I will describe below, the interstitial stem cell population in *Hydra* is strongly influenced by its complex microenvironment.

4.1
Key Elements that Specify Self-renewal and Control Differentiation of Interstitial Stem Cells

The major cellular components of the microenvironment in which *Hydra* interstitial cells grow and make their decisions are epithelial cells and cells of the interstitial cell lineage themselves. The mesoglea may present an additional important acellular component of the microenvironment. A number of transplantation experiments by which stem cells have been introduced into a variety of different host tissues have revealed two environmental parameters as particularly important. The first parameter – nerve cell density in host tissue – positively influences interstitial cell proliferation. The second parameter – interstitial cell density – negatively influences proliferation. Growth of interstitial cells is faster in tissue with reduced interstitial cell numbers than in normal tissue (Yaross and Bode 1978; Sproull and David 1979; Holstein

and David 1990). Thus, stem cell proliferation in *Hydra* is controlled by a feed-back signal from interstitial cells and their derivatives: decreasing the number of stem cells causes an increase in the self renewal probability and leads to recovery of normal stem cell levels (Sproull and David 1979). Conversely, increasing the number of stem cells decreases the self-renewal probability. Although there is evidence suggesting that the feed-back signal is of short range (David and MacWilliams 1978; Sproull and David 1979; Bosch et al. 1991), the nature of the signal(s) by which interstitial cells measure their density is unknown. It may be a diffusible molecule produced by stem cells or directly mediated by cell-cell contact. Interestingly, feedback regulation by stem cell density does not occur when stem cells are transferred in genetically unrelated tissue (David et al. 1991). Since under these conditions the stem cells behave as if the genetically distinct cells are not present, the signalling involved is species specific and not conserved.

4.2 Paracrine Signalling and Feedback Loops During Neuron Differentiation

There is accumulating evidence that the nervous system of *Hydra* (see Fig. 2) is much more complex than previously conceived. It is composed of many subpopulations which are characterized by the expression of different neuropeptides, genes, and antigens (Grimmelikhujizen et al. 1982a, b; Dunne et al. 1985; Koizumi et al. 1988; Hobmayer et al. 1990a, b; Yum et al. 1998; Darmer et al. 1998; Takahashi et al. 2000; Hansen et al. 2000; Hayakawa et al. 2004). Due to the dynamic state of the tissue which is constantly undergoing renewal as a result of continuous growth and differentiation, the nerve net is also in a steady state of production and loss of neurons. To compensate for the loss and to maintain the homeostasis, neurons in *Hydra* arise continuously by differentiation from multipotent interstitial stem cells (David and Gierer 1974). Neuron differentiation from interstitial stem cells involves several sequential events: commitment to differentiation, migration of committed precursors to the site of differentiation, final mitosis and terminal differentiation as neurons. Essential part of the signalling system involved in maintaining the neuron population are peptides (Bosch and Fujisawa 2001; Bosch 2003). Through a systematic screening of peptide signalling molecules from *Hydra* we have discovered two groups of peptides that affect neuron differentiation (Takahashi et al. 1997, 2000). Neuropeptide Hym-355 positively regulates neuron differentiation, while PW peptides such as Hym-33H that share a common C-terminal motif of Leu or Ile-Pro-Trp negatively regulate neuron differentiation (Takahashi et al. 1997, 2000). Pulse-labelling experiments indicate (Fujisawa, personal communication) that PW peptide inhibit early stages of neuron differentiation by inhibiting commitment or migration of precursor

cells. By indirect immunofluorescence staining using anti-Hym-33H antiserum, Fujisawa and colleagues recently could localize the Hym-33H PW peptide in the ectodermal epithelial cells (Fujisawa, personal communication). Thus, PW peptides appear to be part of the microenvironmental factors which directly affect neuron differentiation – underlining the importance of niche cells as source for molecules that activate pathways controlling and specifying stem cell fate. Since both groups of peptides, Hym355 and Hym-33H, counteract each other, we could incorporate the actions of these molecules into a feedback model to explain the homeostasis of a neuron population (Takahashi et al. 2000). An emerging theme is the balance between Hym-355 and PW peptides that determines the differentiation of stem cells to neurons. The model also proposes that neurons interact with epithelial cells via a short-range signal or direct cell-cell communication and induce the latter cells to deliver PW peptides. In any case, the observations provide convincing support for the niche concept and for an intensive spatio-temporal dialog occurring between interstitial stem and niche cells in *Hydra*.

5
The Molecular Regulation of Neuronal Differentiation Involves bHLH Class Transcription Factors

Proneural genes that encode the bHLH class transcription factors play key roles in the formation of the nervous system in both invertebrates and vertebrates. In *Hydra*, Grens et al. (1995) have isolated the bHLH transcription factor gene *achaete-scute* homolog *Cnash*. In bilaterians, *achaete-scute* is involved neurogenesis. The *Hydra* CnASH protein was found to form heterodimers with the *Drosophila* bHLH protein Daughterless and to bind specifically to consensus achaete–scute DNA-binding sites (Grens et al. 1995). Expression of CnASH in ac–sc double mutants of *Drosophila* can rescue the mutant phenotype. Interestingly, *Cnash* originally was thought (Grens et al. 1995) not to be expressed in nerve cells or nerve cell precursors, but exclusively in cell clusters that give rise to cnidocytes , consistent with the idea that cnidocytes are a neuronal cell type. Recently, however, Hayakawa et al. (2004) reported the involvement of CnASH in the differentiation pathway of sensory neurons in the tentacles. The authors observed that in addition to differentiating cnidocytes in the body column, sensory neurons at early stages of differentiation in the tentacles also express CnASH. Since CnASH-positive neurons are distributed from the base to the tip of tentacles, the gene may also be involved in maintenance of the differentiated state. Thus, proneural genes of the achaete–scute (ac–sc) family are involved in neurogenesis in animals which were first in evolution to develop a nervous system.

6
Neural Effector Genes Influence Cnidocyte Differentiation

Cnidocytes which are unique to and characteristic of all cnidarians (David and Gierer 1974; Holstein 1981; Tardent 1995) derive from the same pluripotent interstitial stem cell population as nerve cells (Bosch and David 1987) and in *Hydra* are present as four different types: stenoteles, holotrichous isorhizas, atrichous isorhizas and desmonemes. Although extensive studies at the biochemical (Kurz et al. 1991; Koch et al. 1998; Engel et al. 2001, 2002; Szczepanek et al. 2002) and ultrastructural (Mariscal 1974; Holstein 1981; Holstein et al. 1994) level have revealed the morphogenesis of cnidocyte capsules, little is known about the factors controlling cnidocyte differentiation.

Lindgens et al. (2004) reported the identification of a gene, *Hyzic*, which is expressed in the early cnidocyte differentiation pathway, starting at the level of interstitial stem cells. *Hyzic* is a homolog of the Zn-finger transcription factor gene *zic/odd-paired*, which acts as an early neural effector gene in vertebrates. *Hyzic* expression is restricted to the proliferative stages of cnidoblasts and, up to now, the earliest marker of cnidocyte differentiation. The above mentioned CnASH-positive cells (Grens et al. 1995) are preceded by a differentiation stage expressing *Hyzic* (Lindgens et al. 2004). Since Hyzic also acts before Nowa (Engel et al. 2002), another early cnidocyte differentiation marker gene encoding a major wall protein of the cnidocyte capsule (Ozbek et al. 2004), Lindgens et al. (2004) concluded that Hyzic may determine stem cells to differentiate into cnidocytes . The seemingly puzzling fact that in *Hydra* CnASH is expressed in both differentiating cnidocytes (Grens et al. 1995; Lindgens et al. 2004) as well as in neurons (Hayakawa et al. 2004) adds strong support to the view that (i) cnidocytes are mechanosensory and/or chemosensory cells (Hausmann and Holstein 1985; Brinkmann et al. 1996) and (ii) that genetic cascades of neural development are highly conserved during animal evolution (Sasai 2001).

The first identification of a Dickkopf gene outside the vertebrates (Fedders et al. 2004) with a deduced amino acid sequence closely related to that of chicken *Dkk-3*, *HyDkk-3*, in *Hydra* adds further support to the view that cnidocytes represent neuronal sensory cells. In vertebrates, Dkk-3 in contrast to Dkk-1 and Dkk-4 is expressed in brain and some other tissue, indicating that it might be involved in neuron differentiation or function (Krupnik et al. 1999). In *Hydra*, *HyDkk*-3 is expressed in all four types of cnidocytes at a late stage of differentiation (Fedders et al. 2004). This differentiation step is characterized by changes in morphology and cell behavior that allow extended cell migration of cnidocytes from the gastric region towards the tentacles. Thus, due to the neuronal function of Dkk3 in vertebrates, not yet understood parallels appear to exist between *Dkk*-3 function in *Hydra* and vertebrates.

7
Pathways that may Suppress Activation of the Stem Cell Differentiation Program in *Hydra*

Epigenetic genome modifications are important for specifying pluripotency and lineage commitment (Azuara et al. 2006). In higher organisms, PcG proteins form multiple Polycomb Repressive Complexes (PRCs) which are epigenetic chromatin modifiers involved in maintenance of embryonic and adult stem cells (Ng et al. 2000; Wang et al. 2002; Plath et al. 2003; Valk-Lingbeek et al. 2004). The PRC2 complex, comprising embryonic ectoderm development (EED), enhancer of zest (EZH2), and additional components, initiates gene silencing and catalyzes histone H3 methylation on lysine 27 (H3K27) at target loci (Kirmizis et al. 2004; Kuzmichev et al. 2004). When screening the *Hydra* transcriptome for genes expressed during embryogenesis (Genikhovich et al. 2006), one of the genes found to be expressed strongly during both early embryogenesis and in adult polyps was the embryonic ectoderm development (EED) homolog HyEED. While in early embryos it is ubiquitously expressed, at later stages of embryogenesis HyEED expression becomes restricted to a subset of cells in the endoderm and ectoderm of the embryos, which morphologically resemble interstitial cells (Genikhovich et al. 2006). In adult polyps HyEED is expressed in all interstitial cells, cnidoblasts and in spermatogonia. Terminally differentiated interstitial cell derivatives such as cnidocytes , gland cells or neurons, do not express HyEED (Genikhovich et al. 2006). Semi-thin sections of male polyps hybridized in situ with the *HyEED* probe revealed that *HyEED* transcripts are localized in the proximal zone of the testis, which is known (Tardent 1974; Kuznetsov et al. 2001) to contain spermatogonia and spermatocytes. No *HyEED* transcripts were found in the distal part of testis containing spermatids and mature sperm. Since chromatin remodeling in male germ cells is required for completion of spermatogenesis (Grimes 2004), HyEED appears to play a role in this process. Interestingly, in male polyps *HyEED* is co-expressed with the *Hydra* homologue of *EZH2* suggesting that, similar to mammals and *Drosophila*, the PRC2 complex exists in *Hydra*. Since sperm precursors in the testis expressing *HyEED* show high levels of histone methylation (Genikhovich et al. 2006) and co-express putative *HyEZH2*, *HyEED* – similar to EED proteins in *Drosophila*, *C. elegans* and mouse (Rideout et al. 2001; Leatherman and Jongens 2003; Cao et al. 2002; Plath et al. 2003; Okamoto et al. 2004; Kirmizis et al. 2004) – may be involved in remodeling and silencing sperm chromatin and thereby play an important role in spermatogenesis. In support of the view that HyEED is actively suppressing final differentiation steps in *Hydra* interstitial stem cells, Konstantin Khalturin in my lab recently has produced transgenic *Hydra* which constitutively express HyEED under the control of the

Hydra actin promoter in their interstitial cells (Khalturin and Bosch, in preparation). Preliminary data indicate that interstitial cells expressing a HyEED/GFP fusion protein – in contrast to control cells expressing the GFP protein alone – do not differentiate into cnidocytes or nerve cells but remain located exclusively in the gastric region. HyEED/GFP fusion protein expressing interstitial cells can never be found in the head or foot region where in control polyps most of the terminally differentiated interstitial cells end up as neurons or cnidocytes. Thus, HyEED is not only the earliest embryonic marker of the interstitial cell precursors known to-date, but appears to be causally involved in suppressing terminal differentiation of interstitial cells.

8 Conclusions and Perspectives

Are studies in *Hydra* telling us anything relevant with respect to stem cells in man? Control of asymmetry and cell fate is as critical to human stem cells as it is to *Hydra* stem cells. Recent studies suggest that Cnidaria – in contrast to the well known model organisms *Drosophila* and *C. elegans* – have retained all key regulatory genes required for cell decision making and share most of their genes with human (Kortschak et al. 2003; Technau et al. 2005). I have outlined above that certain key elements of the mechanisms that specify self-renewal and prevent differentiation of stem cells may be conserved through evolution from *Hydra* to man. These elements include paracrine signalling pathways, negative feed back loops that limit the response to mitogenic signals, and pathways that suppress activation of the differentiation program in stem cells. In *Hydra* both the "pan-metazoan" features such as a tissue-level organization, stem cells with an enormous developmental potential, and a net of nerve cells as well as the unexpected molecular equivalence to human cells are complemented by unique biological and experimental opportunities. The model system *Hydra* offers fully worked out cell lineages and a nearly unlimited potential for tissue manipulation combined with a completely transparent tissue consisting mostly of stem cells which continuously undergo self-renewing mitotic divisions. Furthermore, transgenic *Hydra* are paving the way for many applications including in vivo imaging to analyze stem cell behaviour and niche function in an animal that diverged from the main line of metazoan evolution about 560 million years. Thus, since fundamental processes that are relevant for understanding asymmetric division and self-renewal are expected to be conserved in the animal kingdom, the basal metazoan *Hydra* is showing its worth when it comes to unlocking the mystery of "stemness" and deciphering the components controlling pluripotency and lineage commitment.

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References

Akashi K, He X, Chen J, Iwasaki H, Niu C, Steenhard B, Zhang J, Haug J, Li L (2003) Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. Blood 101(2):383–389

Augustin R, Franke A, Khalturin K, Kiko R, Siebert S, Hemmrich G, Bosch TCG (2006) Dickkopf related genes are components of the positional value gradient in Hydra. Dev Biol (in press)

Azuara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merkenschlager M, Fisher AG (2006) Chromatin signatures of pluripotent cell lines. Nature Cell Biol 8(5):532–538

Bashirullah A, Cooperstock RL, Lipshitz HD (1998) RNA localization in development. Ann Rev Biochem 67:335–394

Bode HR, Heimfeld S, Chow M, Huang LW (1987) Gland cells arise by differentiation from interstitial cells in *Hydra attenuata*. Dev Biol 122:577–585

Bosch TCG (2003) Ancient signals: peptides and the interpretation of positional information in ancestral metazoans. Comp Biochem Physiol B Biochem Mol Biol 136(2):185–196

Bosch TCG (2006) Why polyps regenerate and we don't: towards a cellular and molecular framework for Hydra regeneration. Dev Biol (in press)

Bosch TCG, David CN (1986). Male and female stem cells and sex reversal in Hydra polyps. Proc Natl Acad Sci USA 83:9478–9482

Bosch TCG, David CN (1987) Stem cells of *Hydra magnipapillata* can differentiate into somatic cells and germ line cells. Dev Biol 121:182–191

Bosch TCG, David CN (1990) Cloned interstitial stem cells grow as contiguous patches in hydra. Dev Biol 138:513–515

Bosch TCG, Fujisawa T (2001) Polyps, peptides and patterning. BioEssays 23(5):420–427

Bosch TCG, Rollbühler R, Scheider B, David CN (1991) Role of the cellular environment in interstitial stem cell proliferation in hydra. Roux's Arch Dev Biol 200:269–276

Brinkmann M, Oliver D, Thurm U (1996) Mechanoelectric transduction in nematocytes of a hydropolyp (Corynidae). J Comp Phys 178:125–138

Campbell RD (1985) Sex determination in Hydra: roles of germ cells (interstitial cells) and somatic cells. J Exp Zool 234:451–458

Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298:1039–1043

Chen D, McKearin D (2003) Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. Curr Biol 13:1786–1791

Collins AG (1998) Evaluating multiple alternative hypotheses for the origin of Bilateria: an analysis of 18 S rRNA molecular evidence. Proc Natl Acad Sci USA 95:15458–15463

Darmer D, Hauser F, Nothacker HP, Bosch TC, Williamson M, Grimmelikhuijzen CJ (1998) Three different prohormones yield a variety of Hydra-RFamide (Arg-Phe-NH2) neuropeptides in *Hydra magnipapillata* Biochem J 332(2):403–412
David CN, Challoner D (1974) Distribution of interstitial cells and differentiating nematocytes in *Hydra attenuata*. Am Zool 14:537–542
David CN, Gierer A (1974) Cell cycle kinetics and development of *Hydra attenuata*. III. Nerve and nematocyte differentiation. J Cell Sci 16:359–375
David CN, MacWilliams H (1978) Regulation of the self-renewal probability in Hydra stem cell clones. Proc Natl Acad Sci USA 75(2):886–890
David CN, Murphy S (1977) Characterization of interstitial stem cells in hydra by cloning. Dev Biol 58:372–383
David CN, Plotnick I (1980) Distribution of interstitial stem cells in Hydra. Dev Biol 76(1):175–184
David CN, Fujisawa T, Bosch TCG (1991) Interstitial stem cell proliferation in hydra: evidence for strain specific regulatory signals. Dev Biol 148:501–507
Dellaporta SL, Xu A, Sagasser S, Jakob W, Moreno MA, Buss LW, Schierwater B (2006) Mitochondrial genome of *Trichoplax adhaerens* supports Placozoa as the basal lower metazoan phylum. Proc Natl Acad Sci USA 103(23):8751–8756
Deng W, Lin H (1997) Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in Drosophila. Dev Biol 189(1):79–94
Dübel S, Hoffmeister SA, Schaller HC (1987) Differentiation pathways of ectodermal epithelial cells in hydra. Differentiation 35(3):181–189
Dunne J, Javois LC, Huang LW, Bode HR (1985) A subset of cells in the nerve net of Hydra oligactis defined by a monoclonal antibody: its arrangement and development. Dev Biol 109:41–53
Engel U, Pertz O, Fauser C, Engel J, David CN, Holstein TW (2001) A switch in disulfide linkage during minicollagen assembly in Hydra nematocysts. EMBO J 20(12):3063–3073
Engel U, Oezbek S, Engel R, Petri B, Lottspeich F, Holstein TW (2002) Nowa, a novel protein with minicollagen Cys-rich domains is involved in nematocyst formation in Hydra. J Cell Sci 115:3923–3934
Fedders H, Augustin R, Bosch TCG (2004) A Dickkopf-3 related gene is expressed in differentiating nematocytes in the basal metazoan Hydra. Dev Genes Evol 214:72–80
Fujisawa T (1989) Role of interstitial cell migration in generating position-dependent patterns of nerve cell differentiation in Hydra. Dev Biol 133:77–82
Genikhovich G, Kürn U, Hemmrich G, Bosch TCG (2006) Discovery of genes expressed in Hydra embryogenesis. Dev Biol 289(2):466–481
Gierer A, Berking S, Bode H, David CN, Flick K, Hansmann G, Schaller H, Trenkner E (1972) Regeneration of Hydra from reaggregated cells. Nature New Biol 239:98–101
Grell KG (1971) Trichoplax adhaerens F.E. Schulze und die Entstehung der Metazoen. Naturwiss Rundsch 24:160–161
Grens A, Mason E, Marsh JL, Bode HR (1995). Evolutionary conservation of a cell fate specification gene: the Hydra achaete scute homolog has proneural activity in Drosophila. Development 121:4027–4035
Grimes SR (2004) Testis-specific transcriptional control. Gene 343:11–22
Grimmelikhuijzen CJP, Dockray GJ, Schot LPC (1982a) FMRFamide-like immunoreactivity in the nervous system of hydra. Histochemistry 73:499–508

Grimmelikhuijzen CJP, Dierickx K, Boer GJ (1982b) Oxytocin/vasopression-like immunoreactivity in the nervous system of Hydra. Neuroscience 7:3191–3199
Guder C, Pinho S, Nacak T, Hobmayer B, Niehrs C, Holstein TW (2006) An ancient Wnt-Dickkopf antagonism in Hydra. Development 133(5):901–911
Hager G, David CN (1997) Pattern of differentiated nerve cells in hydra is determined by precursor migration. Development 124:569–576
Hansen GN, Williamson M, Grimmelikhuijzen CJP (2000) Two-color double-labeling in situ hybridization of whole-mount Hydra using RNA probes for five different Hydra neuropeptide preprohormones: evidence for colocalization. Cell Tissue Res 301:245–253
Hausmann K, Holstein TW (1985) Sensory receptor with bilateral symmetrical polarity. Naturwissenschaften 72:145–146
Hayakawa E, Fujisawa C, Fujisawa T (2004) Involvement of Hydra achaete-scute gene CnASH in the differentiation pathway of sensory neurons in the tentacles. Dev Genes Evol 214(10):486–492
Heimfeld S, Bode HR (1984) Interstitial cell migration in Hydra attenuata: I. Quantitative description of cell movements. Dev Biol 105:1–9
Hobmayer E, Holstein TW, David CN (1990a) Tentacle morphogenesis in hydra I: the role of head activator. Development 109:887–895
Hobmayer E, Holstein TW, David CN (1990b) Tentacle morphogenesis in hydra II: formation of a complex between a sensory nerve cell and a battery cell. Development 109:897–901
Holstein T (1981) The morphogenesis of nematocytes in *Hydra* and *Forskália*: an ultrastructural study. J Ultrastruct Res 75:276–290
Holstein TW, David CN (1990) Cell cycle length, cell size, and proliferation rate in hydra stem cells. Dev Biol 142:392–400
Holstein TW, Benoit M, von Herder G, Wanner G, David CN, Gaub EH (1994) Fibrous mini-collagens in *Hydra* nematocytes. Science 223:402–404
Kirmizis A, Bartley SM, Kuzmichev A, Margueron R, Reinberg D, Green R, Farnham PJ (2004) Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 18:1592–1605
Koch AW, Holstein TW, Mala C, Kurz E, Engel J, David CN (1998) Spinalin, a new glycine- and histidine-rich protein in spines of Hydra nematocysts. J Cell Sci 111(11):1545–1554
Koizumi O, Heimfeld S, Bode HR (1988) Plasticity in the nervous system of adult hydra. II. Conversion of ganglion cells of the body column into epidermal sensory cells of the hypostome. Dev Biol 129:358–371
Kortschak RD, Samuel G, Saint R, Miller DJ (2003) EST analysis of the cnidarian Acropora millepora reveals extensive gene loss and rapid sequence divergence in the model invertebrates. Curr Biol 13:2190–2195
Krupnik VE, Sharp JD, Jiang C, Robison K, Chickering TW, Amaravadi L, Brown DE, Guyot D, Mays G, Leiby K, Chang B, Duong T, Goodearl AD, Gearing DP, Sokol SY, McCarthy SA (1999) Functional and structural diversity of the human Dickkopf gene family Gene 238(2):301–313
Kurz E, Holstein TW, Petri BM, Engel J, David CN (1991) Mini-collagens in Hydra nematocytes. J Cell Biol 115:1159–1169
Kuzmichev A, Junewein T, Tempst P, Reinberg D (2004) Different Ezh2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol Cell 14:183–193

Kuznetsov S, Lyanguzowa M, Bosch TCG (2001) Role of epithelial cells and programmed cell death in Hydra spermatogenesis. Zoology 104(1):25–31
Leatherman JL, Jongens TA (2003) Transcriptional silencing and translational control: key features of early germline development. BioEssays 25(4):326–335
Leys SP, Ereskovsky AV (2006) Embryogenesis and larval differentiation in sponges. Can J Zool 84:262–287
Lin H, Schagat T (1997) Neuroblasts: a model for the asymmetric division of stem cells. Trends Genet 13:33–39
Lindgens D, Holstein TW, Technau U (2004) Hyzic, the Hydra homolog of the zic/odd-paired gene, is involved in the early specification of the sensory nemtaocytes. Development 131(1):191–201
Littlefield CL (1984) The interstitial cells control the sexual phenotype of heterosexual chimeras of hydra. Dev Biol 102:426–432
Littlefield CL (1985) Germ cells in *Hydra oligactis* males. I. Isolation of a subpopulation of interstitial cells that is developmentally restricted to sperm production. Dev Biol 112(1):185–193
Littlefield CL (1991) Cell lineages in Hydra: isolation and characterization of an interstitial stem cell restricted to egg production in *Hydra oligactis*. Dev Biol 143:378–388
Littlefield CL (1994) Cell-cell interactions and the control of sex determination in hydra. Semin Dev Biol 5:13–20
Mariscal RN (1974) Nematocysts. In: Muscatine L, Lenhoff HM (eds) Coelenterate biology: reviews and new perspectives. Academic Press, New York, pp 129–178
Metschnikoff E (1883) Untersuchungen über die intracelluläre Verdauung bei Wirbellosen Tieren. Arb Zool Inst Wien 5:141–168
Moore KA, Lemischka IR (2006) Stem cells and their niches. Science 311:1880–1885
Morrison SJ, Shah NM, Anderson DJ (1997) Regulatory mechanisms in stem cell biology. Cell 88:287–298
Müller WEG (2001) How was metazoan threshold crossed? The hypothetical Urmetazoa. Comp Biochem Physiol (A) 129:433–460
Müller WA, Teo R, Frank U (2004) Totipotent migratory stem cells in a hydroid. Dev Biol 275:215–224
Ng J, Hart CM, Morgan K, Simon JA (2000) A Drosophila ESC-E(Z) protein complex is distinct from other Polycomb group complexes and contains covalently modified ESC. Mol Cell Biol 20:3069–3078
Nishimiya-Fujisawa C, Sugiyama T (1993) Genetic analysis of developmental mechanisms in Hydra. XX. Cloning of interstitial stem cells restricted to the sperm differentiation pathway in *Hydra magnipapillata*. Dev Biol 157:1–9
Nishimiya-Fujisawa C, Sugiyama T (1995) Genetic analysis of developmental mechanisms in hydra. XXII. Two types of female germ stem cells are present in a male strain of *Hydra magnipapillata*. Dev Biol 172:324–336
Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. Science 303: 644–649
Ozbek S, Pokidysheva E, Schwager M, Schulthess T, Tariq N, Barth D, Milbradt AG, Moroder L, Engel J, Holstein TW (2004) The glycoprotein NOWA and minicollagens are part of a disulfide-linked polymer that forms the cnidarian nematocyst wall. J Biol Chem 279(50):52016–52023

Pires-DaSilva A, Sommer RJ (2003) Evolution of signalling pathways. Natl Rev Genetics 4:39–49

Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, dela Cruz CC, Otte AP, Panning B, Zhang Y (2003) Role of histone H3 lysine 27 methylation in X inactivation. Science 300:131–135

Rideout WM, Eggan K, Jaenisch R (2001) Nuclear cloning and epigenetic reprogramming of the genome. Science 293:1093–1098

Ruppert EE, Barnes RD (1994) Invertebrate zoology, 6th edn. Saunders College Publishing, Fort Worth

Sasai Y (2001) Regulation of neural determination by evolutionarily conserved signals: anti-BMP factors and what next? Curr Opin Neurobiol 11(1):22–26

Schierwater B, Kuhn K (1998) Homology of Hox genes and the zootype concept in early metazoan evolution. Mol Phylogenet Evol 9:375–381

Schmidt T, David CN (1986) Gland cells in Hydra: cell cycle kinetics and development. J Cell Sci 85:197–215

Schofield R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4(1/2):7–25

Seydoux G (1996) Mechanisms of translational control in early development. Curr Opin Genet Dev 6(5):555–561

Shimizu H, Bode HR (1995) Nematocyte differentiation in hydra: commitment to nematocyte type occurs at the beginning of the pathway. Dev Biol 169:136–150

Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414:98–104

Sproull F, David CN (1979) Stem cell growth and differentiation in Hydra attenuate. II. Regulation of nerve and nematocyte differentiation in multiclone aggregates. J Cell Sci 38:171–179

Sugiyama T, Sugimoto N (1985) Genetic analysis of developmental mechanisms in hydra. XI. Mechanism of sex reversal by heterosexual parabiosis. Dev Biol 110:413–421

Szczepanek S, Cikala M, David CN (2002) Poly-gamma-glutamate synthesis during formation of nematocyst capsules in Hydra. J Cell Sci 115(4):745–751

Takahashi T, Muneoka Y, Lohmann J, deHaro LM, Solleder G, Bosch TCG, David CN, Bode HR, Koizumi O, Shimizu H, Hatta M, Fujisawa T, Sugiyama T (1997) Systematic isolation of peptide signal molecules regulating development in hydra: Lwamide and PW families. Proc Natl Acad Sci USA 94:1241–1246

Takahashi T, Koizumi O, Ariura Y, Romanovitch A, Bosch TCG, Kobayakawa Y, Mohri S, Bode HR, Yum S, Hatta M, Fujisawa T (2000) A novel neuropeptide, Hym-355, positively regulates neuron differentiation in Hydra. Development 127:997–1005

Tardent P (1974) Gametogenesis in the genus hydra. Am Zool 14:447–456

Tardent P (1995) The cnidarian cnidocyte, a high-tech cellular weaponry. BioEssays 17:351–362

Technau U, Holstein TW (1996) Phenotypic maturation of neurons and continuous precursor migration in the formation of the peduncle nerve net in Hydra. Dev Biol 177:599–615

Technau U, Rudd S, Maxwell P, Gordon P, Saina M, Grasso LC, Hayward DC, Sensen CW, Saint R, Holstein TW, Ball EE, Miller DJ (2005) Maintenance of ancestral complexity and non-metazoan genes in two basal cnidarians Trends Genet 21(12):633–639

Teragawa CK, Bode HR (1990) Special and temporal patterns of interstitial cell migration in *Hydra vulgaris*. Dev Biol 138:63–81

Teragawa CK, Bode HR (1995) Migrating interstitial cells differentiate into neurons in hydra. Dev Biol 171:286–293

Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M (2004) Stem cells and cancer; the polycomb connection. Cell 118(4):409–418

Wang J, Mager J, Scneider E, Magnuson T (2002) The mouse PcG gene eed is required for Hox gene repression and extraembryonic development. Mamm Genome 13:493–503

Wittlieb J, Khalturin K, Lohmann J, Anton-Erxleben F, Bosch TCG (2006) Transgenic Hydra allow in vivo tracking of individual stem cells during morphogenesis. Proc Natl Acad Sci USA 103:6208–6211

Wolpert LJ (1988) Stem cells: a problem in asymmetry. J Cell Sci Suppl 10:1–9

Xie T, Spradling AC (2000) A niche maintaining germ line stem cells in the Drosophila ovary. Science 290:328–330

Yamashita YM, Fuller MT, Jones DL (2005) Signaling in stem cell niches: lessons from the Drosophila germline. J Cell Sci 118:665–672

Yaross MS, HR Bode (1978) Regulation of interstitial cell differentiation in *Hydra attenuata*. III Effects of i-cell and nerve cell densities. J Cell Sci 34:1–25

Yum S, Takahashi T, Koizumi O, Ariura Y, Kobayakawa Y, Mohri S, Fujisawa T (1988) A novel neuropeptide, Hym-176, induces contraction of the ectodermal muscle in Hydra. Biochem Biophys Res Comm 248:584–590

Asymmetric Cell Divisions in the Early Embryo of the Leech *Helobdella robusta*

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Abstract

The small glossiphoniid leech *Helobdella robusta* is among the best-studied representatives of the super-phylum Lophotrochozoa in terms of early development. The *Helobdella* embryo undergoes a modified version of spiral cleavage, characterized by stereotyped cell lineages comprising multiple examples of equal and unequal divisions, many of which are well-conserved with respect to those of other clitellate annelids, such as the oligochaete *Tubifex*. Here, we review the early development of *Helobdella,* focusing on the variety of unequal cell divisions. We then summarize an experimental analysis of the mechanisms underlying the unequal first cleavage in *Helobdella*, concluding that the unequal first cleavages in *Helobdella* and *Tubifex* proceed by different mechanisms. This result demonstrates the evolvability of the basic cell biological mechanisms underlying well-conserved developmental processes. Finally, we propose a model in which the unequal *second* cleavage in *Helobdella* may be regulated by the polarized distribution of PAR protein homologs, convergent with the unequal *first* cleavage of the nematode *Caenorhabditis elegans* (super-phylum Ecdysozoa).

1 Introduction

This chapter summarizes our current understanding of unequal cell divisions in the development of the leech, *Helobdella robusta*, within the larger context of comparative studies of development and evolution. The general rationale for studying *Helobdella* is as follows.

To understand the evolutionary changes in developmental processes that have given rise to the diverse body plans of modern animals, we must compare the development of extant species, interpreting similarities and differences with respect to the phylogenetic tree by which their ancestors diverged. Similarities represent either convergence or the conservation of developmental processes present in the last common ancestor of the species being compared. Differences yield insights into the divergence of

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developmental mechanisms along different branches of the phylogenetic tree.

To avoid a self-defeating circularity in this undertaking, it is imperative not to use developmentally-derived traits to construct the phylogenetic trees, because to do so would automatically build in assumptions about the very evolutionary processes we are trying to unravel. Constructing phylogenies on the basis of molecular sequence comparisons is not without severe problems of its own, but it does offer an escape from the circularity of using phylogenies based on morphological traits.

Molecular phylogenies have converged on grouping bilaterally symmetric animals into three major clades, Deuterostomia, Ecdysozoa and Lophotrochozoa (Aguinaldo et al. 1997; Ruiz-Trillo et al. 1999). Combining this consensus phylogeny with paleontological evidence, one proposition is that the last common ancestor of these three groups was an unsegmented animal that relied on mucociliary locomotion and even lacked a true coelom (Valentine and Collins 2000). If so, many of the features we associate with modern bilaterian animals may have arisen largely independently within these three lines. On the other hand, others have proposed that the urbilaterian was a segmented eucoelomate with well-developed sensory structures and limbs (Holland 2000; Panganiban and Rubenstein 2002). In either case, but especially in the former, we anticipate that studies of taxa phylogenetically distant from the commonly used models may reveal novel combinations and applications of ancestral cellular and molecular processes, associated with the formation of diverse body plans over ~600 MY of separate evolution.

The model organisms on which most modern studies of development are carried out fall into either Deuterostomia (i.e., vertebrates) or Ecdysozoa (i.e., fly and nematode). In contrast, *Helobdella* is among the best studied and experimentally tractable representatives of Lophotrochozoa, home to at least one half of the present day phyla. Thus, developmental studies of *Helobdella* should be informative for deducing the features of the "ur-bilaterian", the "ur-protostome" and especially, when taken together with studies of molluscs, flatworms and other annelids, for understanding the divergence of developmental mechanisms involved in the evolution of the "spiral cleavers", a diverse group of animals that now seem likely to form a monophyletic group within Lophotrochozoa.

The annelids, or segmented worms, make up one of the major spiralian taxa. The annelids were traditionally regarded as being composed of three monophyletic classes, polychaetes, oligochaetes and leeches. More recent molecular analyses indicate that the leeches are in fact a monophyletic group arising *within* the oligochaetes (Erseus and Kallersjo 2004). Collectively, leeches and oligochaetes are designated as the class Clitellata, arising within the polychaetes. Moreover, the polychaetes themselves may be polyphyletic with respect to other spiralian groups that were traditionally accorded phylum status, such as echiurans, pogonophorans and sipunculans.

The defining feature of spiralian development is the obliquely oriented and unequal cell divisions (spiral cleavage) by which quartets of smaller cells (micromeres) arise near the animal pole by successive rounds of divisions from larger vegetal cells (macromeres) beginning at third cleavage. The oblique divisions mean that each quartet of micromeres is displaced from the animal-vegetal (A-V) axis with respect to the macromeres, usually first in the clockwise direction and then in the counterclockwise direction with respect to the macromeres (Collier 1997).

The stereotypic cleavage patterns seen in spiralian development are often accompanied by highly determinate cell fates (Wilson 1892; Zackson 1984; Weisblat and Shankland 1985; Huang et al. 2002). However, among species known as "equal cleavers", the specific fates of the blastomeres in each quartet are interchangeable until the embryos reach roughly the 32-cell stage, depending on the species, at which point inductive interactions break the initial 4-fold symmetry to establish the second embryonic axis (Collier 1997). This symmetry breaking process is known as "D quadrant specification"; in standard spiralian nomenclature, the four quadrants of the embryo are designated A–D, with D being defined as the quadrant that produces the bilaterally symmetric mesendoderm and post-trochal ectoderm. In "unequal cleavers", determinate cell fates are evident from the start, because the second embryonic axis is established by unequal cleavages that segregate cell fate determinants present in the zygote, first to blastomere CD at the two-cell stage, and thence to the cell defined as macromere D at the four-cell stage.

Presently it is accepted that equal cleavage is ancestral for spiralians and that unequal cleavage has arisen multiple times independently, at least among molluscs (Freeman and Lundelius 1992). The situation is less clear for annelids in this regard, however. So far, no embryological experiments have been published that demonstrate the developmental equipotency of the early quadrants of any of the putative equal cleavers. Evidence in favor of equal cleavage in annelids comes from a recent study of the polychaete *Hydroides* (Arenas-Mena, in press). In this putative equal cleaver, the early expression of a *forkhead*-related gene (for which the non-uniform expression around the blastopore is believed to be important in gastrulation) is expressed uniformly in all four quadrants of the early embryo. On the other hand, it has been suggested on the basis of lineage studies that unequal cleavage arose very early within the annelids and may even be ancestral to the polychaetes; in any case, unequal cleavage is clearly ancestral to the clitellate annelids such as *Helobdella* (Dohle 1999).

2 Summary of *H. robusta* Development

Hermaphroditic like all clitellate annelids, *H. robusta* and related species are capable of both cross- and self-fertilization, and breed year round in laboratory culture, feeding on small freshwater snails. Fertilization is

internal but development arrests in metaphase I of meiosis, and resumes upon zygote deposition. The zygotes are ~400 microns in diameter and are deposited in clutches of 10–100 in transparent cocoons, from which they can easily be removed and cultured to maturity in a simple salt solution. Development to the juvenile has been divided into 11 stages extending over approximately 10 days, but for more precise analyses, embryos may be timed relative to their passage through any easily observed transition (e.g. the initiation of first cleavage) and this is then translated into the time (at 23 °C) after zygote deposition (AZD) (Fernandez et al. 1987; Weisblat and Huang 2001) (Fig. 1).

In brief, the first and second polar bodies form at 50 and 105 min AZD, respectively, in *H. robusta,* after which the male and female pronuclei migrate to the center of the zygote and fuse (karyogamy). During this period (105–180 min AZD), cytoplasmic rearrangements generate animal and vegetal domains of yolk-deficient cytoplasm (teloplasm), enriched for mitochondria and maternal mRNAs (Astrow et al. 1989; Fernandez et al. 1990; Holton et al. 1994). The first cleavage is unequal. The cleavage plane runs parallel to the animal-vegetal axis, thereby yielding a smaller blastomere AB and a larger blastomere CD, which inherits both pools of teloplasm.

Subsequent cleavages are asynchronous and mostly unequal. CD enters cytokinesis at ~375 min AZD, signaling the transition from the two-cell to the three-cell stage. This cleavage segregates teloplasm to cell D at the four-cell stage, then vegetal teloplasm migrates to the animal pole and mixes with that teloplasm, as the third, unequal division forms quartets of vegetal macromeres (A′–D′) and animal micromeres (a′–d′) (Holton et al. 1989). At fourth cleavage (stage 4b) macromere D′ divides along an obliquely equatorial plane. Both daughter cells inherit some of the teloplasm: the animal daughter cell, DNOPQ (2d in classical spiralian terminology), is the precursor of 8 ectodermal stem cells (bilateral pairs of N, O/P, O/P and Q teloblasts) plus 13 additional micromeres; the vegetal daughter, DM (2D in classical spiralian terminology), is the precursor of 2 mesodermal stem cells (M teloblasts) plus 2 micromeres (Bissen and Weisblat 1989; Sandig and Dohle 1988). Macromeres A′–C′ undergo two more rounds of unequal divisions, yielding two sets of micromere trios (a″–c″ and a‴–c‴). The residual macromeres A‴–C‴ are classically regarded as the endodermal precursors, but the gut actually has a more complicated origin (see below).

The teloblasts are segmentation stem cells. Each teloblast undergoes repeated divisions to generate a column (bandlet) of segmental founder cells (m, n, o/p, o/p and q blast cells; 18–122 h AZD). On each side, the five bandlets come together in a parallel array (germinal band). The left and right germinal bands and the space between them are covered by an epithelium derived from the micromeres that arise during cleavage. The germinal bands move over the surface of the embryo, eventually coalescing along the midline (79–135 h AZD) to form the germinal plate, from which segmental tissues arise.

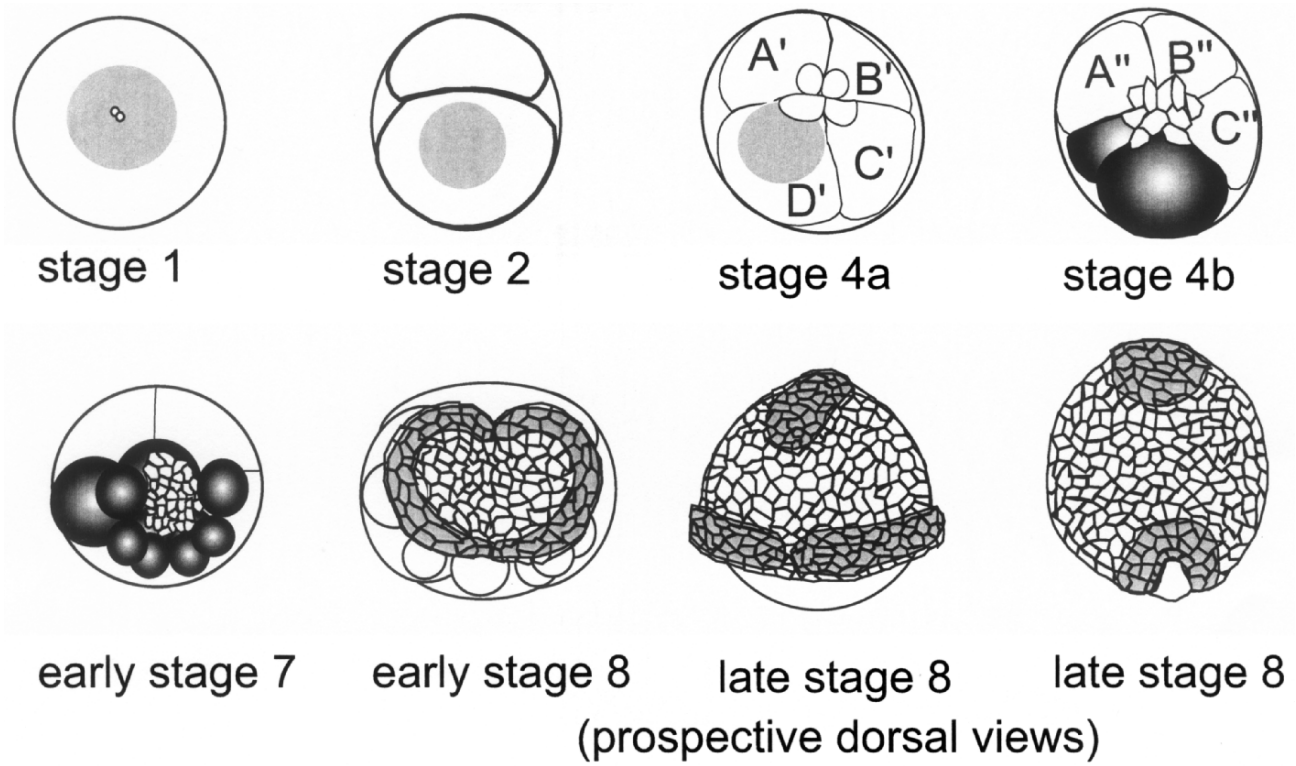

Fig. 1. Overview of *Helobdella* development. *Circles* at stage 1 denote polar bodies at animal pole; *shading* in stages 1–4a denotes teloplasm; *shading* in stages 4b and 7 highlights the origins of teloblasts from cells DM and DNOPQ; *shading* in stage 8 denotes the germinal bands and germinal plate; *expanding meshwork* in stages 4b–8 denotes micromeres and the epibolizing epithelium to which they give rise

Coincident with the movements of the germinal bands, the epithelium spreads over the surface of the embryo. Within the germinal bands and germinal plate, blast cells undergo lineage-specific patterns of cell proliferation, migration and differentiation. The germinal plate expands dorsolaterally around the yolk and eventually coalesces along the dorsal midline, forming the body tube.

The gut forms by cellularization of a syncytial yolk cell (Nardelli-Haefliger and Shankland 1993), which forms by stepwise fusion, first among the macromeres and later still with the teloblast remnants and supernumerary blast cells (~120–160 h AZD) (Desjeux and Price 1999; Liu et al. 1998).The foregut (proboscis, proboscis sheath and esophagus) arises from specific micromere lineages (Huang et al. 2002).

Unequal cell divisions in the embryo of *Helobdella* fall into two categories (Scott Settle, unpublished observations) (Fig. 2). *Slightly unequal divisions*, defined as those that are clearly unequal but in which the ratio of sister cell diameters is less than 3, are seen among the large yolk-rich blastomeres beginning with first cleavage, and also in the stereotyped cell lineages leading from micromeres and blast cells to their definitive prostomial and segmental progeny, respectively. *Highly unequal divisions*, defined as those in which the ratio of sister cell volumes is greater than 3, consist of the micromere-forming divisions scattered throughout cleavage, and the production of blast cells by the repeated stem-cell divisions of the teloblasts. As will be illustrated below, the categorization of two different cell division as slightly unequal for example does not mean that they employ the same mechanism for regulating the position of the spindle apparatus, but nonetheless it's a starting point for addressing the problem. This chapter focuses on the mechanisms at work in slightly unequal cell divisions of the first two rounds of cell division.

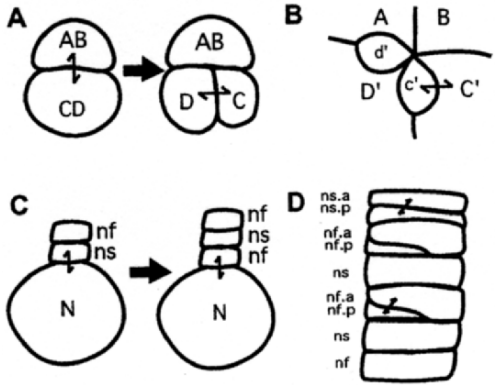

Fig. 2. A–D Unequal cell division in *Helobdella* development. In each panel, recent divisions are indicated by *double-headed arrows*: **A** animal view of the intact embryo at stages 2 and 3 depicts the first two, slightly unequal divisions leading

3
Unequal Cell Division at First Cleavage

In *Helobdella* as in other unequally cleaving spiralians, the chain of events initiated by the unequal first cleavage is critical to the normal development of the body plan. Centrifugation experiments showed that factors permitting the expression of the D quadrant fates are associated with the teloplasm. For instance, when zygotes are compressed to re-orient the mitotic apparatus and both daughters inherit teloplasm at first cleavage, they both make teloblasts (Nelson and Weisblat 1992). And when mild centrifugation is used to distribute teloplasm uniformly to the nominal C and D blastomeres at second cleavage, both these cells may form a full complement of teloblasts (Astrow et al. 1987). Thus, the segregation of teloplasm to cell CD by the unequal first cleavage, and thence to cell D by the unequal second cleavage, is critical for normal development. How are these unequal cell divisions achieved?

The nematode *Caenorhabditis elegans* also undergoes an unequal first cleavage such that, apart from the size difference, the two-cell stage appears very similar to that of *Helobdella*. An elegant body of work is emerging to provide a detailed mechanism for this unequal cleavage . Reviewing that work is beyond the scope of this chapter (Pellettieri and Seydoux 2002) but, in brief, the polarity of the zygote is set by the point of sperm entry, which defines the posterior end, and this initial cue is interpreted to establish posterior and anterior cortical domains in the zygote, marked by PAR1 and a widely conserved complex of proteins including PAR3, PAR6, atypical protein kinase C (aPKC) and one of the Rho family GTPases (CDC42),

Fig. 2. (*Cont'd*) from the zygote to cells AB and CD, and from cell CD to macromeres C and D; AB divides after CD; **B** depiction of the animal pole region, showing the production of micromeres by highly unequal cell divisions at third cleavage (stage 4a). The D quadrant divides first (yielding macromere D′ and micromere d′), then the C quadrant and then A and B divide synchronously; **C** teloblasts are bilaterally paired segmentation stem cells, which produce columns of segmental founder cells (blast cells) by iterated, highly unequal divisions at the rate of about one per hour (stages 6–8). Here, one of the primary neurogenic (N) teloblasts is depicted, which gives rise to two distinct classes of blast cells (nf and ns) in exact alternation; **D** an isolated column of blast cells derived from an N teloblast, showing the first mitoses of the nf and ns blast cells (stage 7–8). The nf and ns blast cells give rise to distinct, segmentally iterated sets of about 70 identifiable neurons, by lineages characterized by unequal cell divisions that are stereotyped according to the timing, orientation and degree of asymmetry. For example, each nf cell divides about 24 h after its birth from the N teloblast, and the anterior daughter (nf.a) is markedly larger than the posterior daughter (nf.p). In contrast, each ns cell divides only about 28 h after its birth, and the anterior daughter (ns.a) is only slightly larger than the posterior daughter (ns.p)

respectively (Nance 2005). Thus, astral microtubules emanating from the anterior spindle pole experience a different biochemical environment at the cell cortex than do those emanating from the posterior spindle pole (Labbe et al. 2003). The associated difference in astral microtubule dynamics results in displacement of the mitotic apparatus toward the posterior of the embryo and leads to the unequal first cleavage (Fig. 3). The posterior localization of a PAR1 homolog is also important in establishing the anterior-posterior polarity in *Drosophila* (Doerflinger et al. 2006), despite the vast differences in the early development of these two ecdysozoan models. Therefore, to ask if this mechanism for establishing zygotic polarity is also used in *Helobdella*, homologs of *par-1* and *par-6* (*Hro-par1* and *Hro-par6*) were cloned and antibodies were raised against them (Ren 2005).

No asymmetric immunostaining was detected in the zygote, suggesting that the mechanisms by which unequal first cleavage is achieved in *Helobdella* differs from that used in *Caenorhabditis elegans*. But by the two-cell stage and beyond, HRO-PAR1 and HRO-PAR6 showed complementary localization patterns, suggesting that the antibodies were recognizing their intended targets and that the proteins in leech are behaving in a biochemically similar manner to their homologs in other organisms. HRO-PAR1 is seen primarily at basolateral membranes, especially in the macromere-macromere junctions. In contrast, HRO-PAR6 is seen at the membrane abutting an intercellular space designated as the blastocoel at the two-cell stage and on both apical and basolateral junctions between micromeres in later stages (Fig. 4).

The failure to detect a pre-established polarity in the *Helobdella* zygote was consistent with the results of previous embryological studies (Nelson and Weisblat 1992). Compressing the zygote so as to re-orient the mitotic apparatus at first cleavage does not disrupt development as long as both pools of teloplasm end up in the same blastomere at first cleavage, suggesting that there was no inherent polarization of the embryo along a prospective second axis prior to first cleavage.

In another approach to the question of the unequal first cleavage, carefully staged embryos were fixed at different time points during mitosis and immunostained for alpha-tubulin (to assess the morphology of the spindle) and gamma-tubulin (as a marker for the centrosomes) (Ren and Weisblat 2006). We found that the paternal centrosome duplicates prior to centration of the pronuclei and gives rise to a symmetric, diastral spindle in prophase and early metaphase (220–245 min AZD). Surprisingly, one centrosome then loses its gamma-tubulin immunoreactivity. Shortly after that, the associated aster becomes greatly reduced in size and the spindle shifts in the direction of the down-regulated aster, setting up the unequal cleavage. Gamma-tubulin immunoreactivity returns to the down-regulated centrosome during telophase, but the spindle remains asymmetric, setting up the unequal cleavage, with the larger aster corresponding to the future CD cell (Fig. 3).

These observations stand in contrast to those obtained previously for a different clitellate annelid, the oligochaete *Tubifex*. Ishii and Shimizu (Ishii and Shimizu 1997), using the same experimental approach, found that the centrosome of the mitotic spindle is maternal in origin and does not duplicate during the first cell cycle. The spindle pole associated with the (gamma-tubulin-positive) centrosome forms a large aster, while the spindle pole that lacks a centrosome (as judged by the absence of gamma-tubulin immunoreactivity) fails to generate an appreciable aster. As a result the first mitotic spindle is essentially monastral and strikingly asymmetric from prophase onwards. The spindle is displaced toward the anastral side of the zygote, resulting in an unequal first cleavage, with the astral half-spindle corresponding to blastomere CD (Fig. 3).

As described in the introduction, the clitellate annelids form a robust clade. The patterns of cell division during cleavage are highly conserved in this clade (Dohle 1999) and no equal cleaving clitellates have been described. So it seems beyond doubt that teloplasm formation and its segregation to the prospective D quadrant by unequal cleavage are unquestionably ancestral traits among clitellates. Thus, the strikingly different mechanisms operating during the unequal first cleavage in *Helobdella* and *Tubifex* must represent changes in the mechanism regulating the unequal first cleavage along one or both branches leading to these species from the ancestral clitellate, despite the fact that the inequality of that cleavage was conserved all along the way. Intriguingly, previous studies have also revealed differences in the cytoskeletal mechanisms underlying teloplasm formation, which is microfilament-dependent in *Tubifex* and microtubule-dependent in *Helobdella* (Astrow et al. 1989; Fernandez et al. 1998; Shimizu 1982).

A priori, it might be postulated that the more derived condition of leeches relative to oligochaetes in terms of adult morphology (e.g. loss of regenerative capabilities and formation of a posterior sucker) predicts that the mechanism governing the unequal first cleavage in *Helobdella* would also be derived with respect to that in *Tubifex*, i.e., that the monastral spindle mechanism is more likely to represent the mechanism of unequal cleavage in the ancestral clitellate. In fact, this conclusion is far from certain, and the relationship between morphological evolution and changes in developmental mechanisms is one of the key issues to be addressed by such comparative studies. Remember that *Tubifex* and *Helobdella* are both "modern" animals, equally far removed from the ancestral clitellate (for a further discussion of this critical issue, see Crisp and Cook 2005). And since we have just seen evidence that a macroscopic process (e.g., unequal first cleavage) can be conserved while the underlying mechanisms evolve, it is impossible to conclude anything about the ancestral process by noting the differences between just two species. Fortunately, *Helobdella robusta* and *Tubifex tubifex* are but two among thousands of clitellate species. Examining additional judiciously chosen representatives should allow us to determine the variety of mechanisms regulating the unequal first cleavage and their phylogenetic origin(s) within this clade.

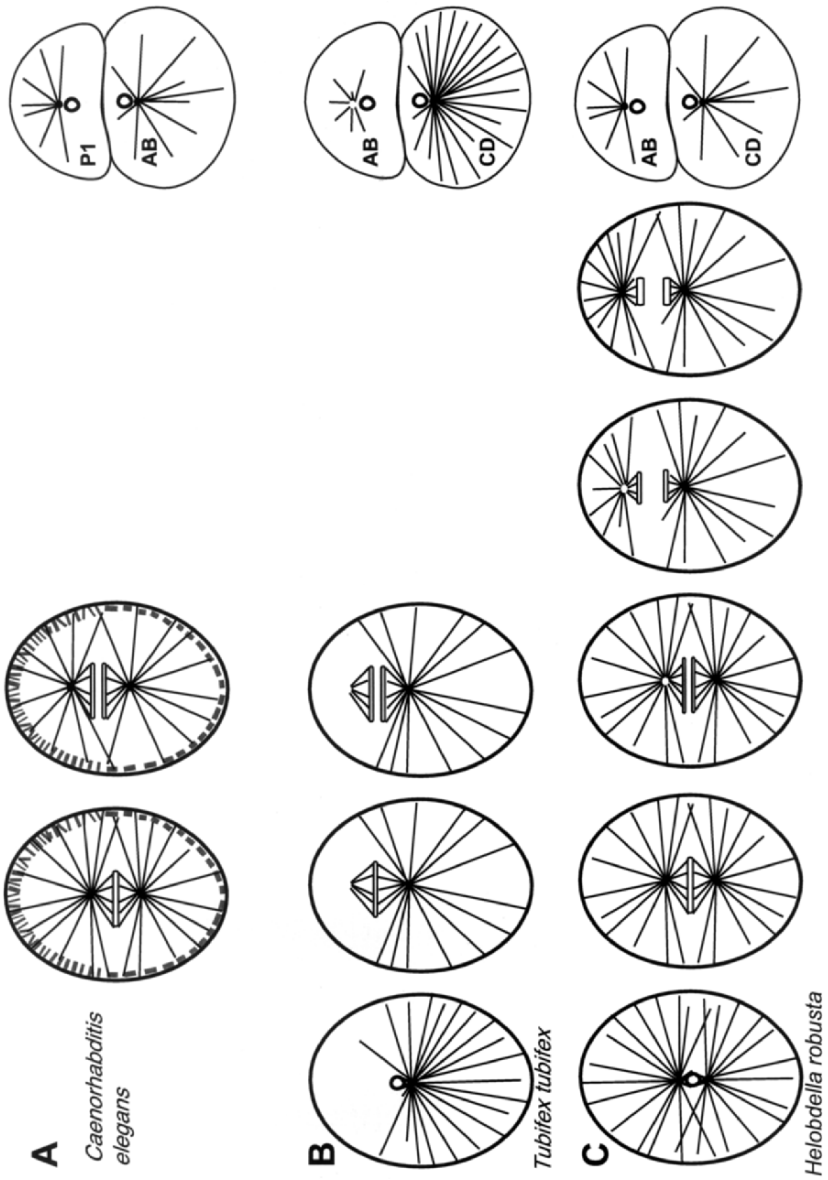
A
Caenorhabditis
elegans
P1
AB
B
Tubifex tubifex
AB
CD
C
Helobdella robusta
AB
CD

Fig. 3. A–C Comparison of the unequal first cleavage in nematode (*C. elegans*), earthworm (*T. tubifex*) and leech (*H. robusta*). Key steps in mitosis are indicated *from left to right* for each species: **A** in *C. elegans* (superphylum Ecdysozoa), events initiated at the point of sperm entry lead to the formation of distinct anterior and posterior domains at the cell cortex, marked by mutually exclusive localization of proteins such as PAR-3 and PAR-6 (*dashes*), and PAR-1 and PAR-2 (*bristles*), respectively. The diastral mitotic apparatus has two gamma-tubulin-positive centrosomes and is positioned symmetrically at the beginning of metaphase. Subsequently it shifts toward the PAR-1/PAR-2 domain, leading to an unequal cell division that yields a larger, anterior AB cell and a smaller, posterior P1 cell; **B** in *T. tubifex* (a clitellate annelid; superphylum Lophotrochozoa) the (maternally derived) centrosome fails to duplicate, so the mitotic apparatus is highly asymmetric from the start of mitosis; **C** *H. robusta* exhibits yet a third mechanism for this unequal first cleavage, despite the fact that it is clearly homologous to that in *Tubifex*. The (paternally derived) centrosome duplicates and the mitotic apparatus is symmetric through early metaphase. Then one centrosome is transiently down-regulated (indicated by the white center in the upper aster) and this is followed by a decrease in the size of the associated aster and the shift of the mitotic apparatus toward that side. Note that there is no biological significance to the fact that the AB cell designation is used in both nematode and annelid

4
Unequal Cell Divisions at Second Cleavage

At second cleavage, blastomere CD also divides in a slightly unequal manner, so that teloplasm is further segregated to cell D at the four-cell stage. There has been no analysis of centrosome dynamics during this division for *Helobdella*, but double staining for tubulin and DNA revealed that the spindle was already positioned eccentrically by metaphase and that the asters associated with the prospective C and D blastomeres remained large, in contrast to the situation during first cleavage.

Other results, from blastomere isolation experiments, provide further evidence that the mechanism regulating the unequal second cleavage are different from those operating at first cleavage (Symes and Weisblat 1992). Specifically, when the fertilization envelope is removed and the AB and CD blastomeres are separated at first cleavage and cultured on an agarose bed, the cells assume more rounded shapes and cell CD tends to divide more equally than in the intact embryo; teloplasm is often inherited by both daughter cells and both may form teloblasts. In other experiments, the isolated CD cells were cultured in agarose wells in the presence of small sephadex beads, which deformed the CD blastomeres in a manner similar to that achieved by cell AB in the normal embryo. The mechanical deformation induced by the bead was sufficient to substantially restore the normal inequality of the CD division and teloplasm segregation. These results suggest that the unequal second cleavage is regulated in part by mechanical cues present in the two-cell stage that are not available to the more symmetrical zygote.

These observations on *Helobdella* complement previous work on *Tubifex*, suggesting that these species may be more similar during second cleavage than during first cleavage. Shimizu (Shimizu 1996) showed that the mitotic apparatus in blastomere CD has two asters, each associated with a gamma-tubulin positive centrosome. Asymmetry becomes evident just after metaphase, when the aster associated with the prospective C blastomere moves toward the membrane adjacent to the AB blastomere. Granted that appearances can be deceiving, but it appears as if the astral microtubules on that side of the prospective C aster have become attached to the cortex on that side of the cell and are undergoing a depolymerization-coupled traction toward the zone of AB/CD apposition, similar to those thought to be operating on kinetochore microtubules during anaphase (Westermann et al. 2006).

Separating the AB and CD blastomeres in *Tubifex* also causes the CD cell to undergo an equal division, as does moving the CD nucleus away from the membrane adjacent to blastomere AB by centrifugation (Takahashi and Shimizu 1997). From these results, it appears that cortical factors induced locally by contact with blastomere AB are required to asymmetrize what is otherwise a symmetric mitotic apparatus in cell CD.

At first, this seems to contradict the conclusion that the deformation of CD is sufficient to asymmetrize the cleavage in *Helobdella*. However, we suggest that these apparently disparate results may in fact just be two different aspects of the same process. On the one hand, the mild mechanical deformation induced by culturing CD blastomeres in the presence of a bead must somehow have biochemical consequences on the mitotic apparatus in order to affect the placement of the cleavage furrow. And, conversely, it may be assumed that co-culturing isolated CD blastomeres in the presence of other "inducing" blastomeres may lead to mechanical deformation of the CD cell as it adheres to the inducing cell. Thus biochemical and mechanical effects may reinforce one another in establishing cortical factors that asymmetrize the mitotic apparatus of cell CD in late metaphase/anaphase.

Why does CD cleave asymmetrically and not AB? A combination of seemingly disparate observations made in *Tubifex* and *Helobdella* suggest the outline of a possible answer.

The first observation is that during the two-cell stage in these embryos an extracellular cavity forms between the separating the apposing faces of the AB and CD blastomeres. This cavity is called the blastocoel, although it should not be concluded from this that it is necessarily homologous to the space of the same name in vertebrate embryos. This blastocoel develops midway through the two-cell stage and is initially surrounded entirely by the apposed AB and CD membranes. Later the blastocoel appears as an extracellular space between the micromeres and macromeres. The presence of the blastocoel at the two-cell stage means that we can distinguish three distinct spatial domains of cell membranes and cortical cytoplasm in the two-cell embryo: domain 1 consists of those membranes making up the outer surface of the embryo; domain 2 consists of the membranes in the region where the AB and CD cells are closely apposed; and domain 3 consists of those membranes making up the walls of the blastocoel itself (Fig. 4).

The second observation, made by Shimizu et al. (Shimizu et al. 1998) is that, during second cleavage, the microtubules of the smaller, essentially anastral mitotic apparatus present in the AB cell seem to have formed extensive contacts with the third domain, i.e. the blastocoel wall. In the larger mitotic apparatus of cell CD, by contrast, the astral microtubules of the aster associated with the prospective C macromere come into close apposition with domain 2 membranes in the region of cell apposition, and avoid the blastocoel wall (Fig. 4).

Finally, the third relevant observation is that, as alluded to in Sect. 2, the complementary localization of homologs of PAR1 (to the region of cell apposition) and PAR6 (to the blastocoel wall) (Ren 2005) suggests a biochemical mechanism by which the astral microtubules can distinguish between these different domains at least in *Helobdella*. Note that if the prospective C aster is attracted preferentially toward the PAR1-positive membrane domain, the presence of the PAR1-deficient blastocoel wall in

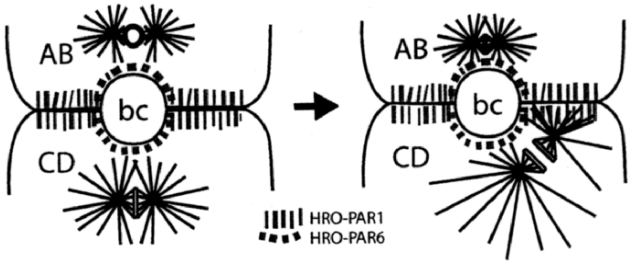

Fig. 4. Proposed mechanism for the unequal second cleavage, of blastomere CD, in *Helobdella*, based on a synthesis of observations made in *Helobdella* and *Tubifex*. By the onset of mitosis in cell CD, a blastocoel (bc) has arisen at the interface of cells AB and CD. Cell membranes surrounding the blastocoel are enriched for HRO-PAR6, while those in direct apposition are enriched for HRO-PAR1. Greater affinity between one aster and the adjacent HRO-PAR1 would result in the displacement of the mitotic apparatus to that side. Why this displacement is invariably toward the right (as viewed from the animal pole) remains to be determined

the center of the embryo ensures that the mitotic apparatus shifts to one side or the other relative to the centrally located blastocoel, thereby setting up the unequal cleavage. Despite the differences in geometry and the fact that this is occurring at second cleavage rather than first, this situation is strikingly similar to that in the *C. elegans* zygote in terms of the movement of the mitotic apparatus relative to the polarized distributions of PAR1 and PAR6 and is presumably similarly governed by the differential effects of the PAR domains on microtubule dynamics (Labbe et al. 2003). We speculate that this represents an independent recruitment of the PAR-mediated cell polarity machinery to regulate an unequal cell division in the leech.

Whether or not this proposed mechanism for the unequal second cleavage will prove true remains to be determined of course. In any event, several questions related to these observations remain such as: how does the clitellate blastocoel form and how are the PAR domains established? What accounts for the different cortical domain preferences of the mitotic apparatus in cell AB vs cell CD? And how is the chirality of the spirally cleaving embryo established?

In considering these questions, the speculations are almost entirely unbounded by any relevant factual observations. Blastocoel formation must involve differential localization of adhesion molecules including tight junctions, and also secretory apparatus and/or ion pumps, assuming that the fluid-filled cavity is inflated by an osmotic imbalance. However the blastocoel is formed, it is interesting to note that it provides an additional mechanical deformation of the membrane. Harking back to the bead experiments, might this be a factor in initiating or maintaining the discrete domains of PAR1 and PAR6 localization?

For *Tubifex*, an obvious difference between the mitotic apparatus in AB and CD cells is that the latter has gamma-tubulin reactive centrosomes and normal asters, while the former has neither of these (Shimizu, 1996). It is tempting to think that this may be important in determining the properties of the microtubules, but from the work in *Helobdella*, it seems that the AB mitotic apparatus has nice asters (Scott Settle, unpublished observations) and presumably centrosomes as well, since it inherits one from the first mitosis (Ren 2005). Other possibilities would be that the PAR1 immunoreactivity we see in the domain of cell-cell apposition is actually confined to cell CD, something that cannot be distinguished by current immunofluorescence observations, or that some other factor, possibly related to the teloplasm and thus present only in cell CD, is required for the mitotic apparatus to respond to the PAR1 and PAR6 domains. A related possibility is that the delay in mitosis of cell AB relative to CD is somehow responsible for the differential response of their mitotic apparatuses to the cortical factors.

Regarding the chirality of second cleavage, this handedness is manifested by the fact that cell CD cleaves so that cell C invariably lies at the counterclockwise side of cell D, when the embryo is viewed from the animal pole. In *Helobdella*, this corresponds to the prospective C aster being the one that shifts toward the PAR1 domain of cell-cell apposition. In principle of course, the elaboration of distinct animal-vegetal and AB-CD axes by the end of first cleavage provides sufficient information to reliably cue the orientation of the handedness of the following cleavages. However, like so many of the other questions in spiralian development, the molecular underpinnings of this process remain to be determined.

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References

Aguinaldo AM, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, Lake JA (1997) Evidence for a clade of nematodes, arthropods and other moulting animals. Nature 387:489–493

Arenas-Mena C (in press) Dev Genes Evol

Astrow S, Holton B, Weisblat D (1987) Centrifugation redistributes factors determining cleavage patterns in leech embryos. Dev Biol 120:270–283

Astrow SH, Holton B, Weisblat DA (1989) Teloplasm formation in a leech, Helobdella triserialis, is a microtubule-dependent process. Dev Biol 135:306–319

Bissen ST, Weisblat DA (1989) The durations and compositions of cell cycles in embryos of the leech, Helobdella triserialis. Development (Cambridge) 106:105–118

Collier JR (1997) Gastropods, the snails. In: Gilbert SF, Raunio AM (eds) Embryology, constructing the organism. Sinauer, Sunderland, pp 189–217
Crisp MD, Cook LG (2005) Do early branching lineages signify ancestral traits? Trends Ecol Evol 20:122–128
Desjeux I, Price DJ (1999) The production and elimination of supernumerary blast cells in the leech embryo. Dev Genes Evol 209:284–293
Doerflinger H, Benton R, Torres IL, Zwart MF, St Johnston D (2006) Drosophila anterior-posterior polarity requires actin-dependent PAR-1 recruitment to the oocyte posterior. Curr Biol 16:1090–1095
Dohle W (1999) The ancestral cleavage pattern of the clitellates and its phylogenetic deviations. Hydrobiologia 402:267–283
Erseus C, Kallersjo M (2004) 18S rDNA phylogeny of Clitellata (Annelida). Zool Scr 33:187–196
Fernandez J, Olea N, Matte C (1987) Structure and development of the egg of the glossiphoniid leech Theromyzon rude: characterization of developmental stages and structure of the early uncleaved egg. Development (Cambridge) 100:211–226
Fernandez J, Olea N, Tellez V, Matte C (1990) Structure and development of the egg of the glossiphoniid leech Theromyzon rude: reorganization of the fertilized egg during completion of the first meiotic division. Dev Biol 137:142–154
Fernandez J, Roegiers F, Cantillana V, Sardet C (1998) Formation and localization of cytoplasmic domains in leech and ascidian zygotes. Int J Dev Biol 42:1075–1084
Freeman G, Lundelius JW (1992) Evolutionary implications of the mode of D quadrant specification in coelomates with spiral cleavage. J Evol Biol 5:205–247
Holland LZ (2000) Body-plan evolution in the Bilateria: early antero-posterior patterning and the deuterostome-protostome dichotomy. Curr Opin Genet Dev 10:434–442
Holton B, Astrow SH, Weisblat DA (1989) Animal and vegetal teloplasms mix in the early embryo of the leech, Helobdella triserialis. Dev Biol 131:182–188
Holton B, Wedeen CJ, Astrow SH, Weisblat DA (1994) Localization of polyadenylated RNAs during teloplasm formation and cleavage in leech embryos. Roux's Arch Dev Biol 204:46–53
Huang FZ, Kang D, Ramirez-Weber FA, Bissen ST, Weisblat DA (2002) Micromere lineages in the glossiphoniid leech Helobdella. Development 129:719–732
Ishii R, Shimizu T (1997) Equalization of unequal first cleavage in the Tubifex egg by introduction of an additional centrosome: implications for the absence of cortical mechanisms for mitotic spindle asymmetry. Dev Biol 189:49–56
Labbe JC, Maddox PS, Salmon ED, Goldstein B (2003) PAR proteins regulate microtubule dynamics at the cell cortex in C. elegans. Curr Biol 13:707–714
Liu NL, Isaksen DE, Smith CM, Weisblat DA (1998) Movements and stepwise fusion of endodermal precursor cells in leech. Dev Genes Evol 208:117–127
Nance J (2005) PAR proteins and the establishment of cell polarity during C. elegans development. Bioessays 27:126–135
Nardelli-Haefliger D, Shankland M (1993) *Lox10*, a member of the *NK-2* homebox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech *Helobdella*. Development 118:877–892
Nelson BH, Weisblat DA (1992) Cytoplasmic and cortical determinants interact to specify ectoderm and mesoderm in the leech embryo. Development 115:103–115

Panganiban G, Rubenstein JL (2002) Developmental functions of the Distal-less/Dlx homeobox genes. Development 129:4371–4386

Pellettieri J, Seydoux G (2002) Anterior-posterior polarity in C. elegans and Drosophila–PARallels and differences. Science 298:1946–1950

Ren X (2005) Isolation and characterization of PAR-1 and PAR-6 homologs in Helobdella robusta. PhD thesis, Dept. of Molecular and Cell Biology, Univ. of California, Berkeley, California

Ren X, Weisblat DA (2006) Asymmetrization of first cleavage by transient disassembly of one spindle pole aster in the leech Helobdella robusta. Dev Biol 292:103–115

Ruiz-Trillo I, Riutort M, Littlewood DT, Herniou EA, Baguna J (1999) Acoel flatworms: earliest extant bilaterian Metazoans, not members of Platyhelminthes. Science 283:1919–1923

Sandig M, Dohle W (1988) The cleavage pattern in the leech Theromyzon tessulatum (Hirudinea, Glossiphoniidae). J Morphol 196:217–252

Shimizu T (1982) Ooplasmic segregation in the Tubifex egg: mode of pole plasm accumulation and possible involvement of microfilaments. Wilhelm Roux's Arch 191:246–256

Shimizu T (1996) Behavior of centrosomes in early Tubifex embryos: asymmetic segregation and mitotic cycle-dependent duplication. Roux's Arch Dev Biol 205:290–299

Shimizu T, Ishii R, Takahashi H (1998) Unequal cleavage in the early Tubifex embryo. Dev Growth Differ 40:257–266

Symes K, Weisblat DA (1992) An investigation of the specification of unequal cleavages in leech embryos. Dev Biol 150:203–218

Takahashi H, Shimizu T (1997) Role of intercellular contacts in generating an asymmetric mitotic apparatus in the *Tubifex* embryo. Dev Growth Differ 39:351–362

Valentine JW, Collins AG (2000) The significance of moulting in Ecdysozoan evolution. Evol Dev 2:152–156

Weisblat DA, Huang FZ (2001) An overview of glossiphoniid leech development. Can J Zool Rev Can Zool 79:218–232

Weisblat DA, Shankland S (1985) Cell lineage and segmentation in the leech. Philos Trans R Soc Lond B Biol Sci 312:39–56

Westermann S, Wang HW, Avila-Sakar A, Drubin DG, Nogales E, Barnes G (2006) The Dam1 kinetochore ring complex moves processively on depolymerizing microtubule ends. Nature 440:565–569

Wilson E (1892) The cell lineage of Nereis. J Morphol 6:361–480

Zackson S (1984) Cell lineage, cel-cell interaction, and egment formation in the ectoderm of a glossiphoniid leech embryo. Dev Biol 104:143–160

Asymmetric Divisions of Germline Cells

Pierre Fichelson and Jean-René Huynh

Abstract

In most vertebrates and invertebrates, germ cells produce female and male gametes after one or several rounds of asymmetric cell division. Germline-specific features are used for the asymmetric segregation of fates, chromosomes and size during gametogenesis. In *Drosophila* females, for example, a germline-specific organelle called the fusome is used repeatedly to polarize the divisions of germline stem cells for their self-renewal, and during the divisions of cyst cells for the specification of the oocyte among a group of sister cells sharing a common cytoplasm. Later during oogenesis of most species, meiotic divisions produce a striking size asymmetry between a large oocyte and small polar bodies. The strategy used to create this asymmetry may involve the microtubules or the actin microfilaments or both, depending on the considered species. Despite this diversity and species-particularities, recent molecular data suggest that the PAR proteins, which control asymmetric cell division in a wide range of organisms and somatic cell types, could also play an important role at different steps of gametogenesis in many species. Here, we review the asymmetric features of germline cell division, from mitosis of germline stem cells to the extrusion of polar bodies after meiotic divisions.

1 Introduction

In several species, the formation of reproductive cells, called gametes, rely on one or several rounds of asymmetric cell division (Deng and Lin 2001; Huynh and St Johnston 2004; Wong et al. 2005). Asymmetric cell division is a process in which one cell divides into two cells with different developmental potentials. This is a fundamental way to generate cell diversity. In the germline, asymmetric division is often used to allow the simultaneous production of a differentiating cell and of a self-renewing stem cell (Deng and Lin 2001; Wong et al. 2005). Depending on the considered species and gender, asymmetric germline stem cell division occurs during a defined time window or throughout the entire life of the organism. The differentiating cells produced after stem cell mitosis undergo several rounds of divisions,

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leading to an increase in germline cell number. These divisions are symmetric in males and ultimately lead to the production of spermatozoids. In contrast, in females of some insects, germline cell divisions are asymmetric and give rise to one oocyte together with several non-reproductive cells (de Cuevas et al. 1997; Huynh and St Johnston 2004). Finally, a widespread phenomenon occurring during late oogenesis is the asymmetric meiotic divisions which lead to the formation of small polar bodies and of a large oocyte, maintaining maternal stores required for embryogenesis (Maro and Verlhac 2002).

Asymmetric cell divisions may involve extrinsic and/or intrinsic factors (Jan and Jan 1998; Knoblich 2001). With extrinsic factors, daughter cells are initially equivalent but adopt different fates as the result of the interactions of the daughter cells with each other or with their environment. With intrinsic factors, unequal amounts of cell-fate determinants are segregated into the two daughter cells which therefore adopt distinct identities. Intrinsic mechanisms can also control the formation of daughter cells with different sizes by acting on spindle displacement, positioning and/or asymmetry. The processes underlying asymmetric cell division have been studied in organisms ranging from bacteria, yeast, worms (*Caenorhabditis elegans*) and flies (*Drosophila*) to mammals (Betschinger and Knoblich 2004).

Here we review the asymmetric features of germline cell division, from the mitosis of germline stem cells (GSCs) to the extrusion of polar bodies after the meiotic divisions. As asymmetric divisions of germline cells have been particularly well documented in *Drosophila*, we focus on this model organism and compare it with data gathered in other organisms whenever possible.

2
Asymmetric Germline Stem Cell Division During *Drosophila* Gametogenesis

Stem cells are characterized by both their ability to make more stem cells, a process called self-renewal, and their capacity to generate specialised cells forming organs (Fuchs and Segre 2000). While symmetric division is required for the expansion of a stem cell population (after an injury for example), asymmetric division allows the simultaneous production of two cell types, a new stem cell and a cell fated to differentiate (Morrison and Kimble 2006). Germline stem cells have been described in organisms where gametes are produced throughout the entire life, such as vertebrate males and *Drosophila* males and females (Li and Xie 2005; Zhao and Garbers 2002). Asymmetric germline stem cell (GSC) division has been documented mainly in *Drosophila* so far and has been shown to rely on extrinsic factors.

More precisely, a micro-environment of somatic cells called a niche controls the maintenance of the GSC fate, by providing specific signals, and the orientation of the GSC division (Wong et al. 2005).

In *Drosophila*, GSCs are located at the anterior apex of the ovary and of the testis (Wong et al. 2005). In the ovary, two to three GSCs are closely associated with somatic cells called cap cells (Fig. 1A). Anterior to these cells lie the terminal filament cells and posterior to the cap cells are the inner sheath cells. In the testis, seven to nine GSCs directly contact the hub cells, which are equivalent to the female cap cells (Fig. 1B). GSC divisions are asymmetric since the two daughter cells adopt different fates. The daughter cell which stays in contact with the cap cells in the female or with the hub cells in the male becomes a GSC, which maintains the stem cell pool, while the posterior daughter cell becomes a cystoblast in the female or a gonioblast in the male. After several mitotic and meiotic divisions, cystoblasts and gonioblast differentiate into cysts giving rise to gametes. Asymmetric GSC division thus permits GSC self-renewal to occur simultaneously with the production of a differentiating cell.

2.1 Extrinsic Features of Asymmetric Germline Stem Cell Division in *Drosophila*

Somatic cells play a fundamental role in controlling asymmetric GSC division. Both the orientation of the mitotic spindle and daughter cell fate determination are dependent upon the surrounding somatic cells. Adherens and gap junctions have been shown to be present at the interface between GSCs and cap/hub cells (Fig. 1A,B) (Gilboa et al. 2003; Song et al. 2002). Loss of function of a major component of adherens junctions, E-Cadherin, leads to a loss of GSCs and to sterility, showing the critical role played by somatic cells-GSC contact for the maintenance of the GSCs (Song et al. 2002). In the male, adherens junctions also orient GSC divisions along the anterior-posterior (A-P) axis by anchoring one centrosome to the anterior cortex. Anchorage occurs through the recruitment of the Adenomatous polyposis coli proteins APC1 and APC2 and of Centrosomin to the adherens junction, which aligns the GSC mitotic spindle along the A-P axis (Yamashita et al. 2003). The anterior daughter cell generated after mitosis stays in contact with the cap/hub cells and adopts a GSC fate while its posterior sibling differentiates as it contacts other somatic cells called escort cells in the female and cyst progenitor cells in the male (Decotto and Spradling 2005). Cyst progenitor cells have been shown to repress the GSC fate and to promote the gonioblast fate via the epidermal growth factor (EGF) pathway (Kiger et al. 2000; Tran et al. 2000). Thus, the asymmetry of GSC division is promoted extrinsically as both fates are induced by two types of specialised somatic cells.

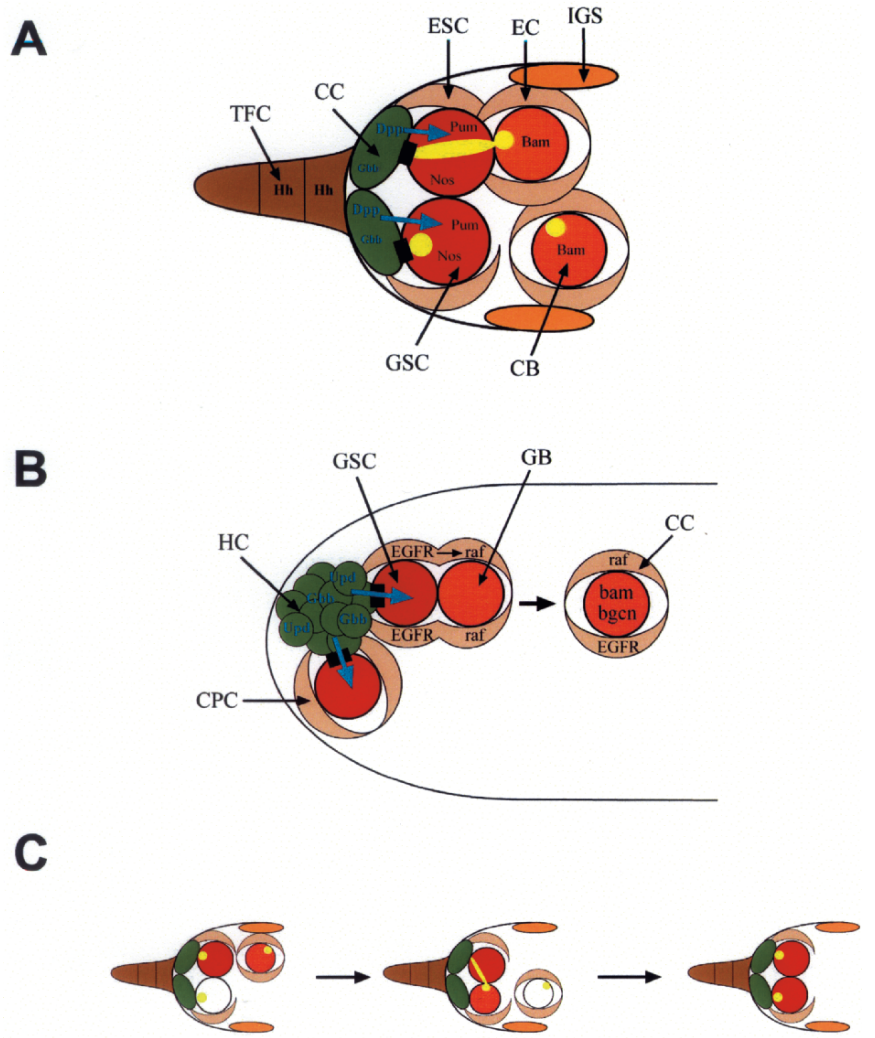

Fig. 1. A–C Asymmetric divisions of germline stem cells: **A** *Drosophila* ovarian niche: germline stem cells (GSC, *in red*) are attached to somatic cap cells (CC, *in green*) by adherens junctions (*black square*). GSCs are surrounded by Escort Stem Cells (ESC). GSCs divide asymmetrically to give rise to another stem cell and to a cystoblast (CB, *in orange*) posteriorly. Cystoblasts are surrounded by somatic Escort Cells (EC). The asymmetry of the division can be visualized by the asymmetric partitioning of the spectrosome (*yellow*). During interphase, the spectrosome is localized close to the adherens junctions in the GSCs. It anchors one pole of the mitotic spindle during mitosis. CCs maintain the GSC fate by secreting Decapentaplegic (Dpp, *in blue*) and Glass Bottom Boat (Gbb, *in blue*). GSCs express nanos (nos) and pumillio (pum), while CBs express bag-of-marbles (bam). Terminal follicle cells (TFC, *brown*) express hedgehog (hh). Inner germarial sheath cells (IGS) are *in*

In contrast to the implication of cyst progenitor cells and possibly escort cells in promoting cyst differentiation, anterior somatic cells (hub and cap cells) play a key role in maintaining the GSC fate by providing a specific microenvironment. This cellular environment, together with the signals emitted by these anterior somatic cells compose what is called a niche that controls the balance between GSC self-renewal and differentiation (Li and Xie 2005; Ohlstein et al. 2004; Spradling et al. 2001). A true niche is able to keep its properties even in the absence of stem cells and must be capable of reprogramming differentiated cells into stem cells. Such has been shown to be the case for the niches found in the *Drosophila* ovary and testis. More precisely, when differentiation of all female GSCs into 4- 8- or 16-cells cysts is triggered by the transient over-expression of the cystoblast-specific gene *bag-of-marbles* (*bam*), the cap cells induce the cyst cells that are in contact to de-differentiate into GSCs (Kai and Spradling 2004). The cells located at the apex of the ovary thus form a true niche which is able to reprogram differentiated cells (cyst cells) into GSCs. In the male, similar experiments have shown that hub cells also form a niche within the testis; not only are hub cells required for GSC maintenance but these cells are also able to reprogram spermatogonies into GSCs (Brawley and Matunis 2004). Asymmetric GSC division thus results from the combination of two categories of extrinsic signals, signals provided by anterior somatic cells, which promote the GSC fate, and signals emitted by posterior somatic cell, which induce differentiation.

What are the signals promoting the GSC vs differentiated cell fate? As mentioned earlier, the EGF pathway promotes gonioblast differentiation in the male germline: loss of function of the EGF receptor or of the downstream effector *raf* leads to an increase in the number of GSCs (Fig. 1B) (Kiger et al. 2000; Tran et al. 2000). Conversely, signals promoting GSC fate have been described in both male and female niches. Niche cells emit several proteins which diffuse within the extracellular space and are received by target cells (GSCs) (Fig. 1A,B). Three signalling pathways have

Fig. 1. (*Cont'd*) *orange*; **B** *Drosophila* testis niche: germline stem cells (GSC, *in red*) are attached to somatic hub cells (HC, *in green*) by adherens junctions (*black square*). GSCs are surrounded by cyst progenitor cells (CPC). GSCs divide asymmetrically to give rise to another stem cell and to a gonioblast (GB, *in orange*) posteriorly. Gonioblasts are surrounded by somatic cyst cells (CC). HCs maintain the GSC fate by secreting Unpaired (Upd, *in blue*) and Glass Bottom Boat (Gbb, *in blue*). Activation of the EGF pathway in CCs represses the GSC fate in favour of the GB fate; **C** Symmetric stem cell division in the ovarian niche: when a GSC (*white*) directly differentiates as a CB, the neighbouring GSC divides orthogonally to the antero-posterior axis, so that both daughter cells remain in the niche. Cells from the niche induce both cells to become GSCs. This division is thus symmetric even though the spectrosome is asymmetrically partitioned (*yellow*)

been shown to be important for GSC maintenance: 1) the Bone morphogenetic protein (BMP) pathway, activated by the ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb), 2) the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway, activated by the Unpaired (Upd) ligand which binds the Domeless receptor and acts upstream of the Hopscotch kinase and the STAT92E transcriptional activator, and 3) the Hedgehog (Hh) pathway (Wong et al. 2005). In female flies, Dpp is expressed by the cap cells and plays an essential role in GSC maintenance (Xie and Spradling 1998). The involvement of the JAK-STAT pathway is more indirect as it acts within the recently identified escort cells which then regulate the progeny of the GSCs (Decotto and Spradling 2005). The situation is different in male flies since it is the Upd and Gbb ligands expressed by the hub cells that both play a key function for GSC maintenance (Kawase et al. 2004; Kiger et al. 2001; Tulina and Matunis 2001).

In the ovary, the BMP pathway has been dissected in a detailed fashion and the main molecular players implicated in signal reception, transduction and target gene regulation have been identified. In female GSCs, Dpp and Gbb bind the Punt and Thick vein receptors, which phosphorylate the DNA-binding protein Mothers against Dpp (Mad). Activation of Mad via its phosphorylation promotes its binding to the Medea protein and induces the Mad-Medea complex to localize to the nucleus. Nuclear Mad-Medea complexes bind a specific regulatory sequence of the cystoblast-specific gene *bam*, which inhibits its transcription and therefore maintains the GSC fate (Chen and McKearin 2005; Song et al. 2004). Conversely in cystoblasts, no BMP signal is received, Mad is not activated, *bam* is transcribed, the GSC-specific RNA-binding proteins Nanos (Nos) and Pumillio (Pum) are inhibited and differentiation is triggered (Szakmary et al. 2005).

In *Drosophila*, GSCs divide asymmetrically due to the major influence of the environment. Upon GSC division, mitotic spindle orientation is controlled by the niche and once division is completed, GSC vs cystoblast (or gonioblast) fate decision depends on the position of the daughter cells relative to the niche. The daughter cell which stays in the niche receives signals promoting the GSC fate while its sibling receives signals inducing differentiation as it is positioned outside the niche.

2.2 Intrinsic Features of Asymmetric Germline Stem Cell Division in *Drosophila*

In addition to the extrinsic factors controlling asymmetric GSC division, one intrinsic asymmetry has also been described upon mitosis, the asymmetric inheritance of a structure present in GSCs called a spectrosome (Huynh 2005; Lin et al. 1994). The spectrosome is made of vesicles attached by cytoskeleton proteins such as α- and β-spectrin, ankyrin and the *Drosophila* homologue of adducin: Hu-li-tai shao (Hts), an actin and spectrin-binding

protein (de Cuevas et al. 1996; Lin et al. 1994; Yue and Spradling 1992). In the adult, the spectrosome is stably anchored at the anterior side of the GSC, in contact with the adherens junctions between the GSC and the overlying cap (hub) cells (Song et al. 2002). During female GSC division, one pole of the mitotic spindle is anchored by the spectrosome thus orientating the division along the A-P axis of the germarium (Deng and Lin 1997). The cystoblast is then produced toward the posterior and the renewed GSC stays at the anterior of the germarium. At the end of telophase, a transient cytoplasmic bridge called ring canal forms between the GSC and the cystoblast. New spectrosome material accumulates in this ring canal and the initial spectrosome elongates from the anterior side of the GSC to fuse with the new spectrosome (de Cuevas and Spradling 1998; Deng and Lin 1997). The cytoplasmic bridge is then severed and one-third of the spectrosome is inherited by the cystoblast where it forms the fusome (see below) while two-thirds remain in the GSC, marking this division as clearly asymmetric (Deng and Lin 1997; Lin and Spradling 1997). The spectrosome relocalizes to the anterior side of the GSC, while the fusome takes a spherical shape at one end of the cystoblast. The function of this asymmetrically distributed organelle regarding GSC vs cystoblast (or gonioblast) cell fate determination remains to be explored. The spectrosome appears important for asymmetric GSC division as it is involved in the anchoring of the mitotic spindle (Deng and Lin 1997). However asymmetric inheritance of the spectrosome upon GSC division does not seem to be sufficient to trigger asymmetric fate decision. When a GSC divides perpendicular to the anterior-posterior axis relative to the niche, both daughter cells become GCSs (Fig. 1C). The division is thus symmetric, despite the asymmetrical behaviour of the fusome (Xie and Spradling 2000). There is so far no evidence of an intrinsic factor which would be sufficient to control the GSC vs differentiated cell fate decision.

Extrinsic factors thus play the major role in establishing asymmetry during GSC division in *Drosophila*. Interestingly, the presence of a niche maintaining GSCs has also been demonstrated in *C. elegans* and in vertebrates (Wong et al. 2005; Zhao and Garbers 2002). As extrinsic signals also regulate GSC fate maintenance in these organisms, the choice between asymmetric and symmetric GSC division depends on the orientation of the mitotic spindle. In *Drosophila*, GSC divisions are generally oriented along the A-P axis. This leads to the formation of daughter cells exposed to different environments, which triggers asymmetric fate decision. Only in cases where a GSC is missing does a neighbour GSC divide symmetrically, perpendicular to the A-P axis, which generates two GSCs. In *C. elegans* and in vertebrates, the orientation of the GSC mitotic spindle appears stochastic. These divisions can be either symmetric, if both daughter cells remain in the niche, or asymmetric in cases where one of the daughter cells exits the niche. Asymmetric vs symmetric fate decision is thus thought to be controlled by proximity to the niche rather than by programmed asymmetric GSC divisions (Lin 1997; Morrison and Kimble 2006).

3
Asymmetric Cell Division During *Drosophila* Oogenesis: Importance of the Fusome for the Specification and Polarisation of the Female Gamete

The differentiating cell generated after GSC division undergoes several rounds of divisions, leading to an amplification of the germline cell population. These divisions are symmetric in males and ultimately generate spermatozoids. In females of some insects, this amplification step takes place but only one gamete is produced per cystoblast. More precisely, in *Drosophila*, the one cell cystoblast goes through four rounds of synchronous mitosis with incomplete cytokinesis to form a cyst of 16 germline cells, which are interconnected by ring canals (Spradling 1993). All the cells within the cyst share the same cystoplasm, but they differ regarding their number of ring canals; for example only two cells, called pro-oocytes, have four ring canals. Once the 16 cell cyst has formed, one of the two pro-oocytes differentiates into the oocyte while the other fifteen cyst cells become nurse cells. An asymmetric fate decision is thus taken within the 16 cell cyst so that only one cell adopts the oocyte fate (Huynh and St Johnston 2004).

Two main models have been proposed to explain how the oocyte is selected. One model is based on the symmetrical behaviour of the two cells with four ring canals (pro-oocytes), and proposes that there is a competition between the two pro-oocytes to become the oocyte (Carpenter 1975, 1994). The "winning" cell would become the oocyte, while the "losing" cell would revert to the nurse cell fate. This process would thus rely on an extrinsic mechanism, such as cell-cell communication within the cyst. However, the factor that could control this fate decision has remained elusive. A second model suggests that the choice of the oocyte is biased by the establishment of some intrinsic asymmetry as early as the first cystoblast division, which is maintained until the overt differentiation of the oocyte (Lin and Spradling 1995; Theurkauf 1994). The formation of a germline-specific organelle called the fusome strongly supports this second model.

The fusome is a large cytoplasmic structure and is an important feature of cyst development both in *Drosophila* and in *Xenopus* (Kloc et al. 2004; Telfer 1975). In *Drosophila* this structure derives from the spectrosome and links all the cells of the cluster through the ring canals. The fusome is made of a continuous network of interconnected ER-derived tubules kept together by components of the sub-membranous cytoskeleton, such as α-spectrin, β-spectrin, and Hts (de Cuevas et al. 1996; Lin et al. 1994; Snapp et al. 2004; Yue and Spradling 1992). The fusome also contains microtubules and microtubule-associated proteins (Grieder et al. 2000; Roper and Brown 2004). It was found in *Drosophila* ovaries that, in the absence of the fusome, cells of the same cluster divide asynchronously and fail to specify an oocyte, despite the presence of ring canals (de Cuevas

et al. 1996; Lin et al. 1994; Yue and Spradling 1992). This demonstrates a key role of the fusome as a channel of communication between the cells for the synchronisation and differentiation of the germline cyst. Interestingly, the fusome is asymmetrically inherited upon the four rounds of cystoblast division. This asymmetric behaviour of the fusome is likely to be essential for oocyte fate determination.

The first mitosis of the cystoblast is very similar to the GSC division (Fig. 2). One pole of the mitotic spindle is anchored by the spherical fusome (de Cuevas and Spradling 1998; Deng and Lin 1997); a new fusome "plug" forms into the arrested furrow at the other end of the cell and comes to fuse with the "original" fusome. However, in contrast to the GSC division, cytokinesis is incomplete and both cells remain linked by a stable ring canal. Furthermore, although it is not known how the plug and the "original" fusome move to fuse together, the "original" fusome does not seem to elongate from one side of the cystoblast, as in the GSC (de Cuevas and Spradling 1998). Cystoblast division is asymmetric as one cell contains the "original" fusome plus half of the plug, whereas the other cell only retains the other half of the fusome plug. At the next division, the two mitotic spindles again orient with one pole close to the fusome and new fusome plugs form in the two ring canals situated at opposite ends (de Cuevas and Spradling 1998; Lin and Spradling 1995; McGrail and Hays 1997; Storto and King 1989). The fusome plugs then move with their ring canal to fuse with the central fusome, which thus remains in the two previous cells. This asymmetric behaviour of the fusome is then repeated in the next two divisions. The oldest cell, therefore, retains the original fusome and accumulates four halves of fusome plugs. Thus, this cell has more fusome than all the other cells and can be identified throughout the divisions. The current model suggests that this cell will become the oocyte (see below). Once the 16 cell-cyst is formed, the fusome starts to break down and disappears. The behaviour of the *Drosophila* fusome during asymmetric cystoblast division has important consequences on the formation and polarisation of the female cyst (de Cuevas et al. 1997; Huynh and St Johnston 2004).

First, upon cystoblast division, the fusome anchors one pole of each mitotic spindle (Deng and Lin 1997; Lin and Spradling 1995). This orientation of the divisions ensures that one cell inherits all the previous ring canals, while a new one is formed at the opposite end of the cell and thus branched off the central fusome. By orienting the mitotic spindle, the fusome therefore directly controls the asymmetry of the division as one cell inherits the old ring canals while the other does not. This orientation also leads to an invariant pattern of interconnections between the cells, with the two central or oldest cells having n ring canals (n being the number of cystoblast divisions), their daughter cells n–1, etc. This pattern is important for the polarisation of the cyst as the oocyte always arises from one of the two cells with the greatest number (n) of ring canals (Buning 1994).

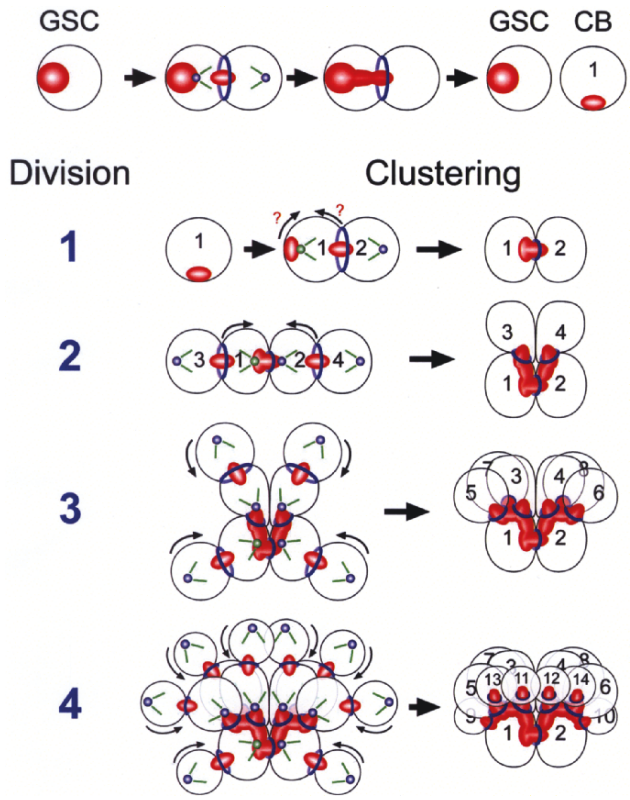

Fig. 2. Asymmetric divisions of female germ cells and formation of the fusome. The spectrosome (*red*) of the GSC anchors one pole of the mitotic spindle and orients the division along the anterior-posterior axis. A new fusome plug forms into the transient ring canal. The spectrosome elongates to fuse with the plug. The cytoplasmic bridge is then severed and one-third of the spectrosome/fusome is inherited by the cystoblast while two-thirds remain in the GSC, marking this division as clearly asymmetric. The spectrosome/fusome (*red*) of the cystoblast (1) interacts with one of the centrosomes (*green and blue spheres*) to anchor one pole of the mitotic spindle (*green lines*), during the first incomplete division. A fusome plug (*red*) forms in the arrested furrow or ring canal (*blue*). The spectrosome (or "original" fusome) and the fusome plug come together to fuse. The direction of these movements is not known (?). The same mechanism is repeated for divisions 2, 3 and 4. 1) One pole of each mitotic spindle is anchored by the fusome. 2) A new fusome plug forms into each ring canal. 3) The ring canals move centripetally for the fusome plugs to fuse with the central fusome (*black arrows*). This behaviour has several crucial consequences: 1) cystocyte (1) has more fusome than the other cystocytes; 2) the same centrosome (*green sphere*) could be inherited by cystocyte (1) from division 1 through division 4; and 3) the fusome always marks the anterior of cystocyte (1), after the clustering of the ring canals

Second, the formation of the fusome provides the strongest evidence in support of an intrinsic mechanism controlling asymmetric fate descision within the cyst (de Cuevas and Spradling 1998). Indeed, the asymmetric inheritance of the "original fusome" during the cyst divisions could play a role in determining which cell will adopt the oocyte fate. This model is supported by analogy with the diving beetle *Dytiscus*, in which oogenesis is very similar to *Drosophila* (Giardina 1901; Telfer 1975). A *Dytiscus* cyst is formed of 15 nurse cells and one oocyte, resulting from four incomplete and synchronous divisions of a cystoblast. However, unlike *Drosophila*, the oocyte can be distinguished as early as the two-cell stage because it contains a large ring of highly amplified rDNA. Moreover, the cell that inherits that ring of rDNA, also inherits the fusome. This also suggests that the early selection of the oocyte could be a general feature among insects (de Cuevas and Spradling 1998; Grieder et al. 2000). Unfortunately, in *Drosophila* most of the fusome has already degenerated by the time oocyte-specific proteins such as BicD or Orb accumulate in a single cell. However, the preferential accumulation of the centrosomes and *oskar*, and *orb* mRNAs in one cell can be detected earlier in cystoblast development, and this is always the cell with the most fusome (Cox and Spradling 2003; Grieder et al. 2000). This is particularly obvious in *egl* and *BicD* mutants, in which the fusome perdures longer, and where the centrosomes clearly accumulate in the cell with the largest piece of fusome remnant (Bolivar et al. 2001). These data strongly suggest that the "original" fusome marks the future oocyte, in support of an intrinsic mechanism underlying oocyte fate determination. It does not rule out the possibility that both pro-oocytes can become the oocyte, but shows that if there is a competition, it is strongly biased by the asymmetric inheritance of the fusome upon division.

What is the link between the asymmetric inheritance of the fusome and the selection of the oocyte? The simplest model is that an oocyte determinant is asymmetrically distributed at each division with the "original" fusome into the future oocyte. It has been proposed that one of the cystoblast centrioles could stay in contact with the fusome during each division, and because of the semi-conservative replication of the centrosome, could be inherited by the oocyte (Theurkauf 1994). Consequently, oocyte determinants could cosegregate with this centriole. Such a mechanism has been shown to mediate the segregation of *dpp* and *eve* mRNAs into specific cells during the asymmetric divisions of the early *Ilyanassa obsoleta* embryo (Lambert and Nagy 2002). Alternatively, the oocyte could inherit more of some protein or activity associated with the fusome, and this early bias could initiate a feedback loop that induces the transport of oocyte determinants towards this cell. Consistent with the second model, it has been suggested that although the fusome starts to degenerate, it acts as a matrix to organise the restriction of oocyte-specific proteins, centrioles and meiosis to a single cell by multiple pathways. However, what molecular mechanisms regulate the different pathways and how these pathways interpret the fusome polarity remains unknown.

4
Asymmetric Meiotic Cell Division Leading to the Formation of Unequal Sized Daughter Cells

Meiosis is a succession of two particular cell divisions without an intervening replication (S) phase, leading to the formation of haploid cells, the gametes in diploid organisms. In the first division, homologous chromosomes become paired and exchange genetic material (via crossing over) before moving away from each other into separate daughter nuclei (reductional division, MI). Sister chromatids separate during the second division (equational division, MII), giving rise to haploid cells. As such, these two divisions are asymmetric since they generate cells with different genetic pools. Here, we focus on female meiotic maturation, which presents an additional asymmetric feature, the formation of unequal sized daughter cells. Before these divisions, the oocyte is arrested in meiotic prophase I in most animal species. A hormonal stimulus usually triggers the reinitiation of meiotic maturation. The timing of fertilization is, however, species-specific (Fig. 3). During female meiotic maturation, both MI and MII produce a small cell called the polar body and a large cell, the oocyte. The polar bodies eventually degenerate while the oocyte conserves the entire maternal stores accumulated during oogenesis.

4.1
Meiotic Spindle Positioning

The size asymmetry observed during MI and MII depends on the localisation and on the orientation of the meiotic spindle. More precisely, size asymmetry results 1) from the migration of the spindle to the periphery of the oocyte and 2) from spindle alignment with an axis perpendicular to the overlying cell cortex. The combination of these two steps minimizes the size of the polar bodies by positioning the plane of division close to the cortex. If either of these two steps is impaired, daughter cell size asymmetry becomes reduced or abolished. Cortical localisation of the spindle is achieved either by the migration of the oocyte nucleus arrested in prophase I (germinal vesicle, GV, stage) or by the migration of the MI spindle, or by both when the breakdown of the GV (GVBD) occurs while it is migrating (Fig. 3). Depending on the species, spindle positioning perpendicular to the overlying cell cortex occurs either directly after migration or requires an extra 90° rotation as described below.

In some species of fishes, amphibian, worms, sea urchin and sea cucumber, the GV is already asymmetrically localized toward the animal pole (Gard 1991; Miyazaki et al. 2005). In *Xenopus*, reinitiation of meiosis triggers the breakdown of the nuclear envelope (GVBD) and the migration of the chromosomes associated with a dense array of microtubules and

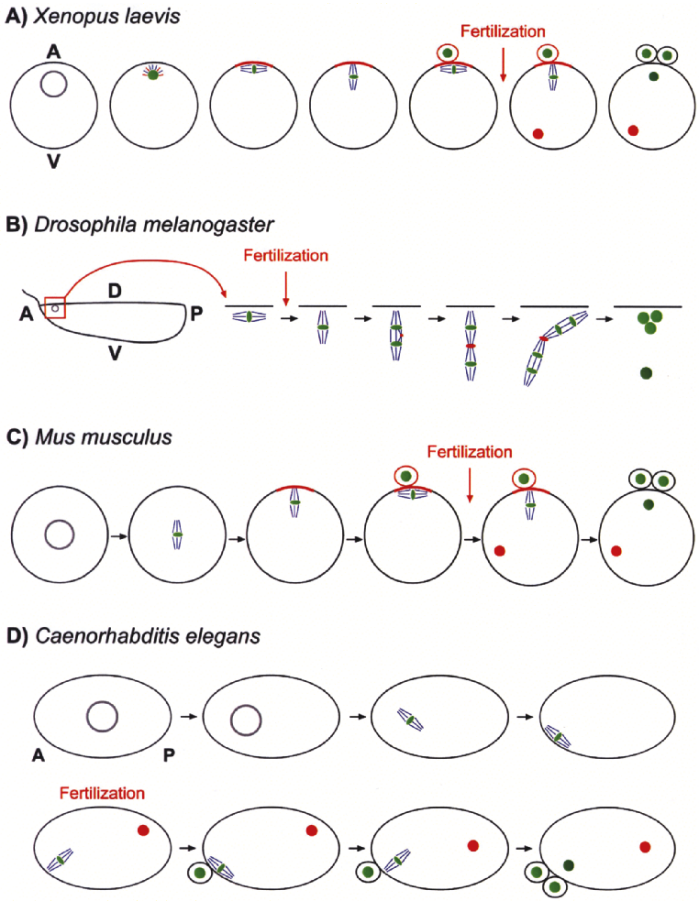

Fig. 3. A–D Asymmetric divisions during meiosis. In most animal species, the oocyte is arrested in meiotic prophase I. A hormonal stimulus usually triggers the reinitiation of meiotic maturation. The timing of fertilization is species-specific. The GV is visualized as a *grey circle*, chromosomes are in *green*, microtubules in *blue*, actin in *red. Red dots* are male pronuclei after fertilization and *dark green dots* are female pronuclei after completion of meiotic maturation: **A** in *Xenopus laevis*: the GV is asymmetrically localized close to the animal pole. Reinitiation of meiosis triggers GVBD and migration of the chromosomes surrounded by the MTOC-TMA. The metaphase I spindle then elongates parallel to the cortex. An actin cap forms above the spindle. The spindle rotates 90°, before expulsion of the first polar body. Fertilization triggers the expulsion of the second polar body and the disappearance of the actin cap; **B** in *Drosophila melanogaster*: the GV is localized asymmetrically at the dorso-anterior corner. After reinitiation of meiosis, the MI spindle lies parallel to the cortex. Fertilization induces a 90° rotation of the MI spindle and two successive mitoses without cytokinesis. The inner most nucleus becomes the female pronucleus (*dark green*); **C** in *Mus musculus*: the GV is localized centrally in the oocyte arrested in prophase I. Reinitiation of meiosis induces

(*Continued*)

actin microfilaments, the MTOC-TMA (Microtubule-Organizing-Center-Transient-Microtubule-Array). Once migration is completed, the MI spindle lies parallel to the cell cortex and rotates 90° to extrude the first polar body; the second polar body extrusion occurs following a similar spindle rotation (Fig. 3A).

In starfish and fly oocytes, the GV is positioned just beneath the cell cortex, which defines the future position of the meiotic spindle (Endow and Komma 1997; Miyazaki et al. 2000). This localisation occurs long before meiosis is reinitiated. After re-entry into meiosis, two polar bodies are sequentially extruded in starfish. In *Drosophila* the situation is atypical (Endow and Komma 1997, 1998; Skold et al. 2005; Tavosanis et al. 1997; Theurkauf and Hawley 1992). The MI spindle first lies parallel to the cell cortex and later rotates 90° (Fig. 3B). Although MI progresses up to anaphase, no polar body is extruded. The MII spindles assemble during MI anaphase and have a shared pole that forms within the MI spindle. Both MII spindles are aligned as a tandem, the inner most nucleus becomes the female pronucleus while the three nuclei next to the cortex assemble into a rosette like structure and degenerate.

In *Ciona*, *C. elegans*, amphibians, some molluscs and mammals, reinitiation of meiosis leads to GVBD and to the formation the MI spindle, which then moves towards the cell cortex (Brunet and Maro 2005; Maro and Verlhac 2002; Pielak et al. 2004; Prodon et al. 2006). In the mouse (Fig. 3C), the MI spindle is assembled around the chromosomes, generally in the center of the oocyte, and migrates towards the cortex (Longo and Chen 1985; Verlhac et al. 2000). Experiments carried in oocytes cultured in vitro show that, once the MI spindle is assembled, its axis defines the path of migration (Verlhac et al. 2000). Migration proceeds towards the nearest part of the cell cortex, following the axis of the spindle, and the first polar body is extruded. After cytokinesis, the MII spindle forms parallel to the overlying cell cortex and the cell cycle arrests in metaphase. MII resumes upon fertilization, the spindle undergoes a 90° rotation and the second polar body is extruded. In *C. elegans* (Fig. 3D), the situation is somewhat different since the MI spindle forms at the periphery of the oocyte and migrates only a short distance following a path perpendicular to the spindle axis (Albertson and Thomson 1993; Yang et al. 2003, 2005). Once the MI spindle has reached the cortex, meiotic maturation

Fig. 3. (*Cont'd*) GVBD and formation of an MI spindle. The spindle migrates in an actin-dependent manner to the closest cortical region. This region then defines the animal pole. An actin cap forms above the "cortical" pole of the MI spindle and the first polar body is extruded. Fertilization causes the MI spindle to rotate 90° and leads to the extrusion of the second polar body; **D** in *C. elegans*: the GV is displaced from the center of the oocyte before GVBD. After GVBD, the MI spindle migrates in a microtubules-dependent manner to the cortex, where it stays parallel to it. Fertilization elicits a 90° rotation of the MI spindle and the subsequent expulsions of two polar bodies

stops until fertilization, which triggers a 90° rotation of the spindle and the sequential extrusion of two polar bodies.

4.1.1 Molecular Mechanisms

During mitosis, movement and orientation of the spindle have been shown to occur through astral microtubules that emanate from centriole-containing centrosomes. However, oocytes rarely have centrioles and in most examined species, female meiotic spindles are devoid of centrosomes and lack astral microtubules. In oocytes, GV or spindle migration and anchoring has been described to rely mainly on microtubules and/or on actin microfilaments, depending on the considered species.

In mice, actin microfilaments are not required for GVBD or MI spindle assembly, but play an essential role regarding spindle migration, as shown by drug treatment (Longo and Chen 1985). In addition, microtubule depolymerization experiments have demonstrated that, in the mouse oocyte, meiotic chromosome migration can occur without microtubules. The lack of microtubules results in the scattering of the chromosomes but migration to the cell cortex is not impaired (Longo and Chen 1985). Therefore microfilaments appear to be the essential factor involved in spindle relocalisation (Sun and Schatten 2006). In agreement with a central role of actin for spindle migration, loss of function of Formin-2, a straight actin filament nucleator, has been shown to lead to defects in MI spindle positioning and to the absence of polar body extrusion (Leader et al. 2002). More precisely, it was recently shown that in Formin-2 mutant oocytes, the MI spindle completely fails to migrate and that the late steps of cytokinesis are impaired (Dumont et al., in press). The *mos*/mitogen activated protein kinase pathway was also shown to be required for MI spindle migration (Verlhac et al. 2000). Interestingly, in *mos*-/- oocyte, polar body extrusion occurs despite the lack of spindle migration. This is due to an 'anaphase rescue' mechanism involving abnormal elongation of the anaphase spindle. Finally, four members of the PAR proteins family have been described to localize on the MI and MII spindles, PAR1, PAR3, PAR4 and PAR6 (Duncan et al. 2005; Moore and Zernicka-Goetz 2005; Szczepanska and Maleszewski 2005; Vinot et al. 2004). The PAR proteins have been shown to control cortical polarity and asymmetric cell division in a wide range of organisms and cell types (Betschinger and Knoblich 2004), suggesting that they could play an important role in the regulation of asymmetric meiotic division. Interestingly, during asymmetric mitotic division, the PAR proteins establish a cortical polarisation that is later interpreted by the spindle, whereas in the mouse oocyte, PAR proteins are first localized on the spindle and only later at the cell cortex (see below), indicating that spindle migration and cortical polarisation could be coupled through a PAR-dependent mechanism. Consistent with this hypothesis, the specific enrichment of PAR6 to the leading pole of the migrating MI

spindle suggests that PAR6 could control spindle migration and interaction with the cell cortex (Vinot et al. 2004). However, in the absence of functional data, one can only speculate about the putative function(s) of the PAR proteins. Interestingly, a recent study has shown that an upstream regulator of the PAR3/PAR6/aPKC complex, the Rho GTPase Cdc42, is required for correct spindle morphology and migration (Na and Zernicka-Goetz 2006). When a dominant negative form of Cdc42 is injected into oocytes, the MI spindle does not migrate, instead it elongates and no polar body is formed. In addition, aPKC, which is normally localized at the spindle poles, becomes located on the whole length of the spindle. The authors indicate that Cdc42 could act via two pathways, controlling respectively spindle morphology and spindle migration.

The lack of microtubule requirement for chromosome migration in the mouse oocyte is in contrast with the situation in *Drosophila*, *Xenopus* and *C. elegans* where microtubules and their associated motors play a key function for GV/spindle migration and anchoring (Gard 1991, 1992; Gard et al. 1995; Januschke et al. 2006; Theurkauf et al. 1992, 1993; Yang et al. 2003, 2005). In *Drosophila*, GV migration depends on a complex network of microtubules as indicated by microtubule depolymerization experiments (Januschke et al. 2006; Theurkauf et al. 1992, 1993). In addition, disruption of molecular motors directed either towards the plus-ends or the minus-ends of the microtubules have been shown to lead to defective GV migration (Januschke et al. 2002). Anchorage of the GV to the oocyte cortex was also demonstrated to require microtubules and the Lis1/dynein complex (Swan et al. 1999). In *Xenopus*, the microtubule, microfilament and intermediary filament networks play an important role controlling asymmetric meiotic division (Gard 1991, 1992; Gard and Klymkowsky 1998). A myosin (Myo10) has been shown to be critical for nuclear anchoring, spindle assembly and anchoring to the cortex by integrating the actin microfilaments and microtubule cytoskeletons (Weber et al. 2004). In *C. elegans*, depletion of microtubules blocks GV and MI spindle migration. In addition, both a microtubule severing enzyme and a microtubule associated kinesin have been involved in the translocation of the MI spindle to the oocyte cortex (Yang et al. 2003, 2005).

In all cases described above, GV or spindle migration creates or reinforces an asymmetry within the oocyte as chromosomes are relocalized to an off-centre position. Coincidently, a second asymmetry is also established, at the cortex of the oocyte.

4.2 Cortical Asymmetry

It is interesting to note that, concomitantly with MI spindle migration, the oocyte cortex becomes asymmetric. In the mouse, before entry into meiosis, at the germinal vesicle (GV) stage, the oocyte cortex presents microvilli

in a uniform fashion. After germinal vesicle breakdown (GVBD), the cortical region above the migrating MI spindle becomes gradually devoid of microvilli and enriched in actin (Brunet and Maro 2005; Longo and Chen 1985; Maro et al. 1984; Sun and Schatten 2006). In addition, cortical granules become excluded from the area surrounding the MII spindle (Deng et al. 2003). The role of the cortical reorganisation observed during mouse meiosis is unclear. The actin rich domain could be involved in anchoring the meiotic spindle and/or chromosomes; this domain has also been suggested to prevent sperm entry next to the female pronucleus. It is noteworthy that actin enrichment above the meiotic spindle is a general feature of meiotic maturation as it has been described in ascidian, *Tubifex*, *Xenopus*, pig, horse, cow and human oocytes, suggesting an essential function (Kim et al. 1997, 1998; Pickering et al. 1988; Sardet et al. 2002; Tremoleda et al. 2001).

Experiments carried in the mouse indicate that chromosomes are involved in remodelling the overlying oocyte cortex by a mechanism acting at a distance that remains to be determined (Maro et al. 1986). Upon meiotic spindle depolymerization, using colcemid, the chromosomes become scattered in subcortical zones. Interestingly, under these experimental conditions, actin microfilaments and myosinIIA accumulate at the cortex overlying or adjacent to each chromosome mass. Thus, cortical remodeling occurs in a chromosome-dependent and microtubule-independent mechanism, possibly involving direct interaction between chromosomes and microfilaments (Longo and Chen 1985). PAR proteins have also been reported to localize asymmetrically during *Xenopus*, mouse and *C. elegans* meiosis (Duncan et al. 2005; Nakaya et al. 2000; Sonneville and Gonczy 2004; Vinot et al. 2004; Wallenfang and Seydoux 2000). In *Xenopus*, it has been shown that 2–3 h after GVBD, aPKC together with PAR3 (ASIP) become specifically localized at the animal cortex of the oocyte, while the vegetal side becomes devoid of these proteins (Nakaya et al. 2000). Possibly acting in concert with PAR proteins, Cdc42 has been involved in controlling asymmetric meiotic cell division in the frog (Ma et al. 2006). Cdc42 is activated at the spindle pole-cortical contact side immediately before polar body formation and inhibition of Cdc42 leads to a failure to extrude a polar body. Interestingly, the Cdc42 cortical activity zone is circumscribed by a cortical RhoA activity zone (Bement et al. 2005). RhoA is also a small GTPase required for both the accumulation of cortical actin during assembly and for the actomyosin contractility of the furrow during cytokinesis (Glotzer 2005). In the *Xenopus* oocyte, concentration of active RhoA depends on microtubules but not on actin and is required for cytokinesis. The authors thus propose that the complementary pattern of Cdc42 and RhoA activities could be an evolutionary conserved process that couples spindle positioning to asymmetric cytokinesis (Ma et al. 2006). In the mouse, both PAR3 and PAR6 have been shown to localize as a crescent overlying the meiotic spindle (Duncan et al. 2005; Vinot et al. 2004). The crescent of PAR3 is present during both MI and MII while the crescent

of PAR6 is detected only during MII. The cortical localization of PAR3 and PAR6 is not dependent on microtubules but requires (at least for PAR3) microfilaments. The function of these two proteins during meiosis is not known, but it is interesting to note that PAR3 is restricted to a subdomain of the actin-rich cortical region which is surrounded by a zone of accumulation of the phosphorylated form of MARCKS (the myristoylated alanine-rich C-kinase substrate protein) (Michaut et al. 2005). Similarly to the situation in *Xenopus*, it has been proposed that the circumferential localization of p-MARCKS could define the position of the contractile ring responsible for polar body abscission, while PAR3 localized centrally and at the ends of the MI and MII spindle could facilitate anchoring the spindle within the forming polar body (Duncan et al. 2005). Finally, in a fashion reminiscent of what was described in mouse oocytes, a crescent of PAR proteins (PAR1 and PAR2) has been shown to be present at the cell cortex overlying the *C. elegans* female pronucleus when progression through meiosis is blocked or delayed (Sonneville and Gonczy 2004; Wallenfang and Seydoux 2000). Altogether, these data indicate that the formation of a cortical crescent of PAR proteins above the meiotic spindle could be a conserved feature involved in the control of polar body emission.

5
Conclusions and Perspectives

Many steps of gametogenesis rely on asymmetric cell division throughout the animal kingdom, but the molecular mechanisms underlying these asymmetric divisions are yet to be further explored. In *Drosophila*, several signalling pathways have been shown to regulate GSC self-renewal vs differentiation in a non-autonomous fashion; however the question whether intrinsic factors could also control asymmetric GSC division remains open. The processes leading to the selection of the oocyte after the mitotic divisions of the insect cystoblast also remain poorly understood. The asymmetric segregation of the fusome upon cystoblast division is likely to play an important function in the selection of the oocyte and future work should shed light on the molecules associated with the fusome that are involved in oocyte fate determination. Finally, the mechanisms leading to the asymmetric meiotic division are still far from clear. The presence of PAR proteins on the meiotic spindle and/or at the cell cortex of the oocytes of many different species raises the exciting possibility that PAR proteins could play a conserved role in the control of polar body extrusion. Considering the essential roles of PAR proteins throughout evolution in cell polarisation, mitotic spindle positioning and anchoring, and in the establishment of mitotic spindle asymmetry (Betschinger and Knoblich 2004), it is tempting to speculate that similar PAR-dependent mechanisms are involved during asymmetric meiotic division.

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References

Albertson DG, Thomson JN (1993) Segregation of holocentric chromosomes at meiosis in the nematode, Caenorhabditis elegans. Chromosome Res 1:15–26

Bement WM, Benink HA, von Dassow G (2005) A microtubule-dependent zone of active RhoA during cleavage plane specification. J Cell Biol 170:91–101

Betschinger J, Knoblich JA (2004) Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol 14:R674–R685 Bolivar J, Huynh JR, Lopez-Schier H, Gonzalez C, St Johnston D, Gonzalez-Reyes A (2001) Centrosome migration into the Drosophila oocyte is independent of BicD and egl, and of the organisation of the microtubule cytoskeleton. Development 128:1889–1897

Brawley C, Matunis E (2004) Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. Science 304:1331–1334

Brunet S, Maro B (2005) Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. Reproduction 130:801–811

Buning J (1994) The insect ovary: ultrastructure, previtellogenic growth and evolution. Chapman and Hall, New York

Carpenter A (1975) Electron microscopy of meiosis in Drosophila melanogaster females. I Structure, arrangement, and temporal change of the synaptonemal complex in wild-type. Chromosoma 51:157–182

Carpenter A (1994) Egalitarian and the choice of cell fates in Drosophila melanogaster oogenesis. In: Marsh J, Goode J (eds) Germline development. Wiley, Chichester, pp 223–246

Chen D, McKearin D (2005) Gene circuitry controlling a stem cell niche. Curr Biol 15:179–184

Cox RT, Spradling AC (2003) A Balbiani body and the fusome mediate mitochondrial inheritance during Drosophila oogenesis. Development 130:1579–1590

de Cuevas M, Lee JL, Spradling AC (1996) α-Spectrin is required for germline cell division and differentiation in the *Drosophila ovary*. Development 124: 3959–3968

de Cuevas M, Spradling AC (1998) Morphogenesis of the Drosophila fusome and its implications for oocyte specification. Development 125:2781–2789

de Cuevas M, Lilly MA, Spradling AC (1997) Germline cyst formation in Drosophila. Ann Rev Genet 31:405–428

Decotto E, Spradling AC (2005) The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. Dev Cell 9:501–510

Deng W, Lin H (1997) Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in Drosophila. Dev Biol 189:79–94

Deng W, Lin H (2001) Asymmetric germ cell division and oocyte determination during Drosophila oogenesis. Int Rev Cytol 203:93–138

Deng M, Kishikawa H, Yanagimachi R, Kopf GS, Schultz RM, Williams CJ (2003) Chromatin-mediated cortical granule redistribution is responsible for the formation of the cortical granule-free domain in mouse eggs. Dev Biol 257: 166–176

Dumont J, Million K, Sunderland K, Rassinier P, Lim H, Leader B, Verlhac M-H (in press) Formin-2 is required for spindle migration and for the late steps of cytokinesis in mouse oocytes. Dev Biol

Duncan FE, Moss SB, Schultz RM, Williams CJ (2005) PAR-3 defines a central subdomain of the cortical actin cap in mouse eggs. Dev Biol 280: 38–47

Endow SA, Komma DJ (1997) Spindle dynamics during meiosis in Drosophila oocytes. J Cell Biol 137:1321–1336

Endow SA, Komma DJ (1998) Assembly and dynamics of an anastral:astral spindle: the meiosis II spindle of Drosophila oocytes. J Cell Sci 111(17): 2487–2495

Fuchs E, Segre JA (2000) Stem cells: a new lease on life. Cell 100:143–155

Gard DL (1991) Organization, nucleation, and acetylation of microtubules in Xenopus laevis oocytes: a study by confocal immunofluorescence microscopy. Dev Biol 143:346–362

Gard DL (1992) Microtubule organization during maturation of Xenopus oocytes: assembly and rotation of the meiotic spindles. Dev Biol 151:516–530

Gard DL, Klymkowsky MW (1998) Intermediate filament organization during oogenesis and early development in the clawed frog, Xenopus laevis. Subcell Biochem 31:35–70

Gard DL, Cha BJ, Schroeder MM (1995) Confocal immunofluorescence microscopy of microtubules, microtubule-associated proteins, and microtubule-organizing centers during amphibian oogenesis and early development. Curr Top Dev Biol 31:383–431

Giardina A (1901) Origine dell'oocite e delle cellule nutrici nel Dytiscus. Int Mschr Anat Physiol 18:417–484

Gilboa L, Forbes A, Tazuke SI, Fuller MT, Lehmann R (2003) Germ line stem cell differentiation in Drosophila requires gap junctions and proceeds via an intermediate state. Development 130:6625–6634

Glotzer M (2005) The molecular requirements for cytokinesis. Science 307:1735–1739

Grieder NC, de Cuevas M, Spradling AC (2000) The fusome organizes the microtubule network during oocyte differentiation in *Drosophila*. Development 127:4253–4264

Huynh JR (2005) Fusome as a cell-cell communication channel of Drosophila ovarian cyst: Landes Biosciences, http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=eurekah.chapter.64665

Huynh JR, St Johnston D (2004) The origin of asymmetry: early polarisation of the Drosophila germline cyst and oocyte. Curr Biol 14:R438–R449

Jan Y, Jan L (1998) Asymmetric cell division. Nature 392:775–778

Januschke J, Gervais L, Dass S, Kaltschmidt JA, Lopez-Schier H, St Johnston D, Brand AH, Roth S, Guichet A (2002) Polar transport in the Drosophila oocyte requires Dynein and Kinesin I cooperation. Curr Biol 12:1971–1981

Januschke J, Gervais L, Gillet L, Keryer G, Bornens M, Guichet A (2006) The centrosome-nucleus complex and microtubule organization in the Drosophila oocyte. Development 133:129–139

Kai T, Spradling AC (2004) Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. Nature 428:564–569

Kawase E, Wong MD, Ding BC, Xie T (2004) Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing bam transcription in the Drosophila testis. Development 131:1365–1375

Kiger A, White-Cooper H, Fuller M (2000) Somatic support cells restrict germline stem cell self-renewal and promote differentiation. Nature 407:750–754

Kiger AA, Jones DL, Schulz C, Rogers MB, Fuller MT (2001) Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science 294:2542–2545

Kim NH, Chung KS, Day BN (1997) The distribution and requirements of microtubules and microfilaments during fertilization and parthenogenesis in pig oocytes. J Reprod Fertil 111:143–149

Kim NH, Chung HM, Cha KY, Chung KS (1998) Microtubule and microfilament organization in maturing human oocytes. Hum Reprod 13:2217–2222

Kloc M, Bilinski S, Dougherty MT, Brey EM, Etkin LD (2004) Formation, architecture and polarity of female germline cyst in Xenopus. Dev Biol 266:43–61

Knoblich JA (2001) Asymmetric cell division during animal development. Nat Rev Mol Cell Biol 2:11–20

Lambert JD, Nagy LM (2002) Asymmetric inheritance of centrosomally localized mRNAs during embryonic cleavages. Nature 420:682–686

Leader B, Lim H, Carabatsos MJ, Harrington A, Ecsedy J, Pellman D, Maas R, Leder P (2002) Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. Nat Cell Biol 4:921–928

Li L, Xie T (2005) Stem cell niche: structure and function. Annu Rev Cell Dev Biol 21:605–631

Lin H (1997) The tao of stem cells in the germline. Ann Rev Genet 31:455–491

Lin H, Spradling AC (1995) Fusome asymmetry and oocyte determination in *Drosophila*. Dev Gen 16:6–12

Lin H, Spradling AC (1997) A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. Development 124:2463–2476

Lin H, Yue L, Spradling AC (1994) The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. Development 120:947–956

Longo FJ, Chen DY (1985) Development of cortical polarity in mouse eggs: involvement of the meiotic apparatus. Dev Biol 107:382–394

Ma C, Benink HA, Cheng D, Montplaisir V, Wang L, Xi Y, Zheng PP, Bement WM, Liu XJ (2006) Cdc42 activation couples spindle positioning to first polar body formation in oocyte maturation. Curr Biol 16:214–220

Maro B, Johnson MH, Pickering SJ, Flach G (1984) Changes in actin distribution during fertilization of the mouse egg. J Embryol Exp Morphol 81:211–237

Maro B, Verlhac MH (2002) Polar body formation: new rules for asymmetric divisions. Nat Cell Biol 4:E281–E283

Maro B, Johnson MH, Webb M, Flach G (1986) Mechanism of polar body formation in the mouse oocyte: an interaction between the chromosomes, the cytoskeleton and the plasma membrane. J Embryol Exp Morphol 92:11–32

McGrail M, Hays TS (1997) The microtubule motor cytoplasmic dynein is required for spindle orientation during germline cell divisions and oocyte differentiation in *Drosophila*. Development 124:2409–2419

Michaut MA, Williams CJ, Schultz RM (2005) Phosphorylated MARCKS: a novel centrosome component that also defines a peripheral subdomain of the cortical actin cap in mouse eggs. Dev Biol 280:26–37

Miyazaki A, Kamitsubo E, Nemoto SI (2000) Premeiotic aster as a device to anchor the germinal vesicle to the cell surface of the presumptive animal pole in starfish oocytes. Dev Biol 218:161–171

Miyazaki A, Kato KH, Nemoto S (2005) Role of microtubules and centrosomes in the eccentric relocation of the germinal vesicle upon meiosis reinitiation in sea-cucumber oocytes. Dev Biol 280:237–247

Moore CA, Zernicka-Goetz M (2005) PAR-1 and the microtubule-associated proteins CLASP2 and dynactin-p50 have specific localisation on mouse meiotic and first mitotic spindles. Reproduction 130:311–320

Morrison SJ, Kimble J (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441:1068–1074

Na J, Zernicka-Goetz M (2006) Asymmetric positioning and organization of the meiotic spindle of mouse oocytes requires CDC42 function. Curr Biol 16:1249–1254

Nakaya M, Fukui A, Izumi Y, Akimoto K, Asashima M, Ohno S (2000) Meiotic maturation induces animal-vegetal asymmetric distribution of aPKC and ASIP/PAR-3 in Xenopus oocytes. Development 127:5021–5031

Ohlstein B, Kai T, Decotto E, Spradling AC (2004) The stem cell niche: theme and variations. Curr Opin Cell Biol 16:693–699

Pickering SJ, Johnson MH, Braude PR, Houliston E (1988) Cytoskeletal organization in fresh, aged and spontaneously activated human oocytes. Hum Reprod 3:978–989

Pielak RM, Gaysinskaya VA, Cohen WD (2004) Formation and function of the polar body contractile ring in Spisula. Dev Biol 269:421–432

Prodon F, Chenevert J, Sardet C (2006) Establishment of animal-vegetal polarity during maturation in ascidian oocytes. Dev Biol 290:297–311

Roper K, Brown NH (2004) A Spectraplakin is enriched on the fusome and organizes microtubules during oocyte specification in Drosophila. Curr Biol 14:99–110

Sardet C, Prodon F, Dumollard R, Chang P, Chenevert J (2002) Structure and function of the egg cortex from oogenesis through fertilization. Dev Biol 241:1–23

Skold HN, Komma DJ, Endow SA (2005) Assembly pathway of the anastral Drosophila oocyte meiosis I spindle. J Cell Sci 118:1745–1755

Snapp EL, Iida T, Frescas D, Lippincott-Schwartz J, Lilly MA (2004) The fusome mediates intercellular endoplasmic reticulum connectivity in Drosophila ovarian cysts. Mol Biol Cell 15:4512–4521

Song X, Zhu CH, Doan C, Xie T (2002) Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. Science 296:1855–1857

Song X, Wong MD, Kawase E, Xi R, Ding BC, McCarthy JJ, Xie T (2004) Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development 131:1353–1364

Sonneville R, Gonczy P (2004) Zyg-11 and cul-2 regulate progression through meiosis II and polarity establishment in C. elegans. Development 131:3527–3543

Spradling AC (1993) Developmental genetics of oogenesis. In: Bate M, Martinez-Arias A (eds) The development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, New York, pp 1–70

Spradling AC, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414:98–104

Storto P, King R (1989) The role of polyfusomes in generating branched chains of cystocytes during Drosophila oogenesis. Dev Genet 10:70–86
Sun QY, Schatten H (2006) Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. Reproduction 131:193–205
Swan A, Nguyen T, Suter B (1999) Drosophila Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. Nat Cell Biol 1:444–449
Szakmary A, Cox DN, Wang Z, Lin H (2005) Regulatory relationship among piwi, pumilio, and bag-of-marbles in Drosophila germline stem cell self-renewal and differentiation. Curr Biol 15:171–178
Szczepanska K, Maleszewski M (2005) LKB1/PAR4 protein is asymmetrically localized in mouse oocytes and associates with meiotic spindle. Gene Expr Patterns 6:86–93
Tavosanis G, Llamazares S, Goulielmos G, Gonzalez C (1997) Essential role for gamma-tubulin in the acentriolar female meiotic spindle of Drosophila. EMBO J 16:1809–1819
Telfer W (1975) Development and physiology of the oocyte-nurse cell syncitium. Adv Insect Physiol 11:223–319
Theurkauf W (1994) Microtubules and cytoplasm organization during Drosophila oogenesis. Dev Biol 165:352–360
Theurkauf WE, Hawley RS (1992) Meiotic spindle assembly in Drosophila females: behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. J Cell Biol 116:1167–1180
Theurkauf W, Smiley S, Wong M, Alberts B (1992) Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport. Development 115:923–936
Theurkauf W, Alberts M, Jan Y, Jongens T (1993) A central role for microtubules in the differentiation of *Drosophila* oocytes. Development 118:1169–1180
Tran J, Brenner T, DiNardo S (2000) Somatic control over the germline stem cell lineage during *Drosophila* spermatogenesis. Nature 407:754–757
Tremoleda JL, Schoevers EJ, Stout TA, Colenbrander B, Bevers MM (2001) Organisation of the cytoskeleton during in vitro maturation of horse oocytes. Mol Reprod Dev 60:260–269
Tulina N, Matunis E (2001) Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science 294:2546–2549
Verlhac MH, Lefebvre C, Guillaud P, Rassinier P, Maro B (2000) Asymmetric division in mouse oocytes: with or without Mos. Curr Biol 10:1303–1306
Vinot S, Le T, Maro B, Louvet-Vallee S (2004) Two PAR6 proteins become asymmetrically localized during establishment of polarity in mouse oocytes. Curr Biol 14:520–525
Wallenfang MR, Seydoux G (2000) Polarization of the anterior-posterior axis of C. elegans is a microtubule-directed process. Nature 408:89–92
Weber KL, Sokac AM, Berg JS, Cheney RE, Bement WM (2004) A microtubule-binding myosin required for nuclear anchoring and spindle assembly. Nature 431:325–329
Wong MD, Jin Z, Xie T (2005) Molecular mechanisms of germline stem cell regulation. Annu Rev Genet 39:173–195
Xie T, Spradling AC (1998) Decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. Cell 94:251–260

Xie T, Spradling AC (2000) A niche maintaining germ line stem cells in the Drosophila ovary. Science 290:328–330

Yamashita YM, Jones DL, Fuller MT (2003) Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. Science 301:1547–1550

Yang HY, McNally K, McNally FJ (2003) MEI-1/katanin is required for translocation of the meiosis I spindle to the oocyte cortex in C elegans. Dev Biol 260: 245–259

Yang HY, Mains PE, McNally FJ (2005) Kinesin-1 mediates translocation of the meiotic spindle to the oocyte cortex through KCA-1, a novel cargo adapter. J Cell Biol 169:447–457

Yue L, Spradling AC (1992) hu-li tai shao, a gene required for ring canal formation during Drosophila oogenesis, encodes a homolog of adducin. Genes Dev 6:2443–2454

Zhao GQ, Garbers DL (2002) Male germ cell specification and differentiation. Dev Cell 2:537–547

Asymmetric Cell Division During Brain Morphogenesis

Takaki Miyata

Abstract

The division patterns of neural progenitor cells in developing vertebrate brains have traditionally been classified into three types: (i) "symmetric" divisions producing two progenitor cells (P/P division), (ii) "symmetric" divisions producing two neurons (N/N division), and (iii) "asymmetric" divisions producing one progenitor cell and one neuron (P/N division). Many studies examining the mechanism(s) regulating P/N divisions have focused on mitotic cleavage orientation and the possible uneven distribution of cell-fate determining molecules such as Numb. Although these two factors may intrinsically determine daughter cell fate arising from M-phase progenitor cells, no unified explanations have yet to be put forth incorporating all available data. In this review, I will discuss recent advances in techniques allowing the more detailed monitoring of daughter cell behavior in a heterogeneously pseudostratified neuroepithelium that demonstrate previously unrecognized asymmetries in P/P divisions. Careful observations of daughter cell behavior suggest that, immediately after their birth at the apical surface of the neuroepithelium, generated cells may not yet be fate committed but rather integrate extrinsic and intrinsic signals during G1 phase before continuing down a developmental pathway.

1 Introduction

1.1 Applicability of Drosophila Models for Vertebrate Brain Formation

The production of two different cell types from one parent cell during animal development is referred to as asymmetric daughter cell output (or "asymmetric cell division" if loosely defined). Recently, significant progress has been made in our understanding of the mechanisms underlying asymmetric divisions in invertebrate model systems, especially *Drosophila* and *C. elegans* (reviewed in Horvitz and Herskowitz 1992; Matsuzaki 2000;

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Lu et al. 2000; Doe and Bowerman 2001; Knoblich 2001; Cowan and Hyman 2004). One widespread mechanism involves the segregation of cell fate determinants to only one daughter cell during progenitor cell mitosis ("intrinsically asymmetric" cell division). The ability of intrinsically asymmetric divisions to generate the vertebrate central nervous system (CNS) has been examined (reviewed in Betshinger and Knoblich 2004; Rogegiers and Jan 2004; Huttner and Kosodo 2005). In particular, studies have examined whether the orientation of cell division determines daughter cell fate and/or if there is a role for the asymmetric distribution of the vertebrate Numb protein in determining cell fate analogous to Numb in *Drosophila* (reviewed in Lu et al. 2000; Doe and Bowerman 2001).

1.2 Apical-basal Divisions are Insufficient to Generate Solely Asymmetric Daughter Cell Output

An early pioneering videomicroscopic study by Chenn and McConnell (1995) identified a close relationship between cleavage plane and daughter cell fate choice. They followed cell divisions at the apical surface of the ventricular zone (VZ) (Fig. 1) in developing ferret cerebral wall slices. They observed that in divisions with a horizontal mitotic spindle and a vertical cleavage (referred to as "horizontal" or "planar" division), both daughter cells tended to remain in the VZ. In contrast, in divisions with a vertical spindle and a horizontal cleavage (referred to as "vertical" or "apical-basal" division), the basally produced daughter cell migrated away while the apical daughter remained in the VZ. When considered with another imaging study using rat retinal explants (Cayouette and Raff 2003), these data suggest that the plane of cell division in the vertebrate CNS primordium can influence the fate of the produced daughter cells. Additionally, in recent functional experiments on cortical and retinal progenitor cells interfering with G protein mediated signaling (heterotrimetric guanine-nucleotide binding regulatory proteins), a factor important for mitotic spindle positioning and orientation during asymmetric cell division in *Drosophila* (reviewed in Betshinger and Knoblich 2004) caused the cleavage plane orientation to shift (from apical-basal to planar) with apparent daughter cell fate changes (Sanada and Tsai 2005; Zigman et al. 2005), further supporting a role for cleavage plane in daughter cell fate determination. These studies all suggest that asymmetric segregation of cell-fate determinants along the apical-basal axis of M-phase progenitor cells might contribute to cell diversification.

In a variety of CNS regions in several animal species, however, the proportion of M-phase cells at the apical surface of the VZ undergoing apical-basal division is not sufficient to fully explain neuronal differentiation: 3–5% in E11–E14 mouse cerebral wall (Smart 1973); 2–5% in

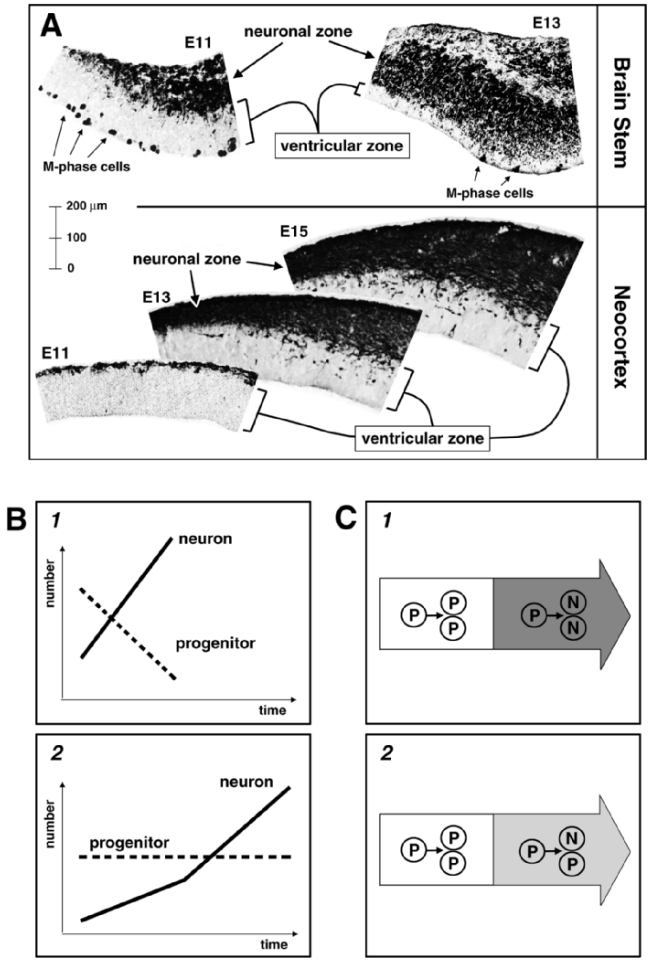

Fig. 1. A–C Continuous neuron production through asymmetric daughter cell output: **A** Photomicrographs depicting the accumulation of βIIItubulin-positive neurons in the outer zone of the embryonic mouse brain stem or neocortical wall are shown. Brain stem sections were also stained for phosphohistoneH3, a marker of M-phase cells. While the ventricular zone (VZ) of the brain stem is reduced between E11 and E13, the neocortical wall maintains VZ thickness from E11 to E15; **B** Schematic representation of the changes in the number of progenitor cells and neurons. Cytogenesis in the brain stem during E11-13 is characterized by an exhaustion of progenitor cells and a rapid but discontinuous increase in the number of neurons (*pattern 1*). In contrast, the number of neurons in the neocortical wall continuously increases during E11–E15 while the number of progenitor cells is maintained (*pattern 2*); **C** Two different patterns of daughter cell output following a common period of the overall "P→P+P" output are possible. If the progenitor pool then undergoes division to produce neurons only ("P→N+N"), it will soon disappear, as in the brain stem (*pattern 1*). Continuous neuron production, as occurs in the neocortical wall, can be achieved through asymmetric daughter cell output ("P→P+N") by the entire progenitor population (*pattern 2*) (Takahashi et al. 1996; Cai et al. 2002).

E10–E14 mouse cerebral wall (Landrieu and Goffinet 1979); approximately 16% between E29 and E36 in ferret (corresponding to E12/13–E14/15 mouse) cerebral wall (Chenn and McConnell 1995); less than 20% between E12–E16 with a sudden increase to about 50% at E14 in mouse cerebral wall (Hayder et al. 2003); <10% in all brain regions of E9–E14 mice (Kosodo et al. 2004); 5–10% in E10–E14 mouse spinal cord (Smart 1972), 3–21% in E18–P4 rat retina (Cayouette et al. 2001); <10% in E3–E5 chick retina (Silva et al. 2002); 0% in 28–45 h zebrafish retina (Das et al. 2003). Therefore, many planar divisions must also produce different daughter cells.

1.3 Diverse Roles for Vertebrate Numb

Vertebrate Numb is immunohistochemically detected in the VZ of mouse (Zhong et al. 1996), rat (Cayouette et al. 2001), and chick (Wakamatsu et al. 1999; Silva et al. 2002) embryos. Although forced expression of Numb in developing chick brains inhibits Notch and consequently accelerates neuronal differentiation (Wakamatsu et al. 1999), in the mouse, Numb and the Numb-related protein Numblike have multiple or complicated functions. Targeted disruption of mammalian Numb and Numblike causes the depletion of the cortical progenitor pool (Petersen et al. 2002, 2004), but the loss of both proteins using a different knock-out approach causes progenitor cell hyperproliferation coupled with defective neuronal differentiation (Li et al. 2003). In the chick neuroepithelium, Numb immunoreactivity is clearly localized to the basal side of metaphase cells (Wakamatsu et al. 1999; Silva et al. 2002), as well as the apical endfeet of interphase progenitor cells (Wakamatsu et al. 1999), and the latter staining pattern resembles meshes generated by adherens junction proteins including N-cadherin, catenins, and ZO1 (Fig. 2). Numb immunoreactivity was seen in M-phase cells in cross-sectional views of embryonic mouse cerebral walls (Zhong et al. 1996; Cayouette et al. 2001), but it was not determined whether only some $ZO1^+$ meshes visualized in an *en face* view of rodent neuroepithelium (Fig. 2) are Numb immunoreactive. If the entire cellular mesh is Numb positive, this would indicate that mammalian Numb is expressed in both M-phase and interphase cells. In a review, Roegiers and Jan (2004) discussed that the lack of precise lineage trees combined with the possible underestimation of asymmetric divisions likely contribute to the current uncertainty regarding the role of asymmetrically distributed Numb in mammalian brain development. They wrote, "Asymmetric divisions leading to two types of progenitor cells, which may be morphologically similar but will nonetheless give rise to different populations of daughter cells, could be mis-characterized as symmetric divisions if there were no means (e.g. molecular markers) to distinguish the two progenitors."

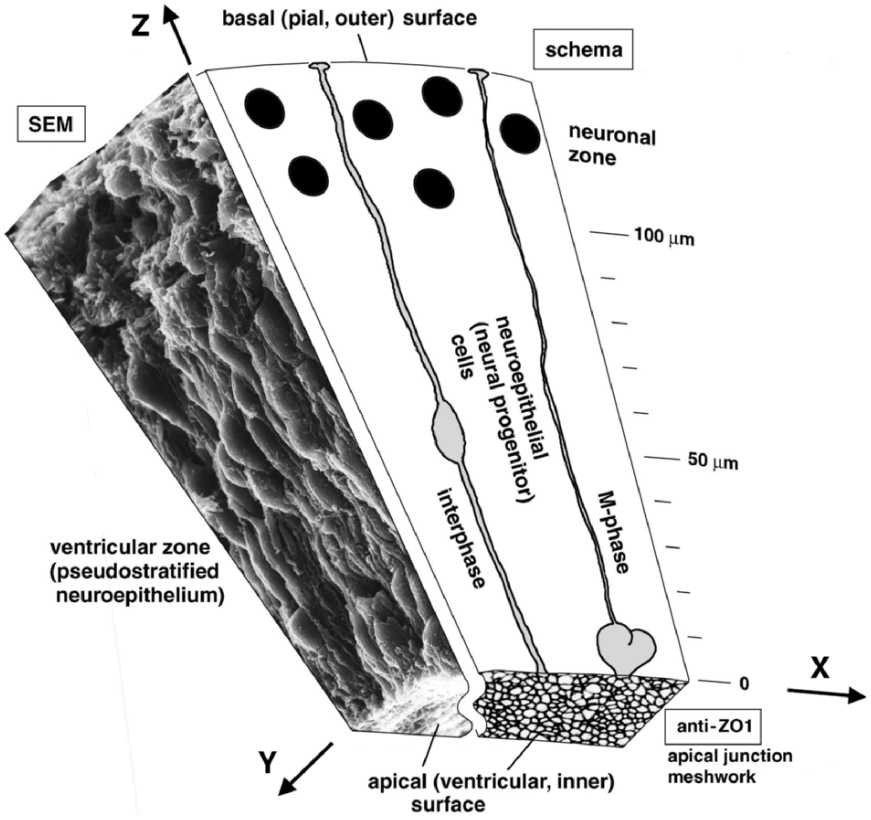

Fig. 2. Bipolar-shaped neural progenitor cells comprise the neuroepithelium. A three-dimensional representation of a portion (approximately 20 μm × 50 μm × 130 μm) of the early embryonic mouse cerebral wall is shown. In the XZ plane, a cross-sectional schematic view depicts two progenitor cells (one in interphase and one in M-phase) spanning the brain wall, and these can be visualized by sporadic labeling with DiI, a lipophilic dye, or green fluorescent protein. Also shown in the XZ plane are neurons accumulating in the outer zone. In the YZ plane, a cross-sectional view obtained by scanning electron microscopy (SEM) shows the ventricular zone filled with progenitor cell somata. An *en face* view in the XY plane of the ventricular (apical) surface of the brain wall consists of an SEM part (*left*) and an immunohistochemically stained part (*right*). Structurally, the ventricular zone (VZ) is a pseudostratified epithelium composed of several layers of nuclei in the VZ with the cytoplasm encircling each nucleus and contacting both the apical and basal surfaces of the brain wall. At the apical surface, the endfeet of progenitor cells form meshes of the adherens junction, which can be visualized by immunostaining for ZO1. Compared to the cross-sectional area of the soma of a progenitor cell, the apex of the same cell is much smaller (roughly 10%) (Smart 1972). As schematically illustrated, most M-phase cells at the apical surface of the early cerebral walls undergo planar division with a vertical cleavage (Smart 1973; Landrieu and Goffinet 1979; Hayder et al. 2003; Kosodo et al. 2004). Live observation is required to directly examine how this narrow apical surface is divided during the planar division and inherited by daughter cells.

1.4
Aims of this Review

In this review, I will address how the germinal zone of the mammalian CNS primordia differs in size (larger) and cellular composition (more heterogeneous) from that of the developing *Drosophila* nervous system. Additionally, detection of an unexpectedly high frequency of asymmetric cell division using live observation of cell cycle-dependent, three-dimensional cellular behaviors will be explained. Although cell-intrinsic mechanisms can play important roles in cell lineage choice, environmental factors may also contribute to this key event. Finally, I will discuss when and how the mitotic fate of a daughter cell is "chosen" through the coordination of extrinsic and intrinsic signals.

2
Cytogenesis During Mammalian Cerebral Cortical Development

2.1
The Neural "Germinal Zone" is a Thick Pseudostratified Neuroepithelium

As shown in Fig. 1, young, βIIItubulin-positive neurons are found in the outer part of embryonic brain walls during the active neuronogenic period. The border between the immunohistochemically defined neuronal territory and the remaining part (VZ) is almost completely parallel to the ventricular surface. The VZ is several cells thick (up to 100 μm thick) and enriched for the somata of progenitor cells (Fig. 2), but some neuronally-differentiating daughter cells about to express βIIItubulin may also exist there. The typical progenitor cell in interphase is bipolar-shaped, spanning the ventricular (apical) and pial (basal) surfaces (Fig. 2) (Hinds and Ruffett 1971; Seymor and Berry 1975). Its apical endfoot makes close contacts with surrounding endfeet through adherens junctions, and these connections can be visualized as meshes in an *en face* view (Fig. 2). Although progenitor cells in early neuroepithelia mostly divide at the surface, mitoses away from the surface increase as development proceeds (Martinez-Cerdeno et al. 2006). Compared to the cross-sectional area of the soma of a progenitor cell, the apex of the same cell is much smaller (Hinds and Ruffett 1971; Smart 1972; Baek et al. 2006).

The nucleus of each daughter cell generated at the apical surface moves away from the ventricular surface during G1 phase, and DNA is synthesized when the cell nucleus is in the outer half of VZ. In cells to divide at the surface, the nucleus moves adventricularly during G2 phase. This to-and-fro movement is known as interkinetic nuclear migration (INM), and a pseudostratified epithelium is generated by the overall arrangement of progenitor (neuroepithelial) cells (Sauer 1935; Sauer and Walker 1959; Sidman et al.

1959; Fujita 1962). In the mouse cerebral wall during the midembryonic period, the estimated length of the cell cycle for the entire progenitor population is about 10–15 h (Takahashi et al. 1995) and the time needed for a daughter cell generated at the ventricular surface to exit the VZ is about 12.5 h (Takahashi et al. 1996). Thus, daughter cell residence in neuronal territory is spatially and temporally distant from mitotic events at the ventricular surface.

2.2 Complexity of Mammalian Germinal Zone and Asymmetric Output

The VZ contains a mixture of progenitor cells at differing phases of the cell cycle as well as immunohistochemically unidentifiable, newly formed neurons. However, the VZ is much more heterogeneous containing progenitor cells with widely different properties: morphology (long vs short), mitosis position (ventricular surface vs immediately basal to the VZ), and lineage (committed vs stem-like) (Smart 1973; reviewed by Temple 1990). This diversity was identified relatively early, prior to the use of *Drosophila* as a model system of neural development, but it has been largely forgotten as studies have tried to apply lessons from the simple *Drosophila* ectoderm to the more complex mammalian neuroepithelium.

In the midembryonic cerebral wall, the VZ is thought to expand the progenitor pool through "symmetric" (P/P) divisions and/or produce both neurons and progenitor cells by "asymmetric" (P/N) divisions. This functional simplification is based on cell cycle kinetics analyses of the overall progenitor population that indicates a stage-dependent shift of the cell output pattern from "symmetric" (both daughter cells synthesize DNA) to "asymmetric" (Takahashi et al. 1996; Cai et al. 2002) (Fig. 1B,C).

2.3 Lessons from Time-lapse Lineage-analysis Studies: Are *All* Divisions "Asymmetric"?

2.3.1 Lineage Trees in Low Cell-density Monolayer Culture

The definitive characteristic of a progenitor cell is its ability to produce daughter cells, and DNA synthesis is a crude, indirect readout of cell fate. Therefore, the true functional characterization of a progenitor cell can only be accomplished by monitoring its division and the type of daughter cells produced. While simple in theory, such an approach is laborious, but long-term (~14 days) videomicroscopic observations by Temple and colleagues demonstrated that clonal monolayer-cultured cortical progenitor cells and their descendant cells divide in various patterns generating large lineage trees, branching mostly into neurons and astrocytes (Qian et al. 1998, 2000; Shen et al. 2002, 2006). These lineage trees contained some P/N divisions, but P/P divisions were much

more predominant. However, the majority of the P/P divisions in monolayer culture were asymmetric producing daughter cells distinct in mitotic activity. For example, one daughter cell could divide to generate paired granddaughter neurons (N/N division), while its sister cell generated paired progenitor cells as granddaughters (another P/P division). Thus, models of "asymmetric" cell output based on population level data (Fig. 1C) must be reexamined with single cell data. Single cell studies also suggest that all P/P divisions may be asymmetric if examined in detail. In many two-cell clones arising from P/P division (both daughter cells are Nestin^{+}) there was asymmetric expression of the epidermal growth factor receptor (EGFR) (Sun et al. 2005). In addition, morphological and molecular differences can be detected between sister neurons that are pair-generated (N/N) in the same culture system (Shen et al. 2002; Kawaguchi et al. 2004), further exemplifying previously unrecognized asymmetries in division patterns other than P/N.

2.3.2 Four-cell Clones in Slice Culture

Sliced embryonic cerebral walls can be maintained in culture using various supporting materials such as collagen gel and filter membranes (Chenn and McConnell 1995; Miyata et al. 2001; Nadarajah et al. 2001; Noctor et al. 2001). This culture system preserves the three-dimensional cellular morphology and apical-basal tissue polarity. In this culture system, fluorescently labeled progenitor cells divide normally for at least two days forming three- or four-cell clones through P/N or P/P divisions, respectively (Saito et al. 2003; Miyata et al. 2004; Noctor et al. 2004). Figure 3 schematically

Fig. 3. (*Cont'd*) mitotic and migratory behaviors of the generated daughter cells (**C** and **D**). In these asymmetric P/P divisions, one of the daughter cells irreversibly moves to the subventricular zone (SVZ), a thin layer immediately basal to the VZ, during cell cycle progression and then generates a pair of neurons, while the other daughter cell divides at the surface to maintain the number of progenitor cells. In thinner (younger) cerebral walls, the basal process inheriting daughter cell moves to the SVZ while the non-inheriting daughter cell elaborates a new process and divides at the surface ($P \rightarrow {}^{BP}P_{NS\text{-}div}+P_{S\text{-}div}$) (**C**), but this occurs much less frequently in a slightly thicker (thus more developmentally advanced) cerebral wall ($P \rightarrow P_{NS\text{-}div}+{}^{BP}P_{S\text{-}div}$) (**D**). In 23% of the total P/P divisions observed, pair-generated daughter cells undergo to-and-fro movements in the VZ followed by division at the apical surface ($P \rightarrow {}^{BP}P_{S\text{-}div}+P_{S\text{-}div}$) (**B**); this pattern was seen in thin cerebral walls before the emergence of the CP. The important factors to conclude from these data are as follows: (1) asymmetric P/P divisions are the predominant division pattern at the apical surface of the cerebral wall just before and during the emergence of the CP, a stage known for asymmetric daughter-cell output at the population level (Takahashi et al. 1996; Cai et al. 2002) and (2) all daughter cells observed, regardless of their final fate, were attached to the apical surface for at least several hours after their birth.

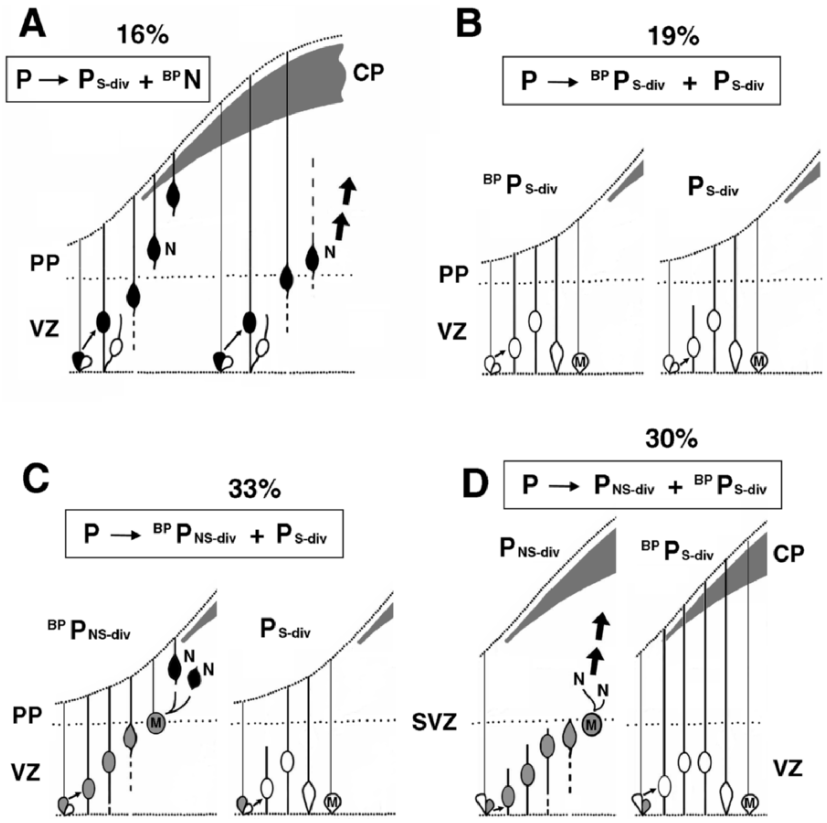

Fig. 3. A–D P/N and P/P divisions observed in E13-14 mouse cerebral wall slice culture: **A** Two distinct types of P/N division observed at the apical surface (Miyata et al. 2001) are schematically illustrated with developmental stage and brain thickness increasing towards the right side of the panel. In each case, a single DiI-labeled progenitor cell generated a daughter cell that remained in the VZ and divided at the apical surface (*white*, judged to be a progenitor cell, $P_{S\text{-}div}$; their mitoses are omitted for clarity) while the other daughter cell (*black*) exited the VZ without exhibiting any signs of mitosis (judged to be a neuron, N). Both daughter neurons, having inherited the basal process from their progenitor cells (therefore this division pattern is designated as $P \rightarrow {}^{BP}N+P_{S\text{-}div}$), are initially bipolar-shaped resembling their progenitor cells, but they exit the VZ after loosing their apical attachment. The daughter neuron born in an early and thin cerebral wall (*left part*), which consists of the VZ and the preplate (PP), uses the inherited process throughout its migration to the neuronal territory (Miyata et al. 2001). However, the process-inheriting neuron born later in a thicker cerebral wall (*right part*) uses its inherited process only until its exit from the VZ. It then retracts its basal process and undergoes further morphological changes before entering the cortical plate (CP) (T. Miyata, unpublished). In later cerebral walls, the basal process tends to be inherited by non-neuronal daughter cells generated by P/N divisions (Noctor et al. 2001); **B–D** diagrammatic representation of three different patterns of P/P divisions leading to the formation of four-cell clones through paired mitoses (M) (Miyata et al. 2004). In each panel, development proceeds to the right and the sequence of morphological changes is separately illustrated for each of the pair-generated daughter cells. Notably, 78% of the P/P division cases are clearly asymmetric with respect to the

depicts several different P/P division patterns observed in E13–E14 mouse cerebral wall slices (Miyata et al. 2004). The majority (77%) of P/P divisions occurring at the apical surface contributed one daughter cell to the subventricular zone (SVZ), a layer immediately basal to the VZ, for non-surface (or basal) division while the other daughter cell was retained in the VZ for division at the apical surface ($P_{S\text{-}div} \rightarrow P_{NS\text{-}div}+P_{S\text{-}div}$). The surface-(apically-) dividing daughter cell subsequently generated at least one mitotic granddaughter cell ($P_{S\text{-}div} \rightarrow$P+P or N) (Fig. 3A,B), but the non-surface-(basally-) dividing daughter cell primarily gave rise to paired granddaughter neurons ($P_{NS\text{-}div} \rightarrow$N+N) (Fig. 3C,D). Therefore, P/P divisions examined at the single cell level in slice culture frequently give rise to asymmetric daughter cells; results consistent with data obtained using monolayer cultures (Qian et al. 1998, 2000; Shen et al. 2002).

These observations suggest that the result of P/N divisions determined at the population level during the midembryonic stage (Takahashi et al. 1996; Cai et al. 2002) (Fig. 1) can be traced back to an initial asymmetric P/P division and the subsequent N/N and P/P or P/N divisions (*i.e.* P→2P+2N or P+3N through two rounds of the cell cycle). Both daughter cells arising from asymmetric P/P divisions must have been generated by a planar division (Smart 1973; Landrieu and Goffinet 1979; Hayder et al. 2003; Kosodo et al. 2004) and contribute to the maintenance of the ventricular surface meshwork through their endfeet (Fig. 2). The mechanisms controlling the loss of their apical attachment by SVZ-directing daughter cells will be discussed later in relation to coupling between fate determination and the choice of mitosis position.

2.3.3 Morphological Asymmetry in Surface-dividing Cells

Experiments using the slice culture system have clearly demonstrated different outcomes for generated paired sister progenitor cells, but they have also revealed an unexpected morphology of M-phase cells. Although classical Golgi and electron microscopic studies suggested that each neuroepithelial cell in the developing cerebral wall loses its basal process while its soma is at the apical surface during M phase (Hinds and Ruffett 1971; Seymour and Berry 1975), intensive monitoring of fluorescently labeled progenitor cells has revealed that the basal process remains and is inherited asymmetrically by one daughter cell (Miyata et al. 2001; Noctor et al. 2001) (Fig. 2). Similar morphologically asymmetric divisions occur in the developing retina (Cayouette and Raff 2003; Das et al. 2003; Saito et al. 2003; Pearson et al. 2005). The inheritor of the basal process can be a neuron or a progenitor (Fig. 3), and it is currently unknown whether the retained basal process is directly involved in the choice of daughter cell fate. Interestingly, however, in P/P divisions in which both daughter cells subsequently divided at the apical surface ($P_{S\text{-}div} \rightarrow P_{S\text{-}div}+P_{S\text{-}div}$) in retinal

and cerebral wall slices, the basal process inheriting daughter cells migrates away from the apical surface more quickly with a more basal trajectory of INM than its non-inheriting sister cell (Saito et al. 2003; Miyata et al. 2004). This phenomenon suggests that if there is an apical-basal concentration gradient of an unidentified molecule, the inheritance of the basal process by a daughter cell would affect the degree of its exposure to such a molecule.

3
Links Between Cell Cycle Progression, Nuclear Migration, and Mitotic Fate Choice in Asymmetric P/P Divisions

3.1
Neuronal-lineage Choice of a Progenitor Cell Precedes its Departure from the Apical Surface

Asymmetric P/P divisions, which are very frequent as shown in Fig. 3, provide valuable insight into the mechanisms underlying daughter cell organization of cell-cycle progression, mitotic fate determination, and nuclear movement (Miyata et al. 2004). Morphological, behavioral, and molecular comparisons between the surface-dividing and non-surface-dividing neocortical progenitor cells (schematically illustrated in Fig. 4A) have revealed several important cell fate-deterministic events. Immunoreactivity for Neurogenin2 (Ngn2), a bHLH transcription factor known to be important for commitment towards the neuronal lineage and the earliest known marker of neuron lineage commitment (Nieto et al. 2001; Schuurmans et al. 2004; reviewed in Guillemot 2005; Guillemot et al. 2006) is observed in a subpopulation (about 32%) of G1-phase progenitor cells. Additionally, $Ngn2^+$ cells possess an apical process during G1 phase, and, as the cell cycle proceeds, Ngn2 protein expression becomes restricted in a lineage-dependent fashion to the SVZ-directing population and is reduced in each cell. Finally, irreversible detachment of the SVZ-directing progenitor cells from the apical surface occurs during S or G2 phase. This temporal mapping provides a framework for efforts to link different molecular and cellular events. These data and the overall model generated are supported by observations by other groups using different technical approaches (Berger et al. 2004; Britz et al. 2006). Retrovirally forced expression of Ngn2 in VZ cells led to the loss of the cellular apical attachment and an increase in non-surface division (Miyata et al. 2004). These data suggest that Ngn2 induces progenitor cell apical detachment (through unexplored mechanisms) rather than the converse. However, detachment might further reinforce the lineage commitment and/or cell cycle inhibition (Fig. 4B).

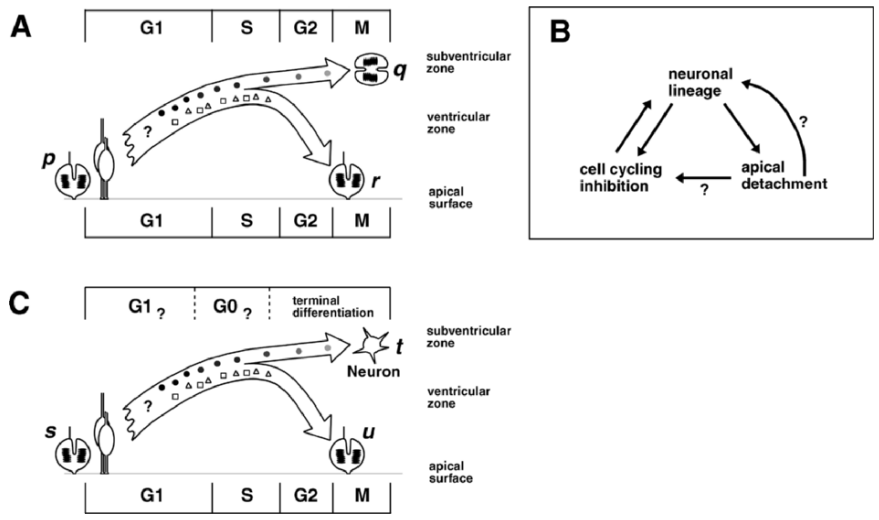

Fig. 4. Daughter cell residence in the VZ is coordinated with cell cycle progression and fate choice: **A** Observation-based diagram of a P/P division giving rise to one daughter cell (*q*) that subsequently undergoes an N/N division in the SVZ while the other daughter cell (*r*) undergoes P/P or P/N division at the apical surface. Neurogenin2 (Ngn2) protein (*solid circle*) is most strongly detected in the SVZ-directing cell during G1 phase (Miyata et al. 2004). Active Notch1 immunoreactivity (*open triangle*) (Tokunaga et al. 2004) and the expression of the destabilized Venus (an EYFP variant, *open square*) driven by the *nestin* enhancer (Sunabori et al. 2004) have a complementary expression pattern with Ngn2. The mechanisms regulating Ngn2 and active Notch1 expression as well as *nestin* enhancer activation remain unclear; these markers are only rarely positive in M-phase cells at the apical surface, suggesting that the asymmetric inheritance of unevenly distributed proteins is likely not responsible for daughter cell fate choice; **B** Schema depicting the relationship between three major events during asymmetric P/P division: cell cycling, mitotic fate choice, and migration. Each arrow indicates an established or suggested hierarchy (or sequence) between the cellular events. Commitment of a daughter cell to a neuronal-lineage progenitor cell precedes its detachment from the apical surface. Deceleration (or finalization) of cell cycling by inhibitors like p27 appears associated with lineage restriction, but the key triggering factor remains unclear; **C** Presumed cellular events occurring during a P/N division, which may be analogous in molecular regulation (*i.e.* Ngn2, Notch, and *nestin* enhancer) to an asymmetric P/P division. Most (if not all) neurons (*t*) may initially be integrated in the apical junction meshwork. Alternatively, all surface-generated daughter cells might initially be developmentally uncommitted, and some might then start taking a neuronal fate at some point during G1 phase by forgoing cell-cycle progression differently from its sister cell (*u*) and the cells *q* and *r*.

3.2
Neuronal-lineage Choice is Coordinated with Cell Cycle Inhibition

The commitment of a progenitor cell to the neuronal lineage eliminates the possibility of generating glial cells in subsequent divisions. The lineage tree arising from the ancestor cell could theoretically consist of many rounds of cell division, but in the early- and mid-embryonic mouse cerebral walls, most of the VZ-exiting (SVZ-directing) progenitor cells undergo terminal mitosis to generate paired neurons (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). The tight correlation between lineage commitment and cell cycle inhibition is at least partially explained by the ability of proneural bHLH factors, including Ngn2, to promote cell cycle withdrawal (reviewed in Ross et al. 2003). Additionally, cell cycle inhibition by $p27^{kip1}$ and PC3(TIS21/BTG2) can lead to lineage restriction through the activation of bHLH proneural genes (Canzoniere et al. 2004). Recently, Guillemot and colleagues demonstrated that $p27^{kip1}$ and Ngn2 are coexpressed in a subset of mouse cerebral wall VZ cells, and $p27^{kip1}$ stabilizes Ngn2 promoting the migration of daughter cells from the VZ and SVZ to more pially located neuronal territories (Nguyen et al. 2006). Transgenic expression of GFP under the control of the TIS21 promoter specifically labels mouse CNS progenitor cells that will generate one or two neurons, but not two progenitor cells, in the next mitotic event (Haubensak et al. 2004). However, a relationship between TIS21 and Ngn2 or $p27^{kip1}$ remains to be determined.

3.3
Is Symmetry Broken During G1 Phase or Earlier?

During S phase and late G1 phase, the staining pattern of Ngn2 immunoreactive cells in the VZ is complimentary to that of cells immunoreactive for activated Notch1 (Tokunaga et al. 2004). This implies that expression of the Hes1/5 proteins, which both function downstream of Notch signaling in maintaining the undifferentiated state of progenitor cells (reviewed in Kageyama and Ohtsuka 1999; Gaiano and Fishell 2002), might also be complimentary with Ngn2. Indeed, an inverse relationship between Hes1 and the proneural bHLH factor Mash1 immunoreactivity has recently been observed in the spinal cord (Baek et al. 2006). In addition, when VZ cells are fluorescently labeled by a nestin enhancer driven transgene, labeled cells are more proliferative and less differentiated in vitro than unlabeled VZ cells, and they tend to be Ngn2 negative during G1-S phase in vivo (Sunabori et al. 2004). The timing of the appearance of these complementary expression patterns between Ngn2 and other markers suggest that daughter cell fate choice is partially if not entirely complete by the end of G1 phase. Additionally, if

Notch-Delta interactions are involved in fate choice, these interactions should occur primarily in G1 phase.

The mechanism(s) controlling Ngn2 protein expression in only a fraction of G1-phase daughter cells in the E13-14 cerebral VZ (32%) remains unknown. While there may be some asymmetric inheritance of Ngn2 from M-phase cells at the surface, this cannot be the major mechanism responsible because only 4–5% of the surface M-phase cells are immunoreactive for Ngn2 (Miyata et al. 2004). Furthermore, the in vitro half-life of Ngn2 is only ~30 min in the absence of p27 and 70–100 min in the presence of p27 (Nguyen et al. 2006). A recent study suggested that Mash1 expression in a subpopulation of VZ cells during early G1-phase precedes Ngn2 expression in the same cells during late G1-phase (Britz et al. 2006), but the mechanism regulating Mash1 expression is unknown. Since EGFR immunoreactivity is asymmetric in 11% of the total M-phase cells in E17 cerebral wall sections and this asymmetry is also seen in E13/14-derived two-cell clones consisting of two Nestin^{+} cells (thus asymmetric P/P division cases) in monolayer culture (Sun et al. 2005), it will be interesting to examine whether EGFR asymmetry is involved in the restricted expression of Ngn2 during G1 phase. Although active Notch1 is not detected in M-phase cells (Tokunaga et al. 2004), Hes1 is seen in some apical M-phase cells (Baek et al. 2006). Whether Hes1 protein expression is heterogeneous among the apical M-phase cell population, within a particular cell, and/or it is expressed in a cell cycle-dependent manner (and if so when the expression is strongest) all remain to be determined. Thus, our current understanding of the contribution of the intrinsic asymmetry of apical M-phase cells to the differential behaviors of pair-generated daughter cells in the cerebral P/P division is quite limited.

The Notch ligand Delta-like1 (Dll1) is important for the maintenance of the undifferentiated state of progenitor cells (Henrique et al. 1997; Grandbarbe et al. 2003; Yoshimatsu et al. 2006). Several studies using different methods support the hypothesis that the Dll1 protein is expressed mainly in the soma and/or cellular processes near the soma in the cells of the VZ (Lindsell et al. 1996; Henrique et al. 1997; Campos et al. 2001). However, a recent study that examined Dll1 expression directly by immunohistochemistry suggests that Dll1 is expressed at much higher levels at the apical surface of the VZ than at other locations (Yoshimatsu et al. 2006). When considered with the fact that all G1-phase daughter cells initially have an apical endfoot (Figs. 3 and 4), these data suggest a model wherein the primary cellular site of Delta-Notch interactions between VZ cells including G1-phase daughter cells regulating cell fate is the endfeet at the apical surface. This model was originally put forth by the Ono group (Minaki et al. 2005; Mizuhara et al. 2005) to explain neuron-progenitor interactions at the ventricular surface of the developing spinal cord, as discussed below.

4
Reevaluation of the P/N Division by Analogy with the Asymmetric P/P Division

4.1
When Does a Surface-born Daughter Cell Become a Neuron?

In the mouse spinal cord at E11–E12, the stage at which neuron production occurs, *dll1* mRNA is detected not only in the somata of VZ cells but also at the apical endfeet that fill $ZO1^+$ rings, and the apical expression of *dll1* mRNA may indicate a nascent neuron (Minaki et al. 2005). This group further showed that dll1 protein is localized to the apical process of VZ cells *in vivo*, and dll1 binds the N-cadherin/β-catenin complex *in vitro* via the scaffolding protein MAGI1 (Mizuhara et al. 2005). This data suggests a model in which apically connected nascent neurons present Dll1 on their apical endfeet leading to Notch activation in the surrounding progenitor cells.

This model relies on the morphological premise that some neurons are at least transiently integrated into the apical junction meshwork. Such integration could be achieved by apical-directed movement of neurons from the SVZ to the surface, a behavior seen in long-term cultured cerebral wall slices (Noctor et al. 2004), and this could, in theory, be a distinct event from the morphology of a daughter neuron immediately after its birth. However, because the G1 phase is essential for regulating P/P divisions, it is likely that the putative Delta/Notch interaction between a nascent neuron and a progenitor cell should also take place during the G1 phase. Therefore, the presence of interactions between an apically generated neuron and the surrounding cells, including its sister cell, should be examined, and the initial morphological relationship of a daughter neuron to the apical surface is of particular interest.

Although the most widely distributed model for the initial morphology of an apically generated daughter neuron predicts its departure from the apical surface immediately after its birth (*i.e.* upon the progenitor cell's apical-basal division by a horizontal cleavage plane) without contributing to the apical junction meshwork (Chenn and McConnell 1995; Hayder et al. 2003), the existence of apically-connected VZ cells that subsequently detach from the apical surface and leave the VZ has clearly been demonstrated in mid-embryonic cerebral wall slices (Miyata et al. 2001, 2004, Noctor et al. 2004). These cells are initially either bipolar-shaped and spanning the cerebral wall (Fig. 3A,C) or pin-like with connections only to the apical surface (Fig. 3D) that subsequently collapse leading to a unipolar shape (Fig. 3A) and isolation (Fig. 3D). Many of these cells do not divide while expressing neuron markers such as Hu, and the non-mitotic Hu^+ cells are considered neurons (Fig. 3A) (Miyata et al. 2001; Noctor et al. 2001). Therefore, results obtained in slice culture are not inconsistent with the

model of Delta-Notch interactions between nascent neurons and progenitor cells at the apical surface.

However, it may not be entirely valid to label a several-hours-old daughter cell, which could be presenting Delta to a neighboring G1-phase progenitor cell, as a "neuron". Importantly, Hu, which becomes positive in 10 h-old (or older) apically generated daughter cells (Miyata et al. 2001), is also expressed in M-phase cells in the SVZ (Miyata et al. 2004), and other molecules are expressed by both lineage-restricted progenitor cells and noncycling (postmitotic) neurons such as βIIItubulin (Ishii et al. 2000), Ngn2 (Mizuguchi et al. 2001; Kawaguchi et al. 2004; Miyata et al. 2004; Hand et al. 2005; Britz et al. 2006; Nguyen et al. 2006), and Tbr2 (Englund et al. 2005). Therefore, it is currently impossible to definitively predict whether an apically connected VZ cell will then become a neuron or a lineage-restricted progenitor cell immediately after its birth. While this may simply reflect technical limitations such as the absence of better marker proteins, it is possible that lineage commitment does not occur at the time of apically-connected daughter cell generation but rather at a later time. The latter possibility will be further discussed below.

4.2 A "Moratorium" Model for Asymmetric Daughter-cell Output from the Apical Surface

Figure 4A,C illustrates P/P and P/N division patterns, respectively, which retain one mitotic daughter cell at the surface for later division (cell *r* in Fig. 4A and cell *u* in Fig. 4C). These two division patterns differ only in the mitotic ability of the SVZ-directing daughter cell (cell *q* that subsequently undergoes N/N division in Fig. 4A vs cell *t* becoming a neuron in Fig. 4C). It is likely that the founding cell of the P/P division (cell *p*, Fig. 4A) and the P/N division (cell *s*, Fig. 4C) are intrinsically different reflecting the observations that progenitor cells change their properties in a stage-dependent manner (Delalle et al. 1999; Takahashi et al. 1999a; Qian et al. 2000; Takizawa et al. 2001; Shen et al. 2006) and they generally become committed by the G2 phase (McConnell and Kaznowski 1991; Dyer et al. 2003; Miyata et al. 2004; Poggi et al. 2005). In the cerebral wall of TIS21-GFP transgenic mice, the former type (cell *p*) is GFP-negative but the latter type (cell *s*, which may correspond to cell *r*) is GFP-positive (Haubensak et al. 2004). The intrinsic properties of an apical M-phase cell will limit the *total* amount of activity that two daughter cells *together* can utilize for cell cycle progression, maintenance of undifferentiated state, and/or lineage restriction. Although one would further expect that this "activity" may also quickly determine the fate of *each* daughter cell through asymmetric inheritance mechanisms, direct evidence to support such "early determination" of the daughter cell has not yet been provided (we have no "early" daughter cell type-specific markers).

Currently available lists of molecular and behavioral similarities among the SVZ-directing daughter cells (*q* and *t* in Fig. 4A,C) coupled with the absence of any signs of early specification of the surface-born daughter cells are consistent with several possibilities. All daughter cells born at the surface likely tentatively enter G1 phase, as evidenced by the diffuse, almost universal immunoreactivity for Ki67, a marker of cycling cells, near the apical surface of the VZ (Chenn and Walsh 2002; Miyata et al. 2004). Additionally, most (if not all) surface-generated daughter cells join the apical junction meshwork. Finally, daughter cells *q* and *t* use common pathways involving Ngn2 and p27, establishing a bias towards the neuronal lineage. Despite these similarities, the choices of "to divide only once to generate two neurons" (cell *q*) or "not to divide" (cell *t*) do occur, probably reflecting the stage-dependent, intrinsic properties of their progenitor cells, *p* and *s*, respectively. At some point during G1 phase, each surface-born daughter cell decides to become a neuron (cell *t*) or to divide (cells *q*, *r*, and *u*); this choice may correspond to the G1/S checkpoint, as previously suggested by Caviness group (Takahashi et al. 1999b).

These multiple possible outcomes can be best understood if a daughter cell enters a "moratorium" period shortly after its generation. During this moratorium, extrinsic signals are still able to alter cell fate decisions of surface-born daughter cells. Two initially identical (or at least very similar) daughter cells can follow completely different developmental pathways after encountering different environmental cues ("extrinsically regulated" asymmetric daughter cell output). The moratorium period therefore connects the extrinsic signals to which a daughter cell is newly exposed with the intrinsic mechanisms present in its progenitor cell. Interactions between these extrinsic and intrinsic pathways regulate the lineage program in the developing retina (Kim et al. 2005; Poggi et al. 2005) and cerebral wall (Sun et al. 2005). Although current efforts to identify the possible intrinsic asymmetry-generating mechanisms (as seen in *Drosophila*) need to be continued, the role of the extrinsic mechanisms should also be examined with particular emphasis on the temporal window of the putative signals in relation to cell cycle progression.

5 Perspective

To understand fully asymmetric daughter cell output during brain development, we must minimize and eventually eliminate the developmental "black box" that exists from the emergence of two compartments within a progenitor cell body at metaphase to the selected expression of transcription factors like the proneural bHLH factors in G1-phase daughter cells. Continuing efforts to visualize the molecular and cellular events associated with mitosis are essential, but greater emphasis should be placed on

analyses examining cellular interactions. Greater use of both 3D tissue culture systems and low-density monolayer cultures retaining apical-basal and planar polarities will greatly facilitate these studies.

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References

Baek JH, Hatakeyama J, Sakamoto S, Ohtsuka T, Kageyama R (2006) Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. Development 133:2467–2476

Berger J, Eckert S, Scardigli R, Guillemot F, Gruss P, Stoykova A (2004) E1-Ngn2/Cre is a new line for regional activation of Cre recombinase in the developing CNS. Genesis 40:195–199

Betschinger J, Koblich JA (2004) Dare to be different: asymmetric cell division in *Drosophila, C. elegans* and vertebrates. Curr Biol 14:R674–R685

Britz O, Mattar P, Nguyen L, Langevin L-M, Zimmer C, Alam S, Guillemot F, Schuurmans C (2006) A role for proneural genes in the maturation of cortical progenitor cells. Cereb Cortex 16:i138–i151

Cai L, Hayes NL, Takahashi T, Caviness VS Jr, Nowakowski RS (2002) Size distribution of retrovirally marked lineages matches prediction from population measurements of cell cycle behavior. J Neurosci Res 69:731–744

Campos LS, Duarte AJ, Branco T, Henrique D (2001) mDll1 and mDll3 expression in the developing mouse brain: role in the establishment of the early cortex. J Neurosci Res 64:590–598

Canzoniere D, Fariolo-Vecchiolo S, Conti F, Ciotti MT, Tata AM, Augusti-Tocco G, Mattei E, Lakshmana MK, Krizhanovsky V, Reeves SA, Giovannoni R, Castano F, Servadio A, Ben-Arie N, Tirone F (2004) Dual control of neurogenesis by PC3 through cell cycle inhibition and induction of Math1. J Neurosci 24:3355–3369

Cayouette M, Raff M (2003) The orientation of cell division influences cell-fate choice in the developing mammalian retina. Development 130:2329–2339

Cayouette M, Whitmore AV, Jeffery G, Raff M (2001) Asymmetric segregation of Numb in retinal development and the influence of pigment epithelium. J Neurosci 21:5643–5651

Chenn A, McConnell SK (1995) Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell 82:631–641

Chenn A, Walsh CA (2002) regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science 297:365–369

Cowan CR, Hyman AA (2004) Asymmetric cell division in *C. elegans*: Cortical polarity and spindle positioning. Ann Rev Cell Dev Biol 20:427–453

Das T, Payer B, Cayouette M, Harris WA (2003) *In vivo* time-lapse imaging of cell divisions during neurogenesis in the developing zebrafish retina. Neuron 37:597–609

Delalle I, Takahashi T, Nowakowski RS, Tsai LH, Caviness VS Jr (1999) CyclinE-p27 opposition and regulation of the G1 phase of the cell cycle in the murine neocortical PVE: a quantitative analysis of mRNA *in situ* hybridization. Cereb Cortex 9:824–832

Doe CQ, Bowerman B (2001) Asymmetric cell division: fly neuroblast meets worm zygote. Curr Opin Cell Biol 13:68–75

Dyer MA, Livesey FJ, Cepko CL, Oliver G (2003) Nat Genet 34:53–58

Englud C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, interposed progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247–251

Fujita S (1962) Kinetics of cellular proliferation. Exp Cell Res 28:52–60

Gaiano N, Fishell G (2002) The role of Notch in promoting glial and neural stem cell fates. Annu Rev Neurosci 25:471–490

Grandbarbe L, Bouissac J, Rand M, Hrabe de Angelis M, Artavanis-tsakonas S (2003) Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. Development 130:1391–1402

Guillemot F (2005) Cellular and molecular control of neurogenesis in the mammalian telencephalon. Curr Opin Cell Biol 17:1–9

Guillemot F, Molnar Z, Tarabykin V, Stoykova A (2006) Molecular mechanism of cortical differentiation. Eur J Neurosci 23:857–868

Hand R, Bortone D, Mattar P, Nguyen L, Ik-Tsen Heng J, Guerrier S, Boutt E, Peters E, Barnes AP, Parras C, Shuurmans C, Guillemot F, Polleux F (2005) Phosphorylation of Neurogenin2 specifies the migration properties and the dendritic morphology of pyramidal neurons in the neocortex. Neuron 48:45–62

Haubensak W, Attardo A, Denk W, Huttner WB (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc Natl Acad Sci USA 101:3196–3201

Hayder TF, Ang E Jr, Rakic P (2003) Mitotic spindle rotation and mode of cell division in the developing telencephalon. Proc Natl Acad Sci USA 100:2890–2895

Henrique D, Hirsinger E, Adam J, Roux IL, Pourquie O, Ish-Horowicz D, Lewis J (1997) Maintenance of neuroepithelial progenitor cells by Delta-Notch signaling in the embryonic chick retina. Curr Biol 7:661–670

Hinds JW, Ruffett TL (1971) Cell proliferation in the neural tube: an electron microscopic and Golgi analysis in the mouse cerebral vesicle. Z Zellforsch 115:226–264

Horvitz HR, Herskowitz I (1992) Mechanism of asymmetric cell division: two Bs not two Bs, that is the question. Cell 68:237–255

Huttner WB, Kosodo Y (2005) Curr Opin Cell Biol 17:648–657

Ishii Y, Nakamura S, Osumi N (2000) Demarcation of early mammalian cortical development by differential expression of fringe genes. Dev Brain Res 119:307–320

Kagayama R, Ohtsuka T (1999) The Notch-Hes pathway in mammalian neural development. Cell Res 9:179–188

Kawaguchi A, Ogawa M, Saito K, Matsuzaki F, Okano H, Miyata T (2004) Differential expression of Pax6 and Ngn2 between pair-generated cortical neurons. J Neurosci Res 78:784–795

Kim J, Wu HH, Lander AD, Lyons KM, Matzuk MM, Calof AL (2005) GDF11 controls the timing of progenitor cell competence in developing retina. Science 308:1927–1930

Knoblich JA (2001) Asymmetric cell division during animal development. Nat Rev Mol Cell Biol 2:11–20

Kosodo Y, Roper K, Haubensak W, Marzesco A-M, Corbeil D, Huttner W (2004) Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. EMBO J 23:2314–2324

Landrieu P, Goffinet A (1979) Mitotic spindle fiber orientation in relation to cell migration in the neo-cortex of normal and reeler mouse. Neurosci Lett 13:69–72

Li HS, Wang D, Shen Q, Schonemann MD, Gorski JA, Jones KR, Temple S, Jan LY, Jan YN (2003) Inactivation of Numb and Numblike in embryonic dorsal forebrain impairs neurogenesis and disrupts cortical morphogenesis. Neuron 40:1105–1118

Lindsell CE, Boulter J, diSibio G, Gossler A, Weinmaster G (1996) Expression patterns of *Jagged*, *Delta1*, *Notch1*, *Notch2*, and *Notch3* genes identify ligand-receptor pairs that may function in neural development. Mol Cell Neurosci 8:14–27

Lu B, Jan L, Jan YN (2000) Control of cell divisions in the nervous system: symmetry and asymmetry. Annu Rev Neurosci 23:531–556

Martinez-Cerdeno V, Noctor SC, Kriegstein A (2006) The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. Cereb Cortex 16:i152–i161

Matsuzaki F (2000) Asymmetric division of *Drosophila* neural stem cells: a basis for neural diversity. Curr Opin Neurobiol 10:38–44

McConnell SK, Kaznowski CE (1991) Cell cycle dependence of laminar determination in developing neocortex. Science 254:282–285

Minaki Y, Mizuhara E, Morimoto K, Nakatani T, Sakamoto Y, Inoue Y, Satoh K, Imai T, Takai Y, Ono Y (2005) Migrating postmitotic neural precursor cells in the ventricular zone extend apical processes and form adherens junctions near the ventricle in the developing spinal cord. Neurosci Res 52:250–262

Miyata T, Kawaguchi A, Okano H, Ogawa M (2001) Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron 31:727–741

Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T, Ogawa M (2004) Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development 131:3133–3145

Mizuguchi R, Sugimori M, Takebayashi H, Kosako H, Nagao M, Yoshida S, Nabeshima Y, Shimamura K, Nakafuku M (2001) Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motor neurons. Neuron 31:757–771

Mizuhara E, Nakatani T, Minaki Y, Sakamoto Y, Ono Y, Takai Y (2005) MAGI1 recruits Dll1 to cadherin-based adherens junctions and stabilizes it on the cell surface. J Biol Chem 280:26499–26507

Nadarajah B, Brunstorm JE, Grutzundler J, Wong ROL, Pearlman AL (2001) Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci 4:143–150

Nguyen L, Besson A, Heng JI-T, Shuurmans C, Teboul L, Parras C, Philpott A, Robertis JM, Guillemot F (2006) $p27^{kip1}$ independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev 20:1511–1524

Nieto M, Shuurmans C, Britz O, Guillemot F (2001) Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron 29:401–413

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714–720

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136–144

Pearson RA, Luneborg NL, Becker DL, Mobbs P (2005) Gap junctions modulate interkinetic nuclear movement in retinal progenitor cells. J Neurosci 16: 10803–10814

Petersen PH, Zou K, Hwang JK, Jan YN, Zhong W (2002) Progenitor cell maintenance requires numb and numblike during mouse neurogenesis. Nature 419:929–934

Petersen PH, Zou K, Krauss S, Zhong W (2004) Continuing role for mouse *Numb* and *Numbl* in maintaining progenitor cells during cortical neurogenesis. Nat Neurosci 7:803–811

Poggi L, Vitorino M, Masai I, Harris WA (2005) Influences on neural lineage and mode of division in the zebrafish retina in vivo. J Cell Biol 171:991–999

Qian X, Goderie SK, Shen Q, Sterm JH, Temple S (1998) Intrinsic programs of patterned cell lineages in isolated vertebrate CNS ventricular zone cells. Development 125:3143–3152

Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S (2000) Timing of CNS cell gereration: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 28:69–80

Roegiers F, Jan YN (2004) Asymmetric cell division. Curr Opin Cell Biol 16:195–205

Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. Neuron 39:13–25

Saito K, Kawaguchi A, Kashiwagi S, Yasugi S, Ogawa M, Miyata T (2003) Dev Growth Differ 45:219–229

Sanada K, Tsai L-H (2005) G protein βγ subunits and AGS3 control spindle orientation and asymmetric cell fate of cerebral cortical progenitors. Cell 122:119–131

Sauer FC (1935) Mitosis in the neural tube. J Comp Neurol 62:377–405

Sauer ME, Walker BE (1959) Radiographic study of interkinetic nuclear migration in the neural tube. Proc Soc Exp Biol Med 101:557–600

Schuurmans C, Armant O, Nieto M, Stenman JM, Britz O, Klenin N, Brown C, Langevin L-M, Seibt J, Tang H, Cunningham JM, Dyck R, Walsh C, Campbell K, Polleux F, Guillemot F (2004) Sequential phases of cortical specification involve *Neurogenin*-dependent and -independent pathways. EMBO J 23:2892–2902

Seymour RM, Berry M (1975) Scanning and transmission electron microscope studies of interkinetic nuclear migration in the cerebral vesicles of the rat. J Comp Neurol 160:105–125

Shen Q, Zhong W, Jan YN, Temple S (2002) Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral ncortical stem cells and neuroblast. Development 129:4843–4853

Shen Q, Wang Y, Dimos JT, Fasano CA, Phoenix TN, Lemischka IR, Ivanova NB, Stifani S, Morrisey EE, Temple S (2006) The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. Nat Neurosci 9:743–751

Sidman RL, Miale IL, Feder N (1959) Cell proliferation and migration in the primitive ependymal zone; an autoradiographic study of histogenesis in the nervous system. Exp Neurol 1:322–333

Silva AO, Ercole CE, McLoon SC (2002) Plane of cell cleavage and numb distribution during cell division relative to cell differentiation in the developing retina. J Neurosci 22:7518–7525

Smart IHM (1972) Proliferative characteristics of the ependymal layer during the early development of the spinal cord in the mouse. J Anat 111:365–380

Smart IHM (1973) Proliferative characteristics of the ependymal layer during the early development of the mouse neocortex: a pilot study based on recording the number, location and plane of cleavage of mitotic figures. J Anat 116:67–91

Sun Y, Goderie SK, Temple S (2005) Asymmetric distribution of EGFR receptor during mitosis generates diverse CNS progenitor cells. Neuron 45:873–886

Sunabori T, Matsuzaki Y, Nagai T, Tokunaga A, Miyata T, Tabata H, Nakajima K, Miyawaki A, Okano H (2004) Visualizing neural progenitor cells with a destabilized fluorescent reporter: Nestin-d4-Venus. Soc Neurosci Abstr 32.9

Takahashi T, Nowakowski RS, Caviness VS Jr (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J Neurosci 15:6046–6057

Takahashi T, Nowakowski RS, Caviness VS Jr (1996) Interkinetic and migratory behavior of a cohort of neocortical neurons arising in the early embryonic murine cerebral wall. J Neurosci 16:5762–5776

Takahashi T, Goto T, Miyama S, Nowakowski RS, Caviness VS Jr (1999a) Sequence of neuron origin and neocortical laminar fate: ralation to cell cycle of origin in the developing murine cerebral wall. J Neurosci 19:10357–10371

Takahashi T, Bhide PG, Goto T, Miyama S, Caviness VS Jr (1999b) Proliferative behavior of the murine cerebral wall in tissue culture: cell cycle kinetics and checkpoints. Exp Neurol 156:407–417

Takizawa T, Nakashima K, Namihira M, Ochiai W, Uemura A, Yanagisawa M, Fujita N, Nakao M, Taga T (2001) DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 1:749–758

Temple S (1990) Characteristics of cells that give rise to the central nervous system. J Cell Sci 97:213–218

Tokunaga A, Kohyama J, Yoshida T, Nakao K, Sawamoto K, Okano H (2004) Mapping spatio-temporal activation of Notch signaling during neurogenesis and gliogenesis in the developing mouse brain. J Neurochem 90:142–154

Wakamatsu Y, Maynard TM, Jones SU, Weston JA (1999) NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. Neuron 23:71–81

Yoshimatsu T, Kawaguchi D, Oishi K, Takeda K, Akira S, Masuyama N, Gotoh Y (2006) Non-cell-autonomous action of STAT3 in maintenance of neural precursor cells in the mouse neocortex. Development 133:2553–2563

Zhong W, Feder JN, Jiang MM, Jan LY, Jan YN (1996) Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. Neuron 17:43–53

Zigman M, Cayouette M, Charalambous C, Schleiffer A, Hoeller O, Dunican D, McCudden CR, Firnburg N, Barres B, Siderovski DP, Knoblich JA (2005) Mammalian inscuteable regulates spindle orientation and cell fate in the developing retina. Neuron 48:539–545

Generating Asymmetry: With and Without Self-Renewal

Ivana Gaziova and Krishna Moorthi Bhat

Abstract

At some point during the history of organismal evolution, unicellular, unipotent and mitotically active cells acquired an ability to undergo a special type of cell division called asymmetric division. By this special type of cell division, these cells could divide to generate two different progeny or to self-renew and at the same time generate a progeny that is committed to become a cell different from the mother cell. This type of cell division, which forms the basis for the functioning of totipotent or multipotent stem cells, underlies the fundamental basis for the developmental evolution of organisms. It is not clear if the asymmetric division without self-renewal preceded the asymmetric division with self-renewal. It is reasonable to assume that the asymmetric division without self-renewal preceded the asymmetric division with self-renewal.

In this review we explore the genetic regulation of these two types of asymmetric divisions using the Drosophila central nervous system (CNS) as a model system. The results from recent studies argue that for cells to undergo a self-renewing asymmetric division, certain "stem cell" proteins must be maintained or up-regulated, while genes encoding proteins responsible for differentiation must be repressed or down-regulated. As long as a balance between these two classes of proteins is maintained via asymmetric segregation and activation/repression, the progeny that receives stem cell proteins/maintains stem cell competence will have the potential to undergo self-renewing asymmetric division. The other progeny will commit to differentiate.

In non-self-renewing asymmetric division, down-regulation of stem cell proteins/competence combined with asymmetric segregation of cell identity specifying factors (either cell-autonomous or a combination of cell autonomous and non-cell autonomous signals) cause the two progeny to assume different differentiated identities. Identification of mutations that confer a stem cell type of division to non-stem cell precursors, or mutations that eliminate asymmetric division, has led the way in elucidating the molecular basis for these divisions. Given that there is a considerable degree of conservation of genes and their function, these studies should provide clear insight into how the self-renewing asymmetric division of stem cells in neural and other lineages is regulated not only in Drosophila but also in vertebrates including humans.

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Progress in Molecular and Subcellular Biology
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Asymmetric Cell Division

1 Introduction

A long time ago during the evolution of living beings, unipotent and mitotically active cells somehow acquired the ability to undergo asymmetric division, with and without self-renewal. Acquisition of these specialized division capabilities changed the history of organismal evolution. These cell divisions formed the basis for the generation of stem cells from which multi-cellular tissues with different functions can be generated, leading to the evolution of complex organisms such as humans.

In this review we will use the example of eukaryotic central nervous system (CNS) to discuss how the above types of cell division, in a few founding neural stem cells, generate a large number of neurons of diverse function. In the CNS, neural progenitor stem cells undergo a series of asymmetric cell divisions to self renew and to generate several rounds of secondary precursors, which then undergo asymmetric division without self-renewal to generate a large number of neurons of distinct identities. Given that a large body of work has been done in the model organism Drosophila, we will focus on how the two types of divisions generate the embryonic CNS in this organism. We will discuss the recent data on these divisions in the post-embryonic brain as well.

1.1 Asymmetric Division in Stem Cells

Asymmetric division is the most important aspect of the biology of stem cells. Because of self-renewing asymmetric division, stem cells are able to maintain their population throughout their lifespan. At the same time, this type of division generates progeny that are committed to a differentiation pathway. While differentiated cells can be reprogrammed to re-initiate development (Campbell et al. 1996) or multi-potential cells can be induced to differentiate into various lineages (reviewed in Hall and Watt 1989), maintenance of a pluripotent stem cell state has been an intriguing and long-standing problem (e.g. Bhat et al. 1988; Tanaka et al. 2002; Golan-Mashiach et al. 2004; reviewed in Szutorisz and Dillon 2005). Questions such as how many genes are involved in maintaining a stem cell state or how self-renewing asymmetric division is regulated remain unanswered.

Classically, stem cells are defined as cells that 1) are pluripotential or multi-potential (i.e., able to generate different cell types, 2) have the ability to self-renew and at the same time generate progeny that are committed to a differentiation pathway, and 3) divide throughout the life span. Some of the most common stem cell lineages include cells in the immune system, germline, skin, intestinal epithelia and brain (reviewed in Hall and Watt 1989). Embryonic cells are also considered stem cells although they do not

exist as stem cells for the entire duration of the life cycle. With the demonstration that even nuclei from differentiated cells of an adult can be re-programmed to re-initiate development (Campbell et al. 1996; Cowan et al. 2005; reviewed in Hochedlinger and Jaenisch 2006), some modification of the above strict definition of stem cells may be in order. In any case, the most important function of stem cells is to generate tissues consisting of different cell types and to have a continuous replacement of those cells. This is achieved through self-renewing asymmetric divisions.

The maintenance of a precursor stem cell state via asymmetric division can be viewed as a reductive process where the activities of some genes maintain this state in one of the two cells. Once these genes are switched off in the other progeny, that cell becomes committed to a differentiation pathway (c.f. Bhat et al. 1988). For example, treatment of embryonal stem cells or embryonal carcinoma cells with retinoic acid (RA) induces these cells to differentiate into neurons, and DMSO induces them to differentiate into muscle cells (c.f. Bhat et al. 1988). RA must be inactivating genes that would prevent differentiation of these cells; at the same time, it must be activating neuron-specific genes. DMSO must also be inactivating the same genes but activating muscle-specific genes.

The other argument would be that RA is activating neuron-specific genes and DMSO is activating muscle-specific genes, thus forcing these cells to differentiate, and into specific lineages. However, several additional studies lend support to the scenario where activities of certain genes maintain an undifferentiated multi-potential state and inactivation of those genes is necessary for the cells to differentiate (Littlefield and Felix, 1982; Bhat et al. 1988; Williams et al. 1988; Ying et al. 2003). For example, a Differentiation Inhibiting Activity (DIA), also known as Leukemia Inhibitory Factor (LIF) has been identified in the immune system. This factor appears to maintain the undifferentiated state of embryonal stem (ES) cells (Williams et al. 1988). In the absence of this gene activity, the cells undergo terminal differentiation (Williams et al. 1988). Similarly, a recent study has shown that bone morphogenetic proteins act in combination with LIF to sustain self-renewal and preserve a multipotential state of (ES) cells (Ying et al. 2003).

In one of our previous studies (Bhat et al. 1988) using a functional selection procedure in a mouse embryonal carcinoma cell line (P19S18), we had cloned several chromosomal loci that get inactivated following induction of differentiation into neurons or muscle cells. These loci contained enhancer elements with the POU protein binding sites. Consistent with these results, several studies have isolated POU genes that are expressed only in the undifferentiated cells but not in differentiated cell types. For example, *oct-4* POU gene, which is expressed in pluripotent stem cells of the mouse early embryo, is turned off when these cells begin to differentiate (Rosner et al. 1990). In vertebrates, genes such as *oct-4* and *nanog* (Chambers et al. 2003; Loh et al. 2006; Mitsui et al. 2003) appear to be part of the pathway indispensable to maintaining stem cell identity/capability. Similarly, SCIP is

expressed in the progenitors of oligodendrocytes, but it is down-regulated when these cells are induced to differentiate (Collarini et al. 1992). These studies reveal only so much as to what is necessary to maintain an undifferentiated stem cell state. The question, however, is how does the self-renewing asymmetric division potential of these stem cells regulated?

1.2 The Drosophila CNS as a Model to Study Asymmetric Divisions

The CNS of the *Drosophila* embryo provides an experimentally advantageous model system to investigate the molecular basis for the asymmetric divisions that generate the metazoan nervous system. In the Drosophila embryo, the ventral nerve cord (VNC) consists of segmentally repeated units called neuromeres; each is divided into two hemineuromeres. Each of the 28 hemineuromeres has ~320 neurons and ~30 glia (Bossing et al. 1996; Schmidt et al. 1997). This complex array of neurons in a hemineuromere is generated by about 30 neuroblast (NBs) (in all of VNC, a total of ~10,000 neurons are generated by ~1000 NBs). These NBs are delaminated from the neuroectoderm in successive waves (Bate 1976; Campos-Ortega and Jan 1991; Doe 1992; Bhat 1999). Once formed, each NB undergoes several "self-renewing" asymmetric divisions to produce a chain of secondary precursor cells called ganglion mother cells or GMCs (Fig. 1). Since an NB appears generally to change its gene expression program following each division, strictly speaking, a NB division may not be a true self-renewal. However, this is a self-renewing division since one of the two cells remains as a NB with the ability to produce GMCs.

Though bipotential, a GMC does not self-renew; instead it divides asymmetrically to generate two different post-mitotic cells: either two neurons, two glial cells or a neuron and a glia (Bate 1976; Bhat and Schedl 1994; Buescher et al. 1998; Wai et al. 1999). The number of divisions each NB undergoes in the nerve cord varies between 3 and perhaps as many as 15. At the end of these divisions, a NB dies, becomes quiescent, or divides symmetrically to generate two GMCs. It is not known if the quiescent NBs in the VNC resume division again in a post-embryonic nerve cord. At least in the brain, quiescent NBs re-enter cell cycle. The ability of NBs and GMCs to divide by asymmetric mitosis is crucial in generating a large number of neurons of distinct identities from a few precursor cells.

1.3 NB4-2→GMC-1 →RP2/sib Lineage as a Model to Study Asymmetric Division

To examine the above issue, we will utilize a typical NB lineage, NB4-2→GMC-1→RP2/sib lineage, in the embryonic CNS. A wealth of information is available for this lineage in terms of genetic regulation of asymmetric

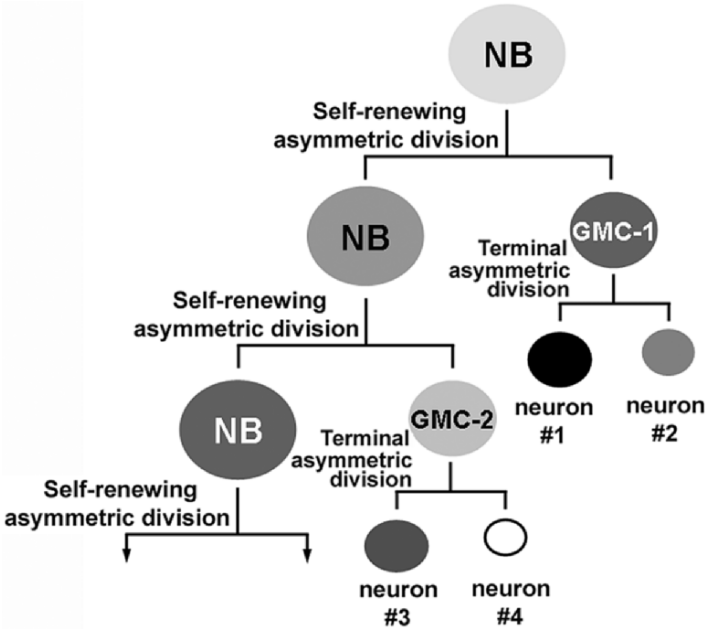

Fig. 1. Neuronal lineage elaboration via asymmetric divisions in the Drosophila CNS

division. We will describe this lineage in detail to give a better picture of the issues under discussion in this review. The NB4-2→GMC-1→RP2/sib lineage is one of the well-studied neuronal lineages in the VNC of the Drosophila embryo (Thomas et al. 1984; Bhat 1999). The NB4-2 generates its first GMC (GMC-1) by self-renewing asymmetric division. This GMC-1 divides to generate an RP2, a motoneuron, and a sib, whose ultimate fate is not known (Fig. 2). There are several well-established ways to distinguish a GMC-1, an RP2 and a sib (Doe 1992; Bhat and Schedl 1994; Buescher et al. 1998; Bhat and Apsel 2004). First, both the nuclear division and cytokinesis of GMC-1 is asymmetric and there is a size difference between a GMC-1 (7.5 μ), an RP2 (~5 μ) and a sib (~3 μ); the nuclear size of a GMC-1 is ~6.5 μ, an RP2 is 4 μ and a sib is 2.5 μ.

Second, there is a level difference in marker gene expression between an RP2 and a sib as well as a difference in the temporal dynamics of expression of these markers: the future RP2 cell has a stronger expression of markers such as Even-skipped (Eve) compared to a future sib; the cell that assumes a sib identity undergoes a size reduction and further down-regulation of expression of RP2-specific marker genes. By ~14 h of development, expression of all those markers is completely lost from the sib. Third, there is a set of marker genes that only a mature RP2 expresses but not the sib or the GMC-1. These include MAP1B (Mab 22C10; Fujita et al. 1982), which allows us to visualize the axon morphology (Fig. 2B–E), or Zfh-1, a transcription factor

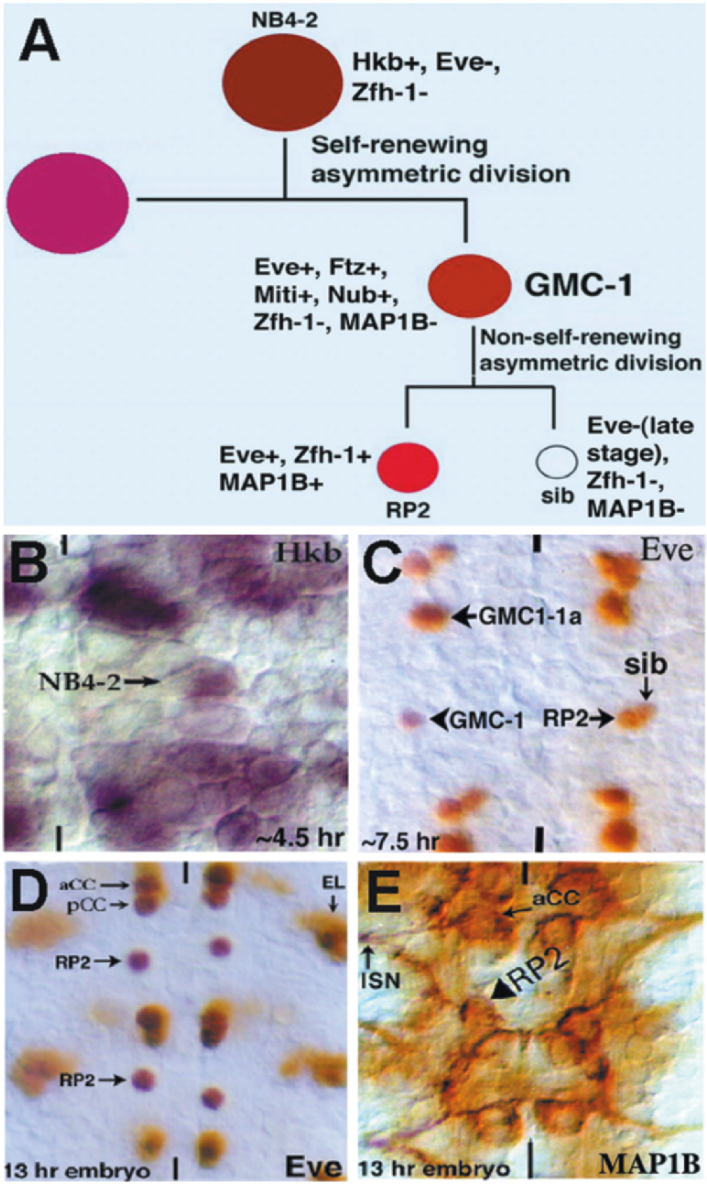

Fig. 2. **A** The NB4-2→GMC-1→RP2/sib lineage. **B** Wild type embryo (~5 h old) stained for Huckebein (Hkb). NB4-2 (*arrow*) and several other NBs have Hkb. **C** Eve stained embryo, the GMC-1 has not yet divided in the left hemisegment whereas in the right hemisegment it is divided into an RP2 and a sib. **D** Eve stained embryo, while the RP2 maintains Eve expression, sib has lost its Eve expression. **E** 22C10 (MAP1B) stained embryo, the RP2 sends out its axon to the intersegmental nerve bundle (ISN)

(Lai et al. 1991). A summary of the lineage development and marker gene expression is given in Fig. 2A.

2 Terminal Asymmetric Division

Since no self-renewal is involved in this asymmetric division, we have named this as terminal asymmetric division. As pointed out above, a GMC normally undergoes a terminal asymmetric division to generate two different neurons but it does not undergo a self-renewing asymmetric division. Recent studies indicate that the three main players namely *inscuteable (insc)*, *numb* (*nb*) and *Notch* (*N*) play a crucial role in this terminal asymmetric division (Buescher et al. 1998; Skeath and Doe 1998; Lear et al. 1999; Schuldt and Brand 1999; Wai et al. 1999; Bhat and Apsel 2004; Yedvobnick et al. 2004). Insc and Numb are cytoplasmic proteins and both are asymmetrically localized in a GMC in two opposing sides: Insc is apical and Numb is basal. Notch is a membrane protein and mediates a signaling cascade to specify a sib fate to one of the two progeny of GMC-1. The asymmetric divisions mediated by these proteins appear to be tied to the asymmetric localization of Insc and Numb in the precursor GMC and their asymmetric segregation between two daughter cells during division (Fig. 3). For instance, as summarized in Fig. 3, during the division of GMC-1 of the RP2/sib lineage, Insc localizes to the apical end of GMC-1, which in turn segregates Numb to the basal end. The cell that inherits Numb is specified as RP2 due to the ability of Numb to block Notch-signaling (which specifies sib fate to a cell), whereas the cell that does not inherit Numb is specified as sib by Notch. Thus, in *insc* mutants, both daughters of the

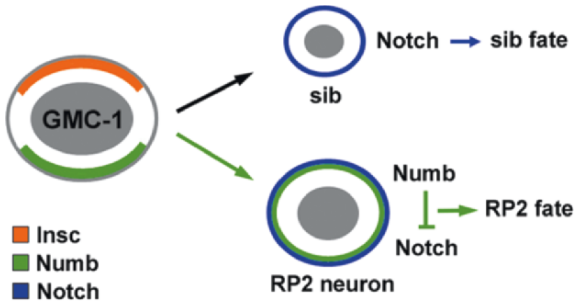

Fig. 3. Insc, Numb, Notch in generating asymmetry. Notch protein is present on the membrane of both daughter cells. In the cell that has Numb, the cleavage of intracellular domain of N (which is the activator of downstream genes) is prevented by Numb. For simplicity, Notch and its interaction with Numb is shown externally

GMC-1 adopt an RP2 fate whereas in *numb* mutants they assume a sib fate (Buescher et al. 1998; Wai et al. 1999). Both progeny of GMC-1 adopt an RP2 fate in *N* mutants (Skeath and Doe 1998; Schuldt and Brand 1999; Yedvobnick et al. 2004).

Numb localization appears to be dependent on Insc localization. In *insc* mutants, Numb is not localized (Buescher et al. 1998); in mutants that alter the localization of Insc such as in gain of function *mitimere (miti)/pdm2* or *nubbin (nub)/pdm1* (Bhat and Apsel 2004) or loss of function for *slit* (which causes an up-regulation of Miti; see Mehta and Bhat 2001), Numb is also not localized, resulting in loss of asymmetric division. While these results show a close relationship between Insc and Numb in Numb localization, it is not mechanistically clear how exactly Insc localizes Numb to the opposite end of the cell. In specifying the RP2 fate, Numb inhibits Notch signaling by mediating the endocytosis of Notch. As a result, the intracellular domain of Notch, which is what activates downstream genes and specifies a sib fate, is not cleaved and does not get translocated to the nucleus (Skeath and Doe 1998; Berdnik et al. 2002; O'Connor-Giles and Skeath 2003). Thus, when Numb is absent as in *numb* mutants, Notch is able to specify both cells to become sib cells.

Several cell-fate determinants are asymmetrically localized at the basal end of a NB during mitosis and segregate into the GMC upon division (a GMC is formed at the basal end). These include Prospero (Pros), Staufen (Stau), Miranda (Mira), Numb, Partner of Numb (Pon) and Brain tumor (Brat) (see Table 1). Basal localization of these proteins appears to be regulated by the apical localization of proteins such as Bazooka (Baz), Insc and Partner-of-Inscuteable (Pins). As a result, the basally localized proteins segregate to a GMC.

The asymmetric localization of these various proteins appears to regulate the specification of identity and/or terminal asymmetric division of progeny GMCs. These localized proteins do not regulate self-renewing asymmetric division of embryonic NBs (see Table 1). For example, asymmetrically localized Pros segregates to GMC during NB division (Knoblich et al. 1995; Spana and Doe 1995). Loss of Pros activity does not affect NB division but causes a loss of GMC identity (see Table 1). Similarly, although Insc is asymmetrically localized in NB4-2, no NB division defects are observed in *insc* loss of function mutants. Instead, it causes GMC to divide symmetrically into two identical neurons (Buescher et al. 1998; see Table 1).

In fact, none of the proteins that have been shown to asymmetrically localize in NB appear to regulate the self-renewing type of asymmetric division that the NBs undergo (Table 1). It seems likely that NBs localize determinants such as Pros to the basal end of the cell so that Pros is inherited by the GMC (a sort of parent cell contribution). Pros is a transcription factor, and becomes nuclear in a GMC and contributes to the identity specification of that GMC (Knoblich et al. 1995). This parent cell contribution principle may also be the case in those instances where the protein is localized but loss

Table 1. A partial list of genes that regulate asymmetric division

Protein	Structure	Localization pattern	Function	Mutant phenotype	References
Bazooka (Baz; DmPAR-3)	PDZ-domain	**NB:** Apical cortex	**NB:** Maintains apical-basal polarity, mitotic spindle orientation and localizes cell fate determinants to the basal cortex through directing Insc to the apical cortex. In MP2, Baz localizes Numb in an Insc-independent manner. Mediates initiation and maintenance of aPKC, Par-6 at the apical end.	**NB:** Apical localization of Par-6, aPKC and Baz (Par) are mutually dependent on each other. Mutation in any of these leads to failure in Insc localization, mis-orientation of NB division and mis-localization of basal cell determinants (Pon/Numb and Mira/Pros).	Kuchinke et al. 1998; Shober et al. 1999; Wodarz et al. 1999; Rath et al. 2002
DmPAR-6	PDZ-domain	**NB:** Apical cortex	**NB:** Establishes apical-basal polarity, present in Baz/Par-6/aPKC (PAR) complex.	**NB:** Similar phenotype as *baz* mutants.	Petronczki and Knoblich 2001
aPKC	Atypical protein kinase C	**NB:** Apical cortex	**NB:** Establishes apical-basal polarity, present in Baz/Par-6/aPKC (PAR) complex; might not be indispensable for Baz/Insc localization. Inactivates Lgl in the apical cortex.	**NB:** Similar phenotype as *baz* mutants. **Larval NB:** Failure to self-renew.	Wodarz et al. 2000; Betschinger et al. 2003, 2005; Rolls et al. 2003; Lee et al. 2006a

(*Continued*)

Table 1. A partial list of genes that regulate asymmetric division—(*Cont'd*)

Protein	Structure	Localization pattern	Function	Mutant phenotype	References
			Promotes NB self-renewal in the larval brain.		
Discs large (Dlg)	Maguk family: PDZ domain, SH3 domain, GUK domain	**NB:** Cortex, apical enrichment	**NB:** Anchors Lgl and Scrib at the cell cortex. Microtubule dependent apical cortex localization of Pins/Gαi (PAR/Insc independent during metaphase). Mitotic spindle asymmetry.	**NB:** Mira/ Pros and Pon/Numb fail to form a basal crescent during metaphase. The linkage between apical proteins and mitotic spindle is affected (division occurs in different axes). NB generates small NB and large GMC (partially penetrant). **Larval NB:** Mutation causes neoplastic overgrowth of larval brains. **GMC:** Loss of GMC identity in the embryonic CNS.	Stewart et al. 1972; Woodhouse et al. 1998; Oshiro et al. 2000; Peng et al. 2000; Albertson and Doe 2003; Bilder 2004; Siegrist and Doe 2005; Lee et al. 2006a

Scribble (Scrib)	LAP family: Leucine rich repeats (LRR), PDZ domain	**NB:** Cortex, apical enrichment	**NB:** Directs Mira to the basal cortex. Mitotic spindle asymmetry.	**NB:** Mira/ Pros and Pon/ Numb fail to form a basal crescent at metaphase. NB generates small NB and large GMC (partially penetrant).	Albertson and Doe 2003; Bilder 2004
Lethal (2) giant larvae (Lgl)	WD40 repeat motifs	**NB:** Cortex, apical enrichment	**NB:** Recruits basal determinants via inactivation of myosin II in basal cortex. Apical restriction of aPKC during the NB metaphase. Mitotic spindle asymmetry.	**NB:** Mira/Pros and Pon/ Numb fail to form a basal crescent at metaphase. NB generates small NB and large GMC (partially penetrant). **Larval NB:** Mutation causes neoplastic over-growth of larval brains. **GMC:** Loss of GMC identity in embryonic CNS. Transformation into NB in the larval brain.	Woodhouse et al. 1998; Oshiro et al. 2000; Peng et al. 2000; Albertson and Doe 2003; Betschinger et al. 2003, 2005; 2006; Bilder 2004; Lee et al. 2006a
Jaguar (Jar)	Myosin VI	**NB:** Basal region during mitosis, segregates to GMC	**NB:** Localization of Miranda to the basal cortex via vesicle transport machinery.	**NB:** Mira and its cargo protein Pros does not localize asymmetrically.	Petritsch et al. 2003

(*Continued*)

Table 1. A partial list of genes that regulate asymmetric division—(*Cont'd*)

Protein	Structure	Localization pattern	Function	Mutant phenotype	References
Zipper (Zip)	Myosin II	**NB:** Apical cortex during anaphase and shifts to the cleavage furrow during telophase	**NB:** Eliminating Mira and its binding partners from the apical cortex.	**NB:** Disruption of Mira/Pros and Pon/Numb localization.	Strand et al. 1995: Peng et al. 2000; Barros et al. 2003
Miranda (Mira)	Coiled coil protein	**NB:** Apical cortex and later during the metaphase basal cortex; segregates to GMC	**NB:** Basal localization and transport of Pros, Brat and Staufen into GMC. Physically interacts with Insc and Numb.	**NB:** Pros, Brat and Staufen fail to form a basal crescent. Mutant larval NB can cause neoplastic overgrowth when transplanted into adult host brain. **GMC:** Loss of GMC identity in the embryonic CNS. Transformation into NB in the larval brain.	Ikeshima-Kataoka et al. 1997; Shen et al. 1997; Matsuzaki et al. 1998; Shen et al. 1998; Caussinus and Gonzalez 2005; Lee et al. 2006b

Prospero (Pros)	Homeodomain transcription factor	**NB:** Apical cortex and later during the metaphase basal cortex; segregates to GMC **GMC:** Nuclear	**GMC:** Induction of GMC specific genes. Regulates glial identity of NB6-4t progeny by activating Glial cell missing (GCM).	**GMC:** Loss of GMC identity in the embryonic CNS. Transformation into neuroblast in the larval brain. Loss of glial cells derived from NB6-4t and other lineages. **Larval NB:** Mutant larval NB can cause neoplastic overgrowth when transplanted into adult host brain.	Doe et al. 1991; Knoblich et al. 1995; Spana and Doe 1995; Manning and Doe 1999; Akiyama-Oda et al. 2000; Li and Vaessin 2000; Freeman and Doe 2001; Caussinus and Gonzalez 2005; Bello et al. 2006; Betschinger et al. 2006
Brain tumor (Brat)	NHL domain, C3HC4 type RING finger	**NB:** Cytoplasmic during pro-metaphase, but localizes to the basal cortex during metaphase; segregates to GMC **GMC:** Cytoplasmic	**NB:** Maintains apico-basal polarity through apical localization of aPKC. **GMC:** Posttranscriptional down-regulation of dMyc in GMCs.	**Larval NB:** Mutation causes neoplastic overgrowth of larval brains. **GMC:** Loss of GMC identity in the embryonic CNS. Transformation into NB in the larval brain.	Woodhouse et al. 1998; Arama et al. 2000; Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b

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Table 1. A partial list of genes that regulate asymmetric division—(*Cont'd*)

Protein	Structure	Localization pattern	Function	Mutant phenotype	References
Staufen (Stau)	*ds* RNA Binding protein	**NB:** Apical cortex and later during the metaphase basal cortex; segregates to GMC	**NB:** Transport of *pros* RNA from NB to GMC.	**NB/GMC:** its function appears to be redundant.	Broadus and Doe 1997; Li et al. 1997; Broadus et al. 1998
Numb	PTB domain	**NB:** Cortex and during mitosis basal cortex; segregates to GMC **GMC:** During mitosis basal cortex; segregates to one of the sibling cells	**GMC:** Inhibits the Notch pathway in Numb inheriting daughter cells.	**GMC:** Loss of asymmetric division (e.g., GMC-1 divides into sib/sib instead of RP/sib). **NB:** Mutant larval NB can cause neoplastic overgrowth when transplanted into adult host brain.	Knoblich et al. 1995; Guo et al. 1996; Spana and Doe 1996; Buescher et al. 1998; Skeath and Doe 1998; Bhalerao et al. 2005; Caussinus and Gonzalez 2005
Partner of Numb (Pon)	Coiled coil protein	**NB:** Cortex and during mitosis basal cortex segregates to GMC	**GMC:** Efficient Numb localization.	**GMC:** Loss of asymmetric division.	Lu et al. 1998, 1999

Inscuteable (Insc)	SH3 binding domain, ankyrin repeats, cyto-skeletal attachment domain	**NB**: Apical cortex **GMC:** Cortex and during mitosis apical cortex	**NB:** Spindle orientation during NB division. Asymmetric localization of Mira/Pros and Pon/Numb, Baz and Pins during the mitosis. **GMC:** Numb localization.	**NB:** Localization of both, apical and basal determinants is disrupted. Divides in random orientation, but the asymmetric division is not affected. **GMC:** Loss of asymmetric division (e.g., GMC-1 divides to RP2/RP2 instead of RP/sib).	Kraut et al. 1996; Li et al. 1997; Buescher et al. 1998; Schaefer et al. 2000; Knoblich et al. 1999; Bhat and Apsel 2004
Partner of Inscuteable (Pins)	GoLoco domain, TPR repeats	**NB:** Apical cortex	**NB:** Maintenance of Insc localization (interdependent). Orientation of NB division. Apical restriction of aPKC during NB metaphase. Asymmetric spindle geometry and unequal cell size division (activates Gβγ through binding to Gαi).	**NB:** Affects mitotic spindle orientation, daughter cell size asymmetry (low penetrance) and Insc and Gαi localization. **Larval NB:** Failure to self renew; NB divide in random orientation. Mutant larval NB can cause neoplastic overgrowth when transplanted into adult host brain. Defective spindle orientation. mitotic Moderate mis-localization of basal determinants. **GMC:** RP2 duplication in NB4-2 lineage.	Parmentier et al. 2000; Schaefer et al. 2000, 2001; Yu et al. 2000, 2005; Cai et al. 2003; Caussinus and Gonzalez 2005; Siegrist and Doe 2005; Lee et al. 2006a

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Table 1. A partial list of genes that regulate asymmetric division—(*Cont'd*)

Protein	Structure	Localization pattern	Function	Mutant phenotype	References
Gαi	Gα subunit of heterotrimeric G-protein	**NB:** Apical cortex	**NB:** In complex with Pins Asymmetric spindle geometry and unequal cell size division.	**NB:** Affects mitotic spindle orientation, daughter cell size asymmetry (low frequency) and localization of Pins and Insc. A *pins*-like phenotype. **GMC:** RP2 duplication.	Parmentier et al. 2000; Schaefer et al. 2000; Cai et al. 2003; Yu et al. 2003; Siegrist and Doe 2005; Wang et al. 2005
Gβ13F	Gβ subunit of heterotrimeric G-protein	**NB:** Cortex	**NB:** Regulation of cell size asymmetry.	**NB:** Affects the stability and/or localization of apical components such as Pins/Gαi (strong effect) and Baz/aPKC/Insc (very weak effect). Regulates generation of daughter cells of the same size (symmetric spindle formation), from which one adopts NB fate and the other GMC fate (frequent phenotype).	Schaefer et al. 2001; Fuse et al. 2003; Yu et al. 2003; Izumi et al. 2004; Wang et al. 2005

Gγ1	Gγ subunit of heterotrimeric G-protein.	**NB:** Cortex	**NB:** Regulation of cell size asymmetry through localization of Gβ13F to the cortex.	**NB:** Gβ13F fails to localize to the cortex; leads to *Gβ13F* -like phenotype.	Fuse et al. 2003; Izumi et al. 2004
Locomotion defects (Loco)	TPR repeats, GoLoco domain, RGS domain.	**NB:** Apical cortex	**NB:** Regulation of spindle orientation and cell size asymmetry through dissociation of Gαi-GDP from Gβγ complex. GDI and GAP activity.	*pins* and *Gαi* like phenotype.	Granderath et al. 1999; Yu et al. 2005

The penetrance of the defects in mutants for various genes appears to vary from 2%~100%. We have not discussed the cell-size asymmetry in this review since mutations that disrupt cell-size asymmetry do not necessarily affect the asymmetric division. PTB domain, the phosphotyrosine binding domain; TPR, the tetratrico peptide repeat; RGS, the regulator of G protein signaling; GDI, guanine nucleotide dissociation inhibitor; GAP, GTPase activating protein

of function has no effect on parental cell division. This possibility is strengthened by the fact that these cell fate proteins are localized to the basal end, which will become a GMC when the NB divides.

3
Self-Renewing Asymmetric Division

Self-renewing asymmetric cell division provides a mechanism for generating a diverse array of cell types from a common mother cell. In the Drosophila CNS, the NBs divide asymmetrically along the apical-basal axis, giving rise to a smaller basally located GMC and a larger apically located NB. While no mutations that affect the self-renewing division of embryonic NBs have been described as yet, we will discuss below the apico-basal polarity of NBs as revealed by the apico-basal localization of several proteins.

3.1
Formation of Polarity in Embryonic NBs

The apico-basal polarity of NBs, in some sense, is already determined at the neuroepithelial stage. This polarity of epithelial cells is established by evolutionarily conserved PAR protein complex consisting of Bazooka (Baz) (homolog of *C. elegans* Par-3), Par-6 and atypical protein kinase C (aPKC). Baz is required for the apical localization of Par-6 and aPKC and vice versa. During the delamination process, a NB forms an apical stalk, which maintains the contact with surrounding epithelial cells. During this time Insc binds to the PAR protein complex via direct interaction with Baz, leading to the asymmetric localization of Insc. During mitosis, Insc recruits Pins (Partner of Insc) and Gαi (heterotrimeric G-protein αi subunit) factors and their localization becomes interdependent as well. The main role of Pins/Gαi complex is in mitotic spindle orientation and generation of cell size asymmetry during neuroblast division, while the primary role of PAR complex is to specify properly the basal cortex localization of cell fate determinants to ensure their segregation into GMC. However, the function of these two pathways in this process is partially redundant, since double mutants between members of the two pathways causes more severe defects in both localization of basal determinants and spindle orientation compared to single mutants. The results discussed above indicate that Insc connects both these pathways.

The PAR protein complex restricts the basal localization of two adaptor proteins Miranda (Mira) and Partner of Numb (PON) through cortically localized tumor suppressors Lethal giant larvae (Lgl), Discs large (Dlg), and Scribble (Scrib). Lgl appears to be the direct phosphorylation target of atypical protein kinase C (aPKC). An inactive phosphorylated form of Lgl

can no longer inactivate Myosin II, which excludes the cell fate determinants from apical cortex. It seems most likely that Dlg localizes Lgl and Srib to the cell cortex. Scrib affects Mira localization as well, but the mechanism by which this is achieved is not yet clear.

Two adaptor proteins, namely Pon and Mira, then recruit other cell fate determinant proteins that specify the identity of the daughter GMC. Pon is a binding protein for Numb. Mira is a binding partner for Prospero (Pros), Brat tumor protein (Brat) and Staufen. Staufen is an RNA binding protein and binds *pros* mRNA. Loss of function for both Pros and Brat affect GMC identity but not the asymmetric division of NBs. It is not clear if these proteins are required for the asymmetric division of GMCs since it requires a temperature sensitive mutant in these genes or supplying the gene product only during the identity specification. Following NB division to generate a GMC, Pros gets translocated to the nucleus in GMC and presumably activates GMC-specific genes; whether Pros also inactivates NB-specific genes in GMCs is not clear since the crucial experiment where expression of a nuclear form of Pros in NB and determining if this alters the behavior of NB has not been done. Brat, however, remains in the cytoplasm of GMC following NB division where it participates in the specification of GMC identity. One possibility is that it down-regulates Myc and thereby inhibits protein synthesis and cell growth. Please refer to Table 1 for more details and specific references.

The above studies make it clear that many cell-fate determinants that are asymmetrically localized to the cortex and basal ends of NB in a crescent shaped manner have been studied exhaustively. However, the asymmetric localization of these proteins in NBs appear to be primarily for the purpose of their asymmetric segregation into one of their two daughter cells (i.e., GMC) thereby leading to distinct identities between the siblings (Buescher et al. 1998; Skeath and Doe 1998; Bhat and Apsel 2004). The major questions, therefore, are what about the self-renewing asymmetric division in NBs? And which genes regulate NB divisions? Two important practical issues make it difficult to study the self-renewing asymmetric division of NBs. First, none of the proteins that have been shown to asymmetrically localize in NB appear to regulate the self-renewing type of asymmetric division of embryonic NBs (Table 1). Loss of function for mutations in these genes does not affect the self-renewing asymmetric division. Second, mutations that affect the self-renewing asymmetric division of NBs have not been identified thus far despite having an exhaustive collection of mutants and use of forward genetics in Drosophila. Since Drosophila is a powerful system for forward genetics, the very lack of identification of mutations that affect NB division pattern has been a serious drawback in studying this interesting problem using NBs.

It seems most likely that NB divisions are under the control of maternally deposited products, at least the initial few divisions. In such instances, traditional genetic screens will not identify genes that regulate self-renewing

asymmetric divisions in NBs. It is possible that the subsequent NB divisions are indeed under the control of zygotic gene products, where the traditional screens should identify these genes. However, it is not easy to score defects in the self-renewing asymmetric divisions of NBs after the initial one or two divisions, mainly due to absence of proper markers/tools to score. Thus, we may have missed zygotic mutations that affect the self-renewing asymmetric division.

The question is how can we identify mutants that affect the self-renewing asymmetric divisions of NB? The best way to address this is by isolating temperature-sensitive (ts) mutants; these mutants will eliminate the problem of maternal contribution thereby allowing one to ascertain the defects of initial NB divisions. However, isolating ts mutants is a tremendous amount of work and therefore this approach is not yet practical. The maternal and zygotic expression of RNA in RNA-interference method (RNAi) may be one way to test if loss of function for a specific gene can cause an abnormal NB division pattern. However, this strategy presents two-fold problems. One, it cannot be used as a forward genetic screen to identify genes (which is the most efficient and unbiased method of gene discovery), and two, results from RNAi can never be fully relied upon unless the results are backed up by true loss of function mutants (if one has those, the need for RNAi does not arise).

Another reason for the failure to identify any mutants that affect NB division pattern might be that the genes that regulate asymmetric division of NBs are functionally redundant. In this scenario, only a gain of function screen can help identify genes that mediate self-renewing asymmetric divisions (see section on MP2 lineage below).

In order to explore the regulation of self-renewing asymmetric division, we reasoned that GMCs have the *potential* to undergo self-renewing asymmetric divisions, but this potential is suppressed by the activity of some proteins. A loss of function for such suppressors of stem cell self-renewing asymmetric divisions would cause GMCs to undergo self-renewing stem cell type of division instead of terminal asymmetric division. Moreover, maternally deposited products often run out by the time GMCs start to divide and their function is taken over by the zygotic genes. A mutation in such a gene will cause a GMC phenotype. Thus, our idea is to identify such proteins in genetically and phenotypically amenable cells such as GMCs and try to understand how stem cells undergo a self-renewing type of asymmetric division.

3.2 Mitimere and Nubbin Regulate Self-Renewing Asymmetric Divisions

Recent studies on two POU genes provide some mechanistic insight into the problem of self-renewing asymmetric division (Bhat and Apsel 2004). It has been shown that the two POU proteins, Nubbin (Nub; also known as

Pdm1) and Mitimere (Miti; also known as Pdm2), are required for the specification of identity of GMC-1 of the RP2/sib lineage (Yang et al 1993; Bhat and Schedl 1994; Bhat et al. 1995; Yeo et al. 1995). Previous work from our laboratory has shown that these two proteins are down-regulated prior to the division of GMC-1 (Bhat and Schedl 1994; Bhat et al. 1995). Consistent with this, a brief ectopic expression of these proteins at high levels prior to GMC-1 division predominantly results in a symmetrical division of GMC-1 to generate two GMC-1s, each of which subsequently divide to generate an RP2 and a sib (Fig. 3; Yang et al 1993; Bhat et al. 1995). These results argue that a down regulation of these two POU proteins is necessary for the GMC-1 to exit from cell cycle and to undergo a terminal asymmetric division.

In line with the hypothesis that certain genes are needed to be down regulated for pluripotent cells to commit to a differentiation pathway, overexpression of *miti/nub* in GMC-1 at high levels for a prolonged period of time results predominantly in multiple self-renewals of GMC-1. Each of these divisions, however, also generates either an RP2 or a sib (Bhat and Apsel 2004). Thus, the GMC undergoes a self-renewing asymmetric division and behaving like a stem cell with the prolonged presence of the POU proteins (Fig. 4) (see Bhat and Apsel 2004 for more details).

The question is how does Miti/Nub confer self-renewing potential to a GMC? The self-renewing asymmetric division in these embryos appears to be due to a failure in the down regulation of Cyclin E (Cyc E) in late GMC-1 and its unequal distribution between two daughter cells (Bhat and Apsel 2004). An overexpression of Cyc E in GMC-1 also causes GMC-1 to undergo a similar type of self-renewing asymmetric division. Moreover, loss of function for *archipelago* (*ago*), which down regulates Cyc E via the degradation of the protein, causes a late GMC-1 to accumulate high levels of Cyc E and its unequal distribution between two daughter cells. This also causes self-renewing asymmetric division of GMC-1 (Bhat and Apsel 2004). When one of the daughter cells of a GMC acquires high levels of Cyc E, it behaves as a GMC with the ability to divide again, while the other differentiates into a neuron. These results provide insight into how cells can undergo a stem cell type of asymmetric division and maintain their multipotency.

A further question is what is the mechanism by which the identity of the committed cell specified? The clue to the above question also comes from our recent study (Bhat and Apsel 2004) and is summarized in Fig. 5. In *miti* or *nub* gain of function embryos, the differentiating progeny can be either an RP2 or a sib when a GMC-1 undergoes a self-renewing asymmetric division. This appears to be related to the localization of Insc and Numb in GMC-1. For example, Insc was found to be non-asymmetric in GMC-1 of embryos over-expressing *miti/nub*, and this effect on Insc distribution was partially penetrant (Bhat and Apsel 2004). What does the above result indicate? In a wild type GMC, Insc is localized to the apical end and Numb is

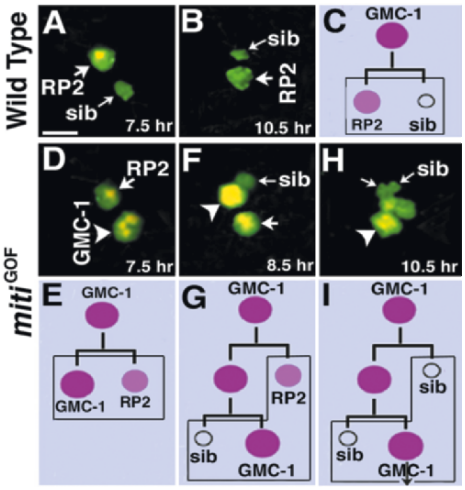

Fig. 4. A–I The GMC-1 adopts a reiterating division pattern in embryos over-expressing *miti* (*miti*^{GOF}). Embryos are stained for Eve. **C**, **E**, **G**, **I** The line drawings are interpretations of the data. **C** Pattern in **A** and **B**. **E** Pattern in **D**. **G** Pattern in **F**. **I** Pattern in **H**. The *boxed area of each line drawing* indicates the cells observed in the corresponding panels. *Scale bar*, ~7.5 μ. **A** and **B**: wild type. The GMC-1 has terminally divided to generate a smaller sib and a larger RP2 (**A**); no additional cells are formed in this lineage in later stages (**B**). **D** and **F**: *hs-miti* transgenic embryo (*miti* transgene is under the control of heat inducible heat shock 70 gene promoter) where the gene was induced at 6.45 h of development for 20 min. In 7.5 h, the GMC-1 appears to have self-renewed (larger of the two) and generated an RP2 (**D**). By 8.5 h, the GMC-1 appears to be dividing to generate a sib; a larger cell, presumably an RP2, has already been generated (**F**). **H** *miti*^{P} embryo where a multi-cell cluster of several smaller cells and one large cell is shown. Note that this (or any) multi-cell cluster in a *miti*^{P} embryo is contributed either by a single GMC-1 or by two GMC-1s formed by the occasional (and most likely the first) symmetrical division of GMC-1

localized to the basal end. Thus, only one cell gets Numb which then prevents Notch signaling from specifying sib fate to the cell; instead, it would adopt an RP2 fate. Whenever the localization of Insc is affected, the localization of Numb is also affected. This would cause both the progeny of GMC-1 inheriting Numb and adopting an RP2 fate. Thus, in those GMC-1s where the Insc localization is non-asymmetric, both the progeny have Numb. The progeny that has higher levels of Cyc E (due to the asymmetric segregation of Cyc E), however, stays as GMC-1; the cell with lower levels of Cyc E but has Numb becomes an RP2 (Fig. 5A).

In those GMC-1s where the localization of Insc is not affected, Numb segregates asymmetrically. Thus, the progeny that has low levels of Cyc E and does not inherit Numb will become a sib. The other cell that has high levels of Cyc E will stay as GMC-1 (Fig. 5B). These results, therefore,

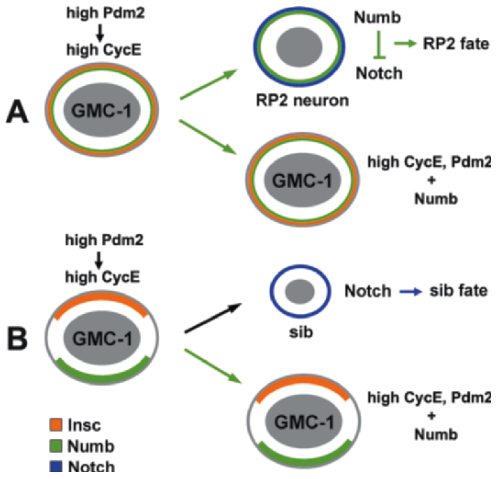

Fig. 5. A,B Self-renewing asymmetric divisions of GMC-1 that generate either an RP2 (**A**) or a sib (**B**). Asymmetric segregation of Cyc E to one of two cells maintain the cell within cell-cycle; If the determinants such as Insc and Numb are non-asymmetric, then an RP2 is generated (**A**) and if they are asymmetric, a sib is generated (**B**)

provide a mechanism by which self-renewing asymmetric divisions of precursor/stem cells can be achieved. They also provide a mechanism for how the identity of the committed cells can be specified.

The above two outcomes have striking similarity to how totipotent stem cells of the immune system can generate a stem cell of the myeloid lineage, a stem cell of the lymphoid lineage (Fig. 6), or a differentiating progeny. For example, the stem cells of the myeloid or lymphoid lineages behave like the GMC-1 in embryos over-expressing *miti* or *nub*. These stem cells will

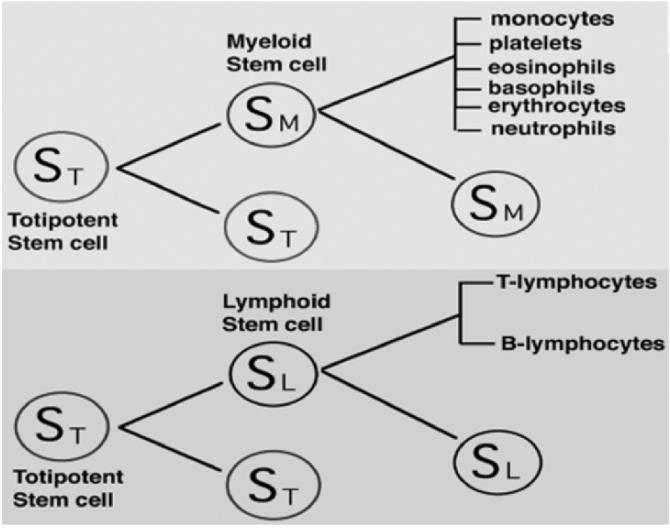

Fig. 6. Lineage elaboration in the vertebrate immune system. The totipotent stem cell self-renews but can generate either a lymphoid stem cell or a myeloid stem cell

self-renew and at the same time they will also generate progeny that are committed to different cell types. A mechanism similar to the one that causes a GMC-1 to self-renew and at the same time to generate either an RP2 or a sib can also operate in these stem cells of the immune system. This is consistent with the idea that during evolution, a pre-existing mechanism is likely to be utilized again to regulate similar processes rather than inventing a new mechanism each time.

Thus, the following scenario emerges: for cells to undergo a stem cell type of self-renewing asymmetric division, certain proteins are required to be up-regulated while others are to be down-regulated. As long as this balance is maintained, a cell will behave as a stem cell and divide by self-renewing asymmetric division. A change in this balance will lead to differentiation.

3.3 Cyclin E in NB Division

Additional evidence for the involvement of Cyc E in asymmetric division comes from studies in a neuroblast, NB6-4. NB6-4 generates segment-specific lineages (Akiyama-Oda et al. 1999). That is, in the thoracic segments, NB6-4 (NB6-4t) divides asymmetrically to produce four to six interneurons and three glial cells; in the abdominal segments, NB6-4a divides terminally and symmetrically to produce two glial cells. Cyc E appears to be sufficient to initiate the asymmetric division of this NB as well as to specify the fate of the thoracic NB6-4 progeny (Berger et al. 2005). During the first asymmetric division of NB6-4t the cell fate determinant Pros segregates exclusively to the glial precursor cell, where it initiates the transcription of *glial cell missing* (*gcm*), a glial cell fate determinant. However, in zygotic *cycE* mutants this asymmetry is disrupted and both progeny inherit Pros. As a result, a homeotic transformation of NB6-4t to NB6-4a occurs with only two Pros positive glial cells forming (Berger et al. 2005). Consistent with this, ectopic expression of *cycE* can cause the opposite transformation. A similar effect of Cyc E was observed in other lineages such as NB1-1 and NB5-4; both produce segment specific progeny (Berger et al. 2005).

Furthermore, as was observed in the GMC-1→Rp2/sib lineage (Bhat and Apsel 2004), expression of Cyc E was found to be asymmetrically maintained in the neuronal precursors of NB6-4t lineage and also in NB6-4t itself prior to its first division (Berger et al. 2005). This asymmetry function of Cyc E seems to be distinct from several other cell cycle regulators such as *string*, *dacapo*, *dE2F* or *cycA* since mutants for these genes do not exhibit any cell fate transformations within the NB6-4 lineage.

4
Embryonic Neuronal Lineages that Require Close Examination to Gain Further Insight into Asymmetric Division

4.1
MP2→dMP2/vMP2 Lineage

MP2 is a very unique NB (Fig. 7). It is formed as an NB under the control of proneural and neurogenic genes, specified by segmentation genes (Doe 1992; Bossing et al. 1996) (therefore it is not a GMC). However, it divides asymmetrically only once like a GMC into a dMP2 and a vMP2 (Fig. 7). This division is terminal. Why this NB does not undergo self-renewing asymmetric divisions like other NBs is not understood.

Given the above properties of MP2, it would be a perfect system to understand the genes that make a NB a stem cell – these genes must be repressed in MP2. Any loss of function mutation that converts an MP2 into a self-renewing asymmetrically dividing cell will be in a gene that represses these genes. One can then go from there to identify stem cell specific genes.

One of the best ways we can use this lineage to identify genes that regulate self-renewing asymmetric division is an Enhancer-Promoter (EP) screen. What is an EP screen? It is a gain of function screen. Gain-of-function mutations can be extremely useful to understand gene function and identify other genes in the pathway. Normally, gain-of function mutants have been isolated as those that are recognized by dominant phenotypes e.g. homeotic mutations in *Drosophila* (Lewis 1978) or as rare induced mutations that alter or increase gene activity (e.g. the *sevenmaker* allele of *rolled*; Brunner et al. 1994). Forced expression of genes has also been generated for specific genes (e.g. expression of *miti* or *nub*, see previous sections), or expression cloning of genes involved in embryonic

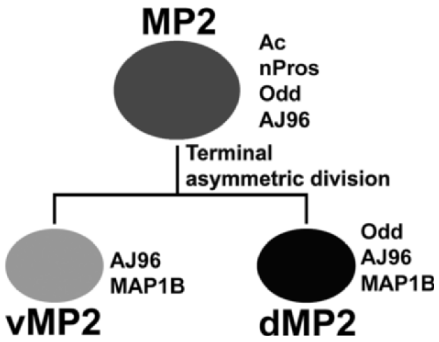

Fig. 7. The MP2→vMP2/dMP2 lineage. Ac, Achaete, nPros, nuclear Prospero, Odd, Odd-skipped, AJ96, an MP2-specific enhancer-trap line

patterning in *Xenopus* (Smith and Harland 1992) and conversion of fibroblasts to myoblasts by ectopic expression of MyoD (Davis et al. 1987). Moreover, it has been estimated that over two-thirds of all *Drosophila*, *C. elegans* and yeast genes show no obvious loss-of-function phenotypes when mutated (Sulston et al. 1992; Dujon et al. 1994), and this is most likely due to functional redundancy. Over-expression of genes can be used to identify such functionally redundant genes and determine their function.

The gain of function EP-screen is based on a combination of principles of P-element hopping, insertional mutagenesis and UASxGAL4 yeast expression system (Brand and Perrimon 1993; Rorth 1996; Rorth et al. 1998). The system is designed to allow conditional expression of genes that are randomly tagged by insertion of a 'target' P-element. The target P-element carries UAS (Upstream Activation Sequence; binding site for a yeast transcriptional activator GAL4) and a basal promoter oriented to direct expression of genomic sequences adjacent to the P-element insertion site. When combined with a source of GAL4, the P-element will direct expression of any gene that happens to lie next to its insertion site.

One can use an MP2-specific GAL4 driver [e.g. Achaete (Ac)-GAL4] to express various genes from the EP insertion lines. The Ac promoter is active in MP2 (and a small number of other lineages). These embryos can then be stained for MP2-lineage specific markers. Those lines where MP2 generates many more vMP2 and/or dMP2 will be the putative asymmetric division genes.

4.2 NB7-3 Lineage

The reason to study the NB7-3 lineage, however, arises from a related but different perspective. This NB lineage is special compared to any of the other NB lineages in the Drosophila CNS (Fig. 8). It generates only four neurons (Bossing et al. 1996) and all of them can be identified by their characteristic position, expression of marker genes and/or their neurotransmitter phenotype (see Fig. 8; Higashijima et al. 1996; Dittrich et al. 1997; Lundell and Hirsh 1998). As shown in Fig. 8, it generates only three GMCs. From two different GMCs, two identical serotonergic neurons (EW1 and EW2) are generated. NB7-3 also generates one motor neuron (GW) and one corazoninergic neuron (which produces the neuropeptide Corazonin) (Bossing et al. 1996; Higashijima et al. 1996; Dittrich et al. 1997; Lundell and Hirsh 1998). Two sibling cells are thought to undergo cell death in this lineage (Fig. 8). The fact that this NB generates two serotonergic neurons from two consecutive GMCs allow us to view its division pattern as closer to a stricter pattern of stem cell division. Mutations that affect the lineage elaboration of this NB will be, therefore, of much importance.

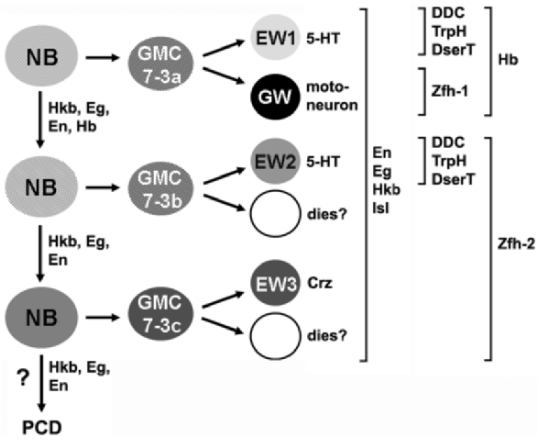

Fig. 8. NB7-3 lineages development and various markers for the lineage. 5-HT, Serotonin; Crz, Corazonin; DDC, Dopa-decarboxylase; DserT, Drosophila Serotonin transporter; Eg, Eagle; En, Engrailed; Hb, Hunchback; Isl, Islet; TrpH, Tryptophan hydroxylase; Zfh-2, Zinc-finger homeodomain 2; PCD, programmed cell death

5
Post-Embryonic CNS NBs and Self-Renewing Asymmetric Division

Recently, there has been some advance on the self-renewing asymmetric division of post-embryonic NBs in the larval brain. At the end of embryogenesis, the embryonic NBs appear to either undergo apoptosis or remain quiescent (Hartenstein and Campos-Ortega, 1984). During the first, second and also in the early third instar larval stages, NBs re-enter cell-cycle (White and Kankel 1978; Truman and Bate 1988; Prokop and Technau 1991; Ito and Hotta 1992; Datta 1995). However, most of these divisions are symmetric to increase the number of NBs in the larval brain (Ceron et al. 2001). This larval-stage expansion phase is followed by a differentiation phase during the late third instar larval and early pupal stages when most of the NBs start to divide asymmetrically and give rise to their neuronal progeny. Thus, post-embryonic NBs appear to function as classical stem cell lineages, with ~45 NBs generating 200,000 neurons.

Post-embryonic NB division has been studied in several proliferative anlagen such as the outer proliferation centre (OPC), inner proliferation centre (IPC), central brain (CB) and ventral thoracic anlagen (VN) (e.g. Tejedor et al. 1995; Park et al. 1998; Ceron et al. 2001; Dumstrei et al. 2003). Analysis of self-renewing asymmetric division of postembryonic NBs indicates that many of the players in the asymmetric divisions of precursor cells in the embryonic CNS also play a similar role in post-embryonic cells. A few new

players have also been identified. For example, several genes that are required to maintain polarity in neural cells were shown to affect self-renewing divisions of larval NBs (Parmenier et al. 2000; Rolls et al. 2003; Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006a, b). Two of them were previously shown to restrict the larval brain size. The larvae defective in *brain tumor* (*brat*) gene display an over-proliferation of neural cells in the brain in a cell autonomous manner (Gateff et al. 1993; Woodhouse et al. 1998). Brat is asymmetrically localized at the basal end along with Pros, and segregate to GMC. Mutations in either of the two cause neoplastic overgrowth of larval brain due to continued symmetric division of NBs (at the expense of GMCs). Brat is a member of evolutionary conserved tumor suppressor family (Arama et al. 2000) and affects the GMC fate by post-transcriptional down-regulation of dMyc in larval NBs (Betschinger et al. 2006).

Mutant *lgl* displays a brain overgrowth phenotype in the larval CNS (Gateff and Schneiderman 1967) as well as formation of tumors in other tissues (Bilder 2004). Lgl plays a role during the asymmetric division by affecting the localization of Mira. This in turn affects the localization of GMC fate determinant Pros (Shen et al. 1997) and Brat (Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b). Another protein that appears to play an important role in self-renewing asymmetric division of post-embryonic NB is aPKC (Rolls et al. 2003). It regulates the self-renewal of NB and its segregation to the progeny of NB ensures the identity specification of the progeny GMC (Lee et al. 2006a). When aPKC is mislocalized, NB divides symmetrically to produce two NBs. Consistent with this result, aPKC null mutants die during the second instar larval stage with reduced number of NBs. This result also suggests that aPKC may also be involved in the symmetric division of NBs. The caveat here is that the reduced number of NBs may be due to a secondary effect.

Because of difficulty from maternal deposition for the analysis of embryonic NB division using traditional genetic mutants, the focus appears to be shifting to the analysis of larval brain NBs. For example, Slack et al. (2006) using a mosaic genetic screen identified several complementation groups, which affect different stages of post-embryonic NB division. These complementation groups include new genes and new alleles of already known players. Another approach will be to isolate temperature-sensitive mutants and analyze them for NB division defects. The advantage here is that these mutations can be used to analyze both embryonic and post-embryonic NB divisions.

6 Conclusions

It is clear that we have gained considerable knowledge about the basic mechanism by which cells undergo asymmetric division. While this review discusses the asymmetric division in the CNS of Drosophila, the general

theme (if not the specifics) should be applicable to pluripotent cells that undergo asymmetric division in all metazoans. Given the power of Drosophila genetics, this organism may be the best one to further elucidate the mechanisms of asymmetric division. While the larval/pupal NBs appear attractive to study the problem, it may be that the embryonic CNS NBs are still a better system for several reasons, such as well-defined markers, de-lineated lineage elaboration patterns as opposed to larval NBs, in which case we do not have any well-defined and lineage-specific markers or tracing of any NB lineages. Once we manage to obtain mutations that affect the NB division in the embryonic CNS, we will have a better handle on the problem. It is also important as next step to take the genes identified in Drosophila and determine their role in the asymmetric division of pluripotent cells in vertebrates. Given the conservation of genes and their function, it is most likely that these genes identified in Drosophila will also play the same or similar role in vertebrates. Studying evolutionary aspect of the asymmetric division such as when in time cells acquired the ability to undergo asymmetric division, which one evolved earlier, the one with self-renewal or the one without self-renewal, will also represent an exciting area of research.

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References

Akiyama-Oda Y, Hosoya T, Hotta Y (1999) Asymmetric cell division of thoracic neuroblast 6-4 to bifurcate glial and neuronal lineage in Drosophila. Development 126:1967–1974

Akiyama-Oda Y, Hotta Y, Tsukita S, Oda H (2000) Mechanism of glia-neuron cell-fate switch in the *Drosophila* thoracic neuroblast 6-4 lineage. Development 127:3513–3522

Albertson R, Doe CQ (2003) Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. Nat Cell Biol 5:166–1670

Arama E, Dickman D, Kimchie Z, Shearn A, Lev Z (2000) Mutations in the beta-propeller domain of the *Drosophila* brain tumor (*brat*) protein induce neoplasm in the larval brain. Oncogene 19:3706–3716

Barros CS, Phelps CB, Brand AH (2003) *Drosophila* nonmuscle myosin II promotes the asymmetric segregation of cell fate determinants by cortical exclusion rather than active transport. Dev Cell 5:829–840

Bate CM (1976) Embryogenesis of an insect nervous system. I. A map of the thoracic and abdominal neuroblasts in *Locusta migratoria*. J Embryol Exp Morphol 35:107–123

Bello B, Reichert H, Hirth F (2006) The *brain tumor* gene negatively regulates neural progenitor cell proliferation in the larval central brain of *Drosophila*. Development 133:2639–2648

Berdnik D, Torok T, Gonzalez-Gaitan M, Knoblich JA (2002) The endocytic protein alpha-Adaptin is required for *numb*-mediated asymmetric cell division in *Drosophila*. Dev Cell 3:221–231

Berger C, Pallavi SK, Prasad M, Shashidhara LS, Technau GM (2005) A critical role for cyclin E in cell fate determination in the central nervous system of *Drosophila melanogaster*. Nat Cell Biol 7:56–62

Betschinger J, Mechtler K, Knoblich JA (2003) The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature 422:326–330

Betschinger J, Eisenhaber F, Knoblich JA (2005) Phosphorylation-induced autoinhibition regulates the cytoskeletal protein Lethal (2) giant larvae. Curr Biol 15:276–282

Betschinger J, Mechtler K, Knoblich JA (2006) Asymmetric segregation of the tumor suppressor *brat* regulates self-renewal in *Drosophila* neural stem cells. Cell 124:1241–1253

Bhalerao S, Berdnik D, Torok T, Knoblich JA (2005) Localization-dependent and -independent roles of numb contribute to cell-fate specification in *Drosophila*. Curr Biol 15:1583–1590

Bhat KM (1999) Segment polarity genes in neuroblast formation and identity specification during *Drosophila* neurogenesis. Bioessays 21:472–485

Bhat KM, Apsel N (2004) A mechanism for the self-renewing asymmetric division of neural precursor cells in the *Drosophila* CNS. Development 131:1123–1134

Bhat KM, Schedl P (1994) The *Drosophila miti-mere* gene, a member of the POU family, is required for the specification of the RP2/sibling lineage during neurogenesis. Development 120:1483–1501

Bhat KM, Poole SJ, Schedl P (1995) The *miti-mere* and *pdm1* genes collaborate during specification of the RP2/sib lineage in *Drosophila* neurogenesis. Mol Cell Biol 15:4052–4063

Bhat KM, McBurney W, Hamada H (1988) Functional cloning of mouse chromosomal loci specifically active in embryonal carcinoma stem cells. Mol Cell Biol 8:3251–3259

Bilder D (2004) Epithelial polarity and proliferation control: links from the *Drosophila* neoplastic tumor suppressors. Genes Dev 18:1909–1925

Bossing T, Udolph G, Doe CQ, Technau GM (1996) The embryonic central nervous system lineages of *Drosophila melanogaster*. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev Biol 179:41–64

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415

Broadus J, Doe CQ (1997) Extrinsic cues, intrinsic cues and microfilaments regulate asymmetric protein localization in *Drosophila* neuroblasts. Curr Biol 7:827–835

Broadus J, Fuerstenberg S, Doe CQ (1998) Staufen-dependent localization of *prospero* mRNA contributes to neuroblast daughter-cell fate. Nature 391:792–795

Brunner D, Oellers N, Szabad J, Biggs WH III, Zipursky SL, Hafen E (1994) A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell 76:875–888

Buescher M, Yeo SL, Udolph G, Zavortink M, Yang X, Tear G, Chia W (1998) Binary sibling neuronal cell fate decisions in the *Drosophila* embryonic central nervous system are nonstochastic and require *inscuteable*- mediated asymmetry of ganglion mother cells. Genes Dev 12:1858–1870

Cai Y, Yu F, Lin S, Chia W, Yang X (2003) Apical complex genes control mitotic spindle geometry and relative size of daughter cells in *Drosophila* neuroblast and pI asymmetric divisions. Cell 112:51–62

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–66

Campos-Ortega JA, Jan YN (1991) Genetic and molecular bases of neurogenesis in *Drosophila melanogaster*. Annu Rev Neurosci 14:399–420

Caussinus E, Gonzalez C (2005) Induction of tumor growth by altered stem-cell asymmetric division in *Drosophila melanogaster*. Nat Genet 37:1125–1129

Ceron J, Gonzalez C, Tejedor FJ (2001) Patterns of cell division and expression of asymmetric cell fate determinants in postembryonic neuroblast lineages of *Drosophila*. Dev Biol 230:125–138

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113:643–655

Collarini EJ, Kuhn R, Marshall CJ, Monuki ES, Lemke G, Richardson WD (1992) Down-regulation of the POU transcription factor SCIP is an early event in oligodendrocyte differentiation in vivo. Development 116:193–200

Cowan CA, Atienza J, Melton DA, Eggan K (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309:1369–1373

Datta S (1995) Control of proliferation activation in quiescent neuroblasts of the *Drosophila* central nervous system. Development 121:1173–1182

Davis RL, Weintraub H, Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000

Dittrich R, Bossing T, Gould AP, Technau GM, Urban J (1997) The differentiation of the serotonergic neurons in the *Drosophila* ventral nerve cord depends on the combined function of zinc finger proteins Eagle and Huckebein. Development 124:2515–2525

Doe CQ (1992) Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. Development 116:855–863

Doe CQ, Chu-LaGraff Q, Wright DM, Scott MP (1991) The *prospero* gene specifies cell fates in the *Drosophila* central nervous system. Cell 65:451–464

Dujon B, Alexandraki D, Andre B, Ansorge W, Baladron V, Ballesta JP, Banrevi A, Bolle PA, Bolotin-Fukuhara M, Bossier P et al. (1994) Complete DNA sequence of yeast chromosome XI. Nature 369:371–378

Dumstrei K, Wang F, Nassif C, Hartenstein V (2003) Early development of the *Drosophila* brain: V. Pattern of postembryonic neuronal lineages expressing DE-cadherin. J Comp Neurol 455:451–462

Freeman MR, Doe CQ (2001) Asymmetric Prospero localization is required to generate mixed neuronal/glial lineages in the *Drosophila* CNS. Development 128:4103–4112

Fujita SC, Zipursky SL, Benzer S, Ferrus A, Shotwell SL (1982) Monoclonal antibodies against the *Drosophila* nervous system. Proc Natl Acad Sci 79:7929–7933

Fuse N, Hisata K, Katzen AL, Matsuzaki F (2003) Heterotrimeric G proteins regulate daughter cell size asymmetry in *Drosophila* neuroblast divisions. Curr Biol 13:947–954

Gateff E, Schneiderman HA (1967) Developmental studies of a new mutant of *Drosophila melanogaster*: Lethal malignant brain tumor (*l(2)gl4*). Am Zool 7:760

Gateff E, Loffler T, Wismar J (1993) A temperature-sensitive brain tumor suppressor mutation of *Drosophila melanogaster*: developmental studies and molecular localization of the gene. Mech Dev 41:15–31

Golan-Mashiach M, Dazard JE, Gerecht-Nir S, Amariglio N, Fisher T, Jacob-Hirsch J, Bielorai B, Osenberg S, Barad O, Getz G, Toren A, Rechavi G, Itskovitz-Eldor J, Domany E, Givol D (2004) Design principle of gene expression used by human stem cells: implication for pluripotency. FASEB J 19:147–149

Granderath S, Stollewerk A, Greig S, Goodman CS, O'Kane CJ, Klambt C (1999) *loco* encodes an RGS protein required for *Drosophila* glial differentiation. Development 126:1781–1791

Guo M, Jan LY, Jan YN (1996) Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17:27–41

Hall PA, Watt FM (1989) Stem cells: the generation and maintenance of cellular diversity. Development 106:619–633

Hartenstein V, Campos-Ortega JA (1984) Early neurogenesis in wildtype *Drosophila melanogaster*. Wilhelm Roux's Arch Dev Biol 193:308–325

Higashijima S, Shishido E, Matsuzaki M, Saigo K (1996) *eagle*, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the *Drosophila* CNS. Development 122:527–536

Hochedlinger K, Jaenisch R (2006) Nuclear reprogramming and pluripotency. Nature 441:1061–1067

Ikeshima-Kataoka H, Skeath JB, Nabeshima Y, Doe CQ, Matsuzaki F (1997) Miranda directs Prospero to a daughter cell during *Drosophila* asymmetric divisions. Nature 390:625–629

Ito K, Hotta Y (1992) Proliferation pattern of postembryonic neuroblasts in the brain of *Drosophila melanogaster*. Dev Biol 149:134–148

Izumi Y, Ohta N, Itoh-Furuya A, Fuse N, Matsuzaki F (2004) Differential functions of G protein and Baz-aPKC signaling pathways in *Drosophila* neuroblast asymmetric division. J Cell Biol 164:729–738

Knoblich JA, Jan LY, Jan YN (1995) Asymmetric segregation of Numb and Prospero during cell division. Nature 377:624–627

Knoblich JA, Jan LY, Jan YN (1999) Deletion analysis of the *Drosophila* Inscuteable protein reveals domains for cortical localization and asymmetric localization. Curr Biol 9:155–158

Kraut R, Chia W, Jan LY, Jan YN, Knoblich JA (1996) Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*. Nature 383:50–55

Kuchinke U, Grawe F, Knust E (1998) Control of spindle orientation in *Drosophila* by the Par-3-related PDZ-domain protein Bazooka. Curr Biol 8:1357–1365

Lai Z, Fortini ME, Rubin GM (1991) The embryonic expression pattern of *zfh1* and *zfh2*, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. Mech Dev 34:123–134

Lear BC, Skeath JB, Patel NH (1999) Neural cell fate in *rca1* and *cycA* mutants: the roles of intrinsic and extrinsic factors in asymmetric division in the *Drosophila* central nervous system. Mech Dev 88:207–219

Lee CY, Robinson KJ, Doe CQ (2006a) Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature 439:594–598

Lee CY, Wilkinson BD, Siegrist SE, Wharton RP, Doe CQ (2006b) Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. Dev Cell 10:441–449

Lewis EB (1978) A gene complex controlling segmentation in *Drosophila*. Nature 276:565–570

Li L, Vaessin H (2000) Pan-neural Prospero terminates cell proliferation during *Drosophila* neurogenesis. Genes Dev 14:147–151

Li P, Yang X, Wasser M, Cai Y, Chia W (1997) Inscuteable and Staufen mediate asymmetric localization and segregation of *prospero* RNA during *Drosophila* neuroblast cell divisions. Cell 90:437–447

Littlefield JW, Felix JS (1982) Rescue of terminally differentiating teratocarcinoma cells by fusion to undifferentiated parental cells. Somatic Cell Genet 8:743–757

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38:431–440

Lu B, Rothenberg M, Jan LY, Jan YN (1998) Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in *Drosophila* neural and muscle progenitors. Cell 95:225–235

Lu B, Ackerman L, Jan LY, Jan YN (1999) Modes of protein movement that lead to the asymmetric localization of partner of Numb during *Drosophila* neuroblast division. Mol Cell 4:883–891

Lundell MJ, Hirsh J (1998) *eagle* is required for the specification of serotonin neurons and other neuroblast 7-3 progeny in the Drosophila CNS. Development 125:463–472

Manning L, Doe CQ (1999) Prospero distinguishes sibling cell fate without asymmetric localization in the *Drosophila* adult external sense organ lineage. Development 126:2063–2071

Matsuzaki F, Ohshiro T, Ikeshima-Kataoka H, Izumi H (1998) miranda localizes staufen and prospero asymmetrically in mitotic neuroblasts and epithelial cells in early *Drosophila* embryogenesis. Development 125:4089–4098

Mehta B, Bhat KM (2001) Slit signaling promotes the terminal asymmetric division of neural precursor cells in the *Drosophila* CNS. Development 128:3161–3168

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642

O'Connor-Giles KM, Skeath JB (2003) Numb inhibits membrane localization of Sanpodo, a four-pass transmembrane protein, to promote asymmetric divisions in *Drosophila*. Dev Cell 5:231–243

Ohshiro T, Yagami T, Zhang C, Matsuzaki F (2000) Role of cortical tumour-suppressor proteins in asymmetric division of *Drosophila* neuroblast. Nature 408:593–596

Park Y, Fujioka M, Jaynes JB, Datta S (1998) *Drosophila* homeobox gene *eve* enhances *trol*, an activator of neuroblast proliferation in the larval CNS. Dev Genet 23:247–257

Parmentier ML, Woods D, Greig S, Phan PG, Radovic A, Bryant P, O'Kane CJ (2000) Rapsynoid/partner of inscuteable controls asymmetric division of larval neuroblasts in *Drosophila*. J Neurosci 20:RC84

Peng CY, Manning L, Albertson R, Doe CQ (2000) The tumour-suppressor genes *lgl* and *dlg* regulate basal protein targeting in *Drosophila* neuroblasts. Nature 408:596–600

Petritsch C, Tavosanis G, Turck CW, Jan LY, Jan YN (2003) The *Drosophila* myosin VI Jaguar is required for basal protein targeting and correct spindle orientation in mitotic neuroblasts. Dev Cell 4:273–281

Petronczki M, Knoblich JA (2001) DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. Nat Cell Biol 3:43–49

Prokop A, Technau GM (1991) The origin of postembryonic neuroblasts in the ventral nerve cord of *Drosophila melanogaster*. Development 111:79–88

Rath P, Lin S, Udolph G, Cai Y, Yang X, Chia W (2002) Inscuteable-independent apicobasally oriented asymmetric divisions in the *Drosophila* embryonic CNS. EMBO 3:660–665

Rolls MM, Albertson R, Shih HP, Lee CY, Doe CQ (2003) *Drosophila* aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. J Cell Biol 163:1089–1098

Rorth P (1996) A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. Proc Natl Acad Sci USA 93:12418–12422

Rorth P, Szabo K, Bailey A, Laverty T, Rehm J, Rubin GM, Weigmann K, Milan M, Benes V, Ansorge W, Cohen SM (1998) Systematic gain-of-function genetics in *Drosophila*. Development 125:1049–1057

Rosner MH, Vigano MA, Ozato K, Timmons PM, Poirier F, Rigby PWJ, Staudt LM (1990) A POU-domain transcription factor in early stem cells and germ cells of mammalian embryo. Nature 345:686–692

Schaefer M, Shevchenko A, Shevchenko A, Knoblich JA (2000) A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in *Drosophila*. Curr Biol 10:353–362

Schaefer M, Petronczki M, Dorner D, Forte M, Knoblich JA (2001) Heterotrimeric G proteins direct two modes of asymmetric cell division in the *Drosophila* nervous system. Cell 107:183–194

Schmidt H, Rickert C, Bossing T, Vef O, Urban J, Technau GM (1997) The embryonic central nervous system lineages of *Drosophila melanogaster*. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. Dev Biol 189:186–204

Schober M, Schaefer M, Knoblich JA (1999) Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. Nature 402:548–551

Schuldt AJ, Brand AH (1999) Mastermind acts downstream of Notch to specify neuronal cell fates in the *Drosophila* central nervous system. Dev Biol 205:287–295

Shen CP, Jan LY, Jan YN (1997) Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila*. Cell 90:449–458

Shen CP, Knoblich JA, Chan YM, Jiang MM, Jan LY, Jan YN (1998) Miranda as a multidomain adapter linking apically localized Inscuteable and basally localized Staufen and Prospero during asymmetric cell division in Drosophila. Genes Dev 12:1837–1846

Siegrist SE, Doe CQ (2005) Microtubule-induced Pins/Galphai cortical polarity in *Drosophila* neuroblasts. Cell 123:1323–1335

Skeath JB, Doe CQ (1998) Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the *Drosophila* CNS. Development 125:1857–1865

Slack C, Somers WG, Sousa-Nunes R, Chia W, Overton PM (2006) A mosaic genetic screen for novel mutations affecting *Drosophila* neuroblast divisions. BMC Genet 7:33

Smith WC, Harland RM (1992) Expression cloning of *noggin*, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. Cell 70:829–840

Spana EP, Doe CQ (1995) The *prospero* transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. Development 121:3187–3195

Spana EP, Doe CQ (1996) Numb antagonizes Notch signaling to specify sibling neuron cell fates. Neuron 17:21–26

Stewart M, Murphy C, Fristrom JW (1972) The recovery and preliminary characterization of X chromosome mutants affecting imaginal discs of *Drosophila melanogaster*. Dev Biol 27:71–83

Strand D, Unger S, Corvi R, Hartenstein K, Schenkel H, Kalme A, Merdes G, Neumann B, Krieg-Schneider F, Coy JF et al. (1995) A human homologue of the *Drosophila* tumour suppressor gene *l(2)gl* maps to 17p11.2-12 and codes for a cytoskeletal protein that associates with nonmuscle myosin II heavy chain. Oncogene 11:291–301

Sulston J, Du Z, Thomas K, Wilson R, Hillier L, Staden R, Halloran N, Green P, Thierry-Mieg J, Qiu L et al. (1992) The *C. elegans* genome sequencing project: a beginning. Nature 356:37–41

Szutorisz H, Dillon N (2005) The epigenetic basis for embryonic stem cell pluripotency. Bioessays 27:1286–1293

Tanaka TS, Kunath T, Kimber WL, Jaradat SA, Stagg C, Usuda M, Yokota T, Niwa H, Rossant J, Ko MS (2002) Gene expression profiling of embryo-derived stem cells reveals candidate genes associated with pluripotency and lineage specificity. Genome Res 12:1921–1928

Tejedor F, Zhu XR, Kaltenbach E, Ackermann A, Baumann A, Canal I, Heisenberg M, Fischbach KF, Pongs O (1995) *minibrain*: a new protein kinase family involved in postembryonic neurogenesis in *Drosophila*. Neuron 14:287–301

Thomas JB, Bastiani MJ, Bate M, Goodman CS (1984) From grasshopper to *Drosophila*: a common plan for neuronal development. Nature 310:203–207

Truman JW, Bate M (1988) Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. Dev Biol 125:145–157

Wai P, Truong B, Bhat KM (1999) Cell division genes promote asymmetric interaction between Numb and Notch in the *Drosophila* CNS. Development 126:2759–2770

Wang H, Ng KH, Qian H, Siderovski DP, Chia W, Yu F (2005) Ric-8 controls *Drosophila* neural progenitor asymmetric division by regulating heterotrimeric G proteins. Nat Cell Biol 7:1091–1098

White K, Kankel DR (1978) Patterns of cell division and cell movement in the formation of the imaginal nervous system in *Drosophila melanogaster*. Dev Biol 65:296–321

Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336:684–687

Wodarz A, Ramrath A, Kuchinke U, Knust E (1999) Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. Nature 402:544–547

Wodarz A, Ramrath A, Grimm A, Knust E (2000) *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. J Cell Biol 150:1361–1374

Woodhouse E, Hersperger E, Shearn A (1998) Growth, metastasis, and invasiveness of *Drosophila* tumors caused by mutations in specific tumor suppressor genes. Dev Genes Evol 207:542–550

Yang X, Yeo S, Dick T, Chia W (1993) The role of a *Drosophila* POU homeo domain gene in the specification of neural precursor cell identity in the developing embryonic central nervous system. Genes Dev 7:504–516

Yedvobnick B, Kumar A, Choudhury P, Opraseuth J, Mortimer N, Bhat KM (2004) The asymmetric division function of Mastermind is separable and distinct from its neurogenic function during *Drosophila* neurogenesis. Genetics 166:1281–1289

Yeo SL, Lloyd A, Kozak K, Dinh A, Dick T, Yang X, Sakonju S, Chia W (1995) On the functional overlap between two *Drosophila* POU homeo domain genes and the cell fate specification of a CNS neural precursor. Genes Dev 9:1223–1236

Ying Q-L, Nichols J, Chambers I, Smith A (2003) BMP Induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115:281–292

Yu F, Morin X, Cai Y, Yang X, Chia W (2000) Analysis of *partner of inscuteable*, a novel player of *Drosophila* asymmetric divisions, reveals two distinct steps in inscuteable apical localization. Cell 100:399–409

Yu F, Cai Y, Kaushik R, Yang X, Chia W (2003) Distinct roles of Galphai and Gbeta13F subunits of the heterotrimeric G protein complex in the mediation of *Drosophila* neuroblast asymmetric divisions. J Cell Biol 162:623–633

Yu F, Wang H, Qian H, Kaushik R, Bownes M, Yang X, Chia W (2005) Locomotion defects, together with Pins, regulates heterotrimeric G-protein signaling during *Drosophila* neuroblast asymmetric divisions. Genes Dev 19:1341–1353

Cell Commitment by Asymmetric Division and Immune System Involvement

Antonin Bukovsky

Abstract

Asymmetric division is a fundamental means of generating cell diversity and may involve extrinsic or intrinsic factors. Here we review observations on symmetric and asymmetric expression of estrogen receptor alpha (ERA) and beta (ERB) during regeneration of trophoblast cells in human placenta and possibly other estrogen-responsive cell types. This is a type of differentiation from committed progenitor cells. Asymmetric segregation of ERA in dividing villous cytotrophoblast cells, accompanied by appearance of ERB in differentiating daughter cells and resulting syncytiotrophoblast, suggests a unique role of estrogen receptors in asymmetric division of estrogen responsive cells.

We also review observations on asymmetric division of ovarian surface epithelium (OSE) stem cells resulting in formation of germ cells differentiating into oocytes in fetal and adult human ovaries. Besides germ cells, the OSE stem cells also give rise to primitive ovarian granulosa (follicular) cells, which are required for the formation of new primary follicles and preservation and differentiation of oocytes. This dual potential of OSE stem cells (germ or granulosa cells) is a type of differentiation from uncommitted and possibly totipotent adult stem cells. A possible role of immune system related cells (monocyte-derived cells and T lymphocytes -cellular signaling) and hormones in the stimulation of OSE differentiation toward germ cells by asymmetric division, and in the continuation of ovarian follicular renewal during prime reproductive period in human females is also reviewed. Follicular renewal ceases after prime reproductive period, possibly due to the diminution of cellular signaling required for asymmetric division of OSE stem cells into the germ cells. The primary follicles persisting in premenopausal ovaries appear to accumulate genetic alterations, a cause of exponentially growing chromosomal abnormalities in the progeny of mothers between 38 years of age and menopause.

1 Introduction

The type of progenitor cell division (symmetric vs asymmetric) determines the fate of daughter cells. The neuronal progenitor cells have been reported to produce the following three types of divisions: 1) symmetric proliferative,

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resulting in two progenitor daughter cells, 2) asymmetric mono-differentiative, resulting in progenitor and differentiating daughters, and 3) symmetric differentiative, where the mother progenitor cell produces two differentiating daughters (Huttner and Kosodo 2005). These pathways of cell divisions are relatively simple, because mother progenitor cells are already committed toward neuronal differentiation.

A more complex situation exists in the lineage commitment of mammalian bone marrow hematopoietic stem cells, where a single progenitor cell type forms six distinct cell types, such as neutrophils, monocytes, erythroblasts, megakaryocytes, and B- and T-lymphoid lineages. Whereas the mechanism of lymphoid cell differentiation from bone marrow progenitors remains unclear, it appears that the progenitor mother cells give rise to the neutrophil/monocyte or erythroblast/megakaryocyte daughter cells by the first asymmetric division, which then produce granddaughter cells with final commitment by second asymmetric division (Takano et al. 2004). Nevertheless, it remains unclear why some mother stem cells persist in a quiescent state, some decide to renew themselves by symmetric division, and some decide to activate certain genes during asymmetric division and produce proteins characteristic for differentiating daughter cells.

Asymmetric division is a fundamental means of generating cell diversity and may involve extrinsic or intrinsic factors. With extrinsic factors, daughter cells are equivalent after separation but adopt different fates due to their interactions with the environment. With intrinsic factors, unequal amounts of cell-fate determinants are distributed into the two cells prior to their separation (Jan and Jan 1998). Among the intrinsic factors underlying asymmetric division, the interactions of the Numb differentiation-associated protein with Notch proliferation-associated signaling is the model most widely studied, ranging from bacteria, yeast, and Drosophila to mammals (Shen and Temple 2002). Mammalian cells known to exhibit asymmetric division include diverse cell types such as neuronal cells, thymocytes, satellite cells involved in myogenesis, hematopoietic stem cells, hair follicle cells, and the list is growing (Jan and Jan 1998; Shen and Temple 2002; Conboy and Rando 2002; French et al. 2002; Takano et al. 2004; Sugiyama-Nakagiri et al. 2006). We also described asymmetric division of placental villous trophoblast cells, associated with changes in estrogen receptor alpha (ERA) and beta (ERB) expression (Bukovsky et al. 2003a) and ovarian surface epithelium cells (OSE) with changes in expression of major histocompatibility complex class I antigens (MHC-I), cytokeratin, and meiotically expressed protein during oogenesis in fetal and adult human ovaries (Bukovsky et al. 2004, 2005a).

Here we review observations on symmetric and asymmetric expression of ERA and ERB during regeneration of trophoblast cells in human placenta (Bukovsky et al. 2003a) and possibly other estrogen-responsive cell types. This is a type of differentiation from committed progenitor cells. We

also review observations on asymmetric division of OSE cells resulting in formation of germ cells differentiating into oocytes in fetal and adult human ovaries (Bukovsky et al. 1995, 2004, 2005a). Besides germ cells, the OSE cells also give rise to primitive ovarian granulosa (follicular) cells, which are required for the formation of new primary follicles and preservation of oocytes. This dual potential of OSE cells (germ or granulosa cells) is a type of differentiation from uncommitted and possibly totipotent (Bukovsky et al. 2005b) stem cells.

A possible role of monocyte-derived cells (MDC) and T lymphocytes in the stimulation of OSE differentiation toward germ cells by asymmetric division, and in the continuation of ovarian follicular renewal during prime reproductive period in human females (Bukovsky et al. 1995, 2005a; Bukovsky 2006a) is also reviewed.

2
Asymmetric Division of Estrogen Responsive Cells

Estrogenic steroids regulate cellular function in a wide variety of tissues. During human pregnancy, the production of 17-beta-estradiol (E2) rises steadily to eighty fold at term (Lobo 1997), and estrogens influence various aspects of placental function in humans and non-human primates (Shanker and Rao 1997; Pepe and Albrecht 1999). The human placenta has been found specifically to bind estrogens (Younes et al. 1981; Kneussl et al. 1982), and we demonstrated the expression of ERA protein in human placenta. Its immunoreactivity was confined to villous cytotrophoblast (CT), vascular pericytes, and amniotic fibroblasts, and, in vitro, the E2 stimulated development of large syncytiotrophoblast aggregates (Bukovsky et al. 2003b).

2.1
Human Placental Trophoblast

Human placenta consists of villous units containing stromal tissue with fetal vessels and CT cells. The latter differentiate into syncytiotrophoblast (ST), the syncytial layer with multiple nuclei, which covers villous structures and enables exchange of nutritives and gases between maternal blood and fetal microcirculation (Castellucci and Kaufmann 1995).

Villous CT cells are a type of progenitor cell which divides during pregnancy including the term placenta (Arnholdt et al. 1991; Esterman et al. 1997; Yamada et al. 2001). However, if the division is symmetric, i.e., resulting in two identical daughter cells, both daughters should either proliferate or differentiate. The former situation might result in placental site

trophoblastic tumor or choriocarcinoma (Shih and Kurman 1998), the latter in the loss of the ability of the villous trophoblast to proliferate. However, ten years ago, biologists began to gain insight into the cellular mechanisms by which a cell divides into two cells of different developmental potentials, a process known as asymmetric division.

2.1.1 Asymmetric Division of Villous CT Cells and ERA Segregation

Villous CT cells lie under the ST, where they occasionally divide and contribute to the ST regeneration. This enables the placenta to grow during pregnancy and be preserved in the functional state. Dividing villous CT cells showed asymmetric segregation of ERA. Prior to separation, the cell nuclei more distant from ST exhibited high ERA and no ERB, while cell nuclei associated with ST showed diminution of ERA and appearance of ERB expression (Bukovsky et al. 2003a).

Our ERA and ERB dual color immunohistochemistry experiments showed occasional symmetric division of CT resulting in two identical daughter cells with strong nuclear ERA expression. Such dividing cells exhibited parallel long axis to the plane of ST and relatively wide ST (approximately 8 μm). However, asymmetric division showed perpendicular orientation of dividing cells toward the ST layer. The ERA expression persisted in the daughter cell more distant from the ST. The differentiating daughter cell showed coexpression of both ERs during early stages of differentiation. Such differentiation of CT was associated with very narrow (approximately 2 μm) ST, which may need a complementation by additional CT cells to function. However, the progenitor and differentiating daughter cells showed in vivo differences in nuclear ER immunoreactivity prior to their complete separation. While the progenitor daughter cell persisting in villous stroma showed high nuclear ERA, the differentiating daughter cell associated with ST showed marked diminution of ERA immunoreactivity and an increase of differentiation-associated ERB protein.

These observations parallel studies that postmitotic neuronal cells may exhibit asymmetric distribution of the differentiation-associated Numb protein prior to their separation. When the progenitor cells undergo asymmetric division (20% in neuronal tissue cell culture) they produce neuronal daughter cells to which Numb protein segregates preferentially, and such daughter cells show an enhanced differentiation of neuronal processes (Shen et al. 2002). Interestingly, Numb is firstly associated with one pole of the plasma membrane of a dividing cell, and subsequently with one of the two resulting nuclei (Jan and Jan 1998; Shen et al. 2002). Our study indicates that preservation of nuclear ERA in one of two postmitotic cells is associated with the ability of CT to replicate and diminution of ERA and appearance of nuclear ERB dictates a generation of a daughter cell that is committed to differentiate (Fig. 1).

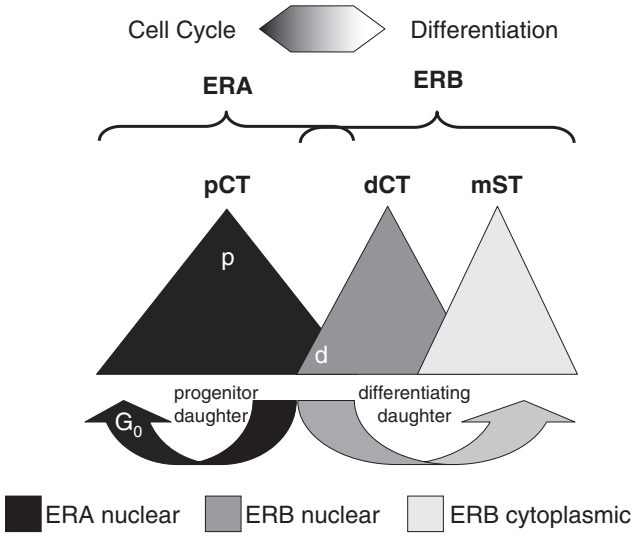

Fig. 1. Simplified schematic view of ERA and ERB differential expression during asymmetric division and differentiation of villous trophoblast cells in vivo (postmitotic cell surface and cytoplasmic ERA expression (Bukovsky et al. 2003b) not included). pCT, parental CT; dCT, differentiating CT; mST, mature ST; p, postmitotic parental cells; d, postmitotic daughter cells. Details in text. Adapted from Bukovsky et al. (2003a) – A.B. copyright

The model presented in Fig. 1 can be used to explain, at least in part, the role of ERs in proliferation and differentiation of other estrogen responsive cell types. However, it has to be considered that ERs are certainly not the only proteins which are involved in decisions of estrogen responsive cells to proliferate or differentiate. At least cell cycle related proteins, either suppressors or facilitators, and growth factors and cytokines produced by stromal cells (Cooke et al. 1997; Kanai et al. 1998; Buchanan et al. 1998; Diel 2002; Klotz et al. 2002) should be considered in addition to the promitotic effect of E2 mediated via ERA. Figure 1 may not be applicable for transformed cells with abnormal ER variants, or cells exhibiting only one type of ER. For example, expression of ERB may only indicate that differentiation but not proliferation is estrogen-dependent.

Former studies also indicated that the decision between symmetric and asymmetric division in vivo depends on the mitotic spindle orientation. During fly neurogenesis, the neuroblasts delaminate from a monolayer of ectodermal cells. The ectodermal cells at the surface of the Drosophila embryo divide with the axes of their mitotic spindle parallel to the plane of the ectodermal monolayer. In contrast, a neuroblast divides along the

apical–basal axis, so that the axes of its mitotic spindles are perpendicular to the plane of the ectodermal layer. Thus, the mitotic spindle of the neuroblast has to reorient by 90 degrees from the plane of the ectodermal layer (Kraut et al. 1996).The orientation of the mitotic spindle correlates with the asymmetric distribution of Numb (Jan and Jan 1998).

Our observations showed that similar perpendicular orientation, i.e., 90° from the plane of the ST layer toward the villous core, applies for asymmetrically dividing villous CT (Bukovsky et al. 2003a). Hence, in chorionic villi, one pole of dividing CT cells is associated with mature ST and the other with the mesenchymal villous core (stroma). It is possible to speculate that the poles of dividing CT cells are influenced differently – the juxta-syncytial pole toward differentiation and juxta-mesenchymal toward proliferation. Therefore, the extrinsic factors (type of neighboring cells) may dictate asymmetric segregation of intrinsic factors determining the fate of dividing cells. In other words, asymmetric division in vivo may be a result of the influence of both extrinsic and intrinsic factors. If so, the extrinsic factors should be viewed as inducers and intrinsic factors as effectors of asymmetric division.

Based on available data (Kraut et al. 1996; Jan and Jan 1998; Shen et al. 2002; Cayouette and Raff 2002; Shen and Temple 2002; Petersen et al. 2002) and our observations, a possible sequence of events involved in asymmetric division of villous CT cells is given in Fig. 2A. Note an involvement of stromal (stimulation of CT division) and ST signaling (stimulation of cell differentiation). Panel 2B indicates that symmetric division of ST may result from the presence of stromal and absence of ST signaling.

However, perpendicular orientation of dividing trophoblast cells in vivo cannot be achieved in vitro, because the complex villous structure, and the villous core in particular, is absent. Although ERA>ERB transition was also observed during trophoblast differentiation in culture, the cultured trophoblast cells are expected to undergo symmetric division toward the differentiated phenotype rather than asymmetric division. Indeed, early cultures show proliferating CT cells, which are gradually transformed into the differentiating CT cells and syncytial aggregates during the culture (Bukovsky et al. 2003b), possibly due to the prevalence of differentiation signals in the absence of stromal signaling (Fig. 2C). This view is supported by gradual diminution of promitotic cyclin E and an increase of antimitotic p27 proteins during trophoblast culture (McKenzie et al. 1998). Finally, regarding the placenta, it is important to consider the stage of pregnancy. For early pregnancy, the scheme in Fig. 1 can possibly be extended to other cell types (decidua, extravillous trophoblast), in contrast to the term placenta where only villous CT cells are proliferating.

We recently showed that exogenous E2 markedly stimulates development of large syncytial aggregates in trophoblast cultures (Bukovsky et al. 2003b). We also observed that this effect can be abolished with pure anti-estrogen ICI 182,780 (unpublished data). These observations indicate that trophoblast

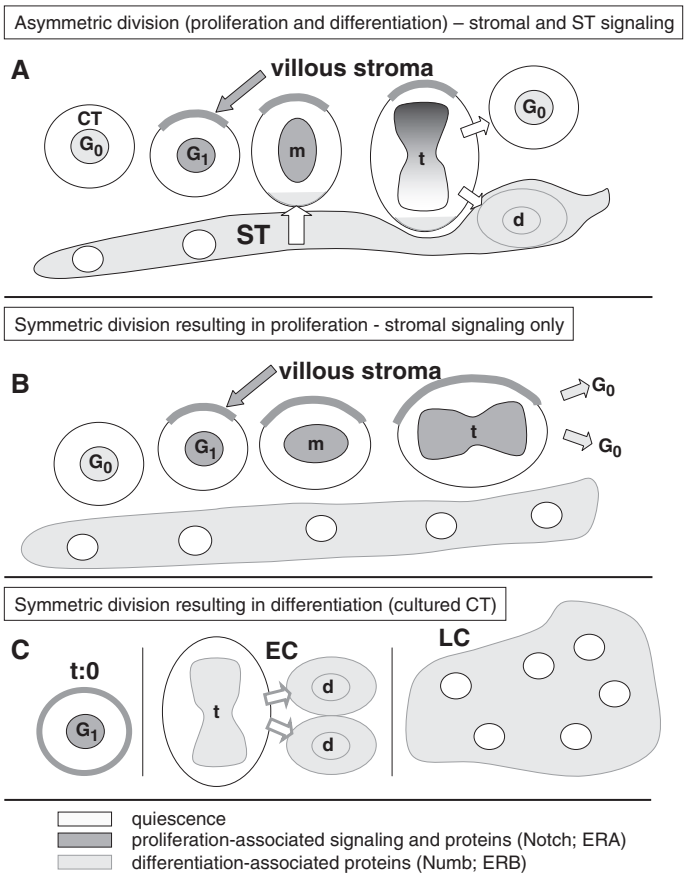

Fig. 2. A–C In vivo (A and B) and in vitro division pathways of human placental trophoblast: **A** structure-related arrangement of asymmetric division (proliferation and differentiation) of villous CT cells; **B** symmetric division resulting in proliferation; **C** symmetric division resulting in differentiation. m, metaphase; t, telophase; d, differentiating cell; EC, early trophoblast culture; LC, late culture. Relevant references and details are given in text. Adapted from Bukovsky et al. (2003a) – A.B. copyright

differentiation is estrogen-dependent. Since trophoblast differentiation is associated with transition from ERA to ERB expression, with temporary co-expression of both, one may assume that both ERs are involved in estrogen stimulated trophoblast differentiation. Since production of placental hormones is characteristic for mature syncytium (Kliman et al. 1986; Castracane and Goldzieher 1986; Petraglia et al. 1995; Shanker and Rao 1997; Pepe and Albrecht 1999), which shows in vivo cytoplasmic ERB

expression only, one may also assume that estrogens stimulate production of placental hormones by ST, via the extranuclear (cytoplasmic) ERB.

2.2
Conclusion – Asymmetric Division of Estrogen Responsive Cells

In conclusion, our observations concur with recent views on the importance of ERA in the proliferation and ERB in maturation of estrogen responsive cells. Asymmetric segregation of ERA in dividing villous CT cells, accompanied by appearance of ERB in differentiating daughter cells, suggests a unique role of ERs in asymmetric division of estrogen responsive cells. High levels of estrogens during pregnancy may promote, via ERs, trophoblast proliferation and differentiation. Since ST is a major source of placental hormones, which ensure optimal conditions for the fetus and mother, the cytoplasmic ERB expression may be involved in a direct (extranuclear) stimulation of placental hormonal production.

3
Asymmetric Division During Initiation of Oogenesis in Fetal and Adult Human Ovaries

The 50-year-old and currently prevailing view that all oocytes and primary follicles in adult mammalian ovaries originate from fetal oogenesis is apparently contradictory to Darwinian evolutionary theory. Why should adult mammalian females carry their oocytes from the fetal period of life (storage theory), as compared to the invertebrates (flies), lower vertebrates (fish and frogs), and males of all species, including mammals, with persisting gametogenesis (continued formation theory) including adulthood?

It is now well documented that mammalian primordial germ cells in developing embryonic gonads originate from uncommitted (totipotent) somatic stem cells, and that their sex commitment is determined by local gonadal environment -signals produced by neighboring somatic cells (Alberts et al. 2002). Once committed to become female germ cells, these primordial germ cells are believed to multiply in human fetal ovaries and differentiate into definitive oocytes persisting until menopause. Yet the children born to women after the age of 35, but not before, are known to accumulate genetic alterations, which may originate from accumulation of alterations in persisting eggs. This indicates that until 35 years of age fresh oocytes and primary follicles are formed replacing aged ones in ovulatory ovaries. We observed that, even in midpregnancy human fetal ovaries, new germ cells are formed by asymmetric division of OSE stem cells (Bukovsky et al. 2005a, 2006). Such fetal oocytes associate with granulosa cells and

give rise to fetal primary follicles. Similar asymmetric division of OSE stem cells and follicular renewal was detected during the prime reproductive period in human females (Bukovsky et al. 1995, 2004).

Altogether, asymmetric division of OSE appears to give rise to new germ cells differentiating into oocytes and forming new primary follicles by association with OSE-derived granulosa cells in midpregnancy human fetal ovaries and after menarche until the end of the prime reproductive period (follicular renewal until approximately 38±2 years of age) (Bukovsky et al. 2004, 2005a, 2006). Hence, it appears that the human fetal primary follicles are capable of persisting for about 12–14 years (until menarche) and, accordingly, the last primary follicles formed by the end of the third decade of life also persist for a similar period of time, until menopause. Since persisting fetal oocytes are replaced by new waves of follicular renewal after menarche, the prime reproductive period is associated with the availability of the fresh eggs for a healthy progeny. However, after termination of follicular renewal persisting primary follicles accumulate endogenous and environmentally induced genetic alterations resulting in increased incidence of abnormal fetal karyotypes: 1 of 20 (5%) at 38–40 years, to 1:16 (6.3%) at 41–43 years, and finally 1:4.5 (22.2%) in 44–46 years (Sachs et al. 1977).

3.1
Ovarian Surface Epithelium Stem Cells in Human Fetal Ovaries

Human fetal OSE contains numerous germ cells (10μm in diameter) from seven weeks of intrauterine life until the neonatal period, and it has been suggested that these cells are extruded into the peritoneal cavity (Motta and Makabe 1982, 1986). This could happen after the cessation of oogenesis [six to seven month of fetal life (Simkins 1932; Peters and McNatty 1980)], when germ cells emerging in OSE may be prevented from entering the cortex by the developing ovarian tunica albuginea (TA) (Bukovsky et al. 2006). However, former observations indicate that the OSE is a source of germ cells differentiating into oocytes in human fetal ovaries (Simkins 1928, 1932; Van Wagenen and Simpson 1965), and we did not observe germ cells leaving the ovary in midpregnancy human fetuses (Bukovsky et al. 2005a).

Our observations in ovaries of midpregnancy human fetuses (Bukovsky et al. 2005a) indicate a presence of small (10 μm) germ cells within OSE (asterisks, Fig. 3A), but such cells are smaller when compared to those positioned under the OSE (white arrowhead). This indicates that the OSE-derived germ cells enter ovarian cortex and differentiate into oocytes. In deeper ovarian cortex (oc, Fig. 3A), germ cells with well defined cytoplasm and plasma membrane show a further increase in size (black arrowhead).

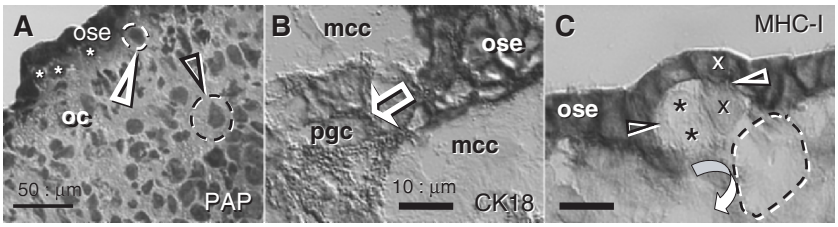

Fig. 3. A–C Origin of granulosa and germ cells from OSE cells in midpregnancy human fetal ovaries: **A** Papanicolaou's staining (PAP) shows surface epithelium (ose) containing small germ cells (*asterisks*), showing increase in size in the adjacent (*white arrowhead*) and distant (*black arrowhead*) ovarian cortex (oc); **B** CK18 staining of a cluster of primitive granulosa cells (pgc) descending from the OSE (*arrow*) between mesenchymal cell cords (mcc); **C** major histocompatibility complex class I (MHC-I) expression diminishes during asymmetric division (*white arrowhead*, *white and black X*; no nuclear counterstain) of OSE cells. Symmetric division (*black arrowhead*) of germ cells follows (*asterisks*).Tadpole-like germ cell (*dashed line*) enters (*arched arrow*) ovarian cortex. *Bar* in (B) for (B) and (C). Adapted from Bukovsky et al. (2005a), with permission of Humana Press, Inc

3.1.1 Origin of Primitive Granulosa Cells from Proliferating Ovarian Surface Epithelium Stem Cells

The OSE in human fetal ovaries is a source of follicular granulosa cells. Primitive granulosa cells (pgc, Fig. 3B) are formed by proliferation (symmetric division) of OSE cells forming sprouts extending into the ovary between mesenchymal cell cords (mcc). These granulosa cells associate with oocytes in the deeper ovarian cortex to form follicles (Bukovsky et al. 2005a). Note that primitive granulosa cells (arrow, Fig. 3B) show a diminution of cytokeratin 18 (CK 18) immunoexpression when compared to the OSE cells (ose). The mesenchymal cell cords in human fetal ovaries are rich in Thy-1 differentiation protein (Thy-1 DP) produced by ovarian stromal cells (Bukovsky et al. 2005a), and the Thy-1 DP accompanies early differentiation of cells in various tissues (Bukovsky et al. 2001).

3.1.2 Origin of Germ Cells by Asymmetric Division of OSE Stem Cells

In addition, new germ cells emerge in OSE of human midpregnancy fetal ovaries. Such germ cells are formed by asymmetric division of OSE cells. Such asymmetric division (white arrowhead, Fig. 3C) results in a smaller progenitor daughter, which keeps major histocompatibility complex class I (MHC-I) expression (white X) of OSE cells, and a larger differentiating daughter showing MHC-I depletion (black X). The asymmetrically

originating germ cell then undergoes single symmetric division (black arrowhead) resulting in two differentiating daughters (asterisks). Such symmetric division of each germ cell is required for a premeiotic phenomenon, which is known as "crossing over" of chromosomes (Alberts et al. 2002). Next the germ cells enlarge in size, achieve the "tadpole like" shape (dashed line), and leave the OSE by entering the ovarian cortex (arched arrow, Fig. 3C).

3.1.3 Monocytes and T Lymphocytes Accompany Asymmetric Division of OSE Stem Cells in Human Fetal Ovaries

Primitive MDC expressing CD14 (m, Fig. 4A) showed association (black arrowhead) with fetal OSE cells (asterisk) and were found to accompany (arrowhead, Fig. 4B) intraepithelial germ cells (asterisks). Asymmetric division (arrowhead, Fig. 4C) of OSE cells resulting in smaller proliferative (white x) and larger differentiating (black X) daughters was also accompanied by T lymphocytes (t, Fig. 4C); activated MDC expressing HLA-DR showed extensions (arrowhead, Fig. 4D) toward emerging germ cells (asterisk).

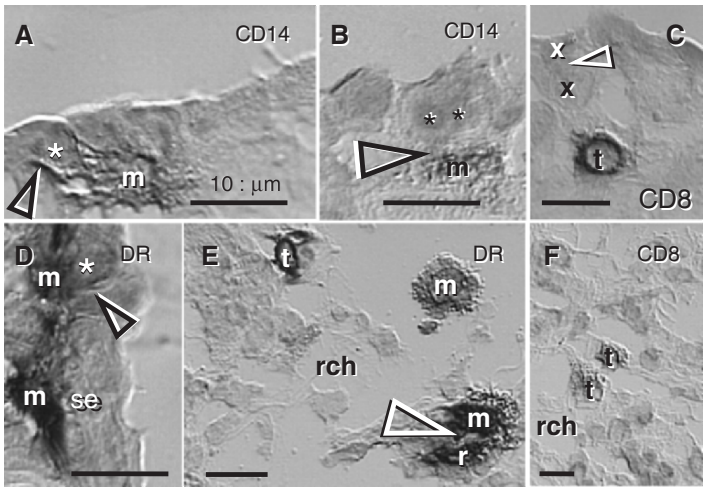

Fig. 4. A–F Localization of MDC and T cells in midpregnancy human fetal ovaries: **A** CD14 MDC (m) exhibiting extensions (*arrowhead*) among OSE cell, which appear to initiate asymmetric division (asterisk); **B** germ cells undergoing symmetric division (*asterisks*) and association of CD14 MDC (*arrowhead*); **C** CD8 T cell (t) accompanies asymmetric division (*arrowhead*) of OSE cells (*white and black X*); **D** interaction (*arrowhead*) of HLA-DR (DR) activated MDC with the OSE appears to initiate asymmetric division (*asterisk*); **E** rete ovarii contains HLA-DR+ resident MDC (r) and MDC and T cells migrating through rete channels (rch). Note interaction (arrowhead) of migrating MDC with resident cell; **F** CD8 T cells in rete channels. Bar in (A) for (A–F), Adapted from Bukovsky et al. (2005a), with permission of Humana Press, Inc

3.1.4 Role of Rete Ovarii

At the embryonic age of nine weeks, female gonads show a marked development of rete cords with lumen formation, and the rete reaches the center of the ovary at 12 weeks. The first follicles are formed after the fourth fetal month and follicle formation always begins in the innermost part of the cortex, close to the rete ovarii. This structure is essential for follicular development. If it is removed before formation of follicles has started, follicles will not form (Byskov et al. 1977). Yet, the exact mechanism by which the rete may contribute to the fetal ovarian development, remains a mystery.

Our observations indicate that activated MDC (HLA-DR+ tissue MDC) reside in rete cords (r, Fig. 4E), and T cells and monocyte type cells (t and m, Fig 4E; t, Fig. 4F) migrate through rete channels, show interactions (arrowhead, Fig. 4E) with resident MDC, and MDC associate with OSE (see above). The rete cords, like mesenchymal cell cords, also show prominent Thy-1 DP expression (Bukovsky et al. 2005a).

Fetal oogenesis is terminated at the beginning of the third trimester of intrauterine life, and a mesenchymal layer of TA is formed under the OSE (Simkins 1932; Peters and McNatty 1980), possibly by epithelial-mesenchymal transition of OSE cells (Bukovsky et al. 2006), since the mesenchymal cells of the TA are capable of expressing cytokeratin and exhibit mesenchymal-epithelial transition back into OSE cells in adult ovaries (see below).

3.1.5 Conclusion on the Role of OSE in Human Fetal Ovaries

These observations indicate that the OSE is a source of germ and primitive granulosa cells. Hence, as in adult ovaries [(Bukovsky et al. 2004) and below], the midpregnancy human OSE stem cells are bipotent progenitors with a commitment for both cell types. It is possible that tissue MDC residing in rete cords carry a memory on the characteristics of germ cells populating the ovary during the embryonal period of life. Such memory could be transferred to monocytes and T cells migrating through the rete channels in midpregnancy ovaries, and the migrating cells reaching the OSE may stimulate transformation of some OSE stem cells into germ cells via asymmetric division. In addition, the mesenchymal cell cords rich in Thy-1 DP may participate in the transformation of OSE cells into primitive granulosa cells. In this way, different potentials of OSE cells may be realized, depending on the local influence of migrating and resident mesenchymal cells. Pluripotency of progenitor cells is not unusual. It persists in bone marrow throughout life, and the “one cell, two fates” phenomenon has also been described for vascular progenitor cells (Yamashita et al. 2000).

3.2
OSE Stem Cells in Adult Human Ovaries

In adult human ovaries mitoses of OSE cells are rare (Motta et al. 1980), and new OSE cells originate from mesenchymal-epithelial transition (open arrow, Fig. 5A) of cytokeratin positive fibroblast-like (fb, Fig 5A) TA cells (Bukovsky et al. 1995, 2004). These cells show a transition through the mesenchymal/epithelial stage (mes/ep, Fig. 5A) and differentiate (arched arrow) into OSE cells (ose).

3.2.1
Origin of Granulosa Cell Nests from OSE in Adult Ovaries

Proliferation of the TA flap over the ovarian surface (taf, Fig. 5A,C) could result in a bi-laminal OSE layer descending into the ovarian cortex (arrow, Fig. 5B); note lack of OSE at the surface (arrowhead). The layer fragments into nests of primitive granulosa cells deeper in the ovarian cortex (arrowheads, Fig. 5C). These nests of primitive granulosa cells are essential for the assembly with oocytes and formation of new primary follicles in adult human ovaries, since unassembled new oocytes are unable to survive and they degenerate (Bukovsky et al. 2004).

When compared to human fetal ovaries, where OSE cells proliferate and form granulosa cells which are present among oocytes and available to form primary follicles [resembling a situation in adult rat ovaries (Bukovsky et al. 2005a)], in adult human ovaries granulosa cell nests are required for formation of new primary follicles during follicular renewal (Bukovsky et al. 2004).

3.2.2
Origin of Germ Cells in Adult Ovaries by Asymmetric division of OSE Stem Cells

While differentiation of granulosa cells from OSE has a different pattern in fetal and adult ovaries, there were some similarities in the development of germ cells. Like in fetal ovaries, the new germ cells differentiated from OSE by asymmetric division (arrowheads, Fig. 5D). Note cytoplasmic expression of PS1, a meiotically expressed zona pellucida carbohydrate antigen (Skinner and Dunbar 1992), in proliferating OSE cell daughters (small white x) and nuclear PS1 expression in differentiating daughters (large white X). Figure 5E shows a double staining for PS1 and cytokeratin (CK), where proliferating daughter (small white x) keeps the CK expression while differentiating daughter (large white X) loses CK and expresses PS1 only [for color see (Bukovsky et al. 2004) – http://www.rbej.com/content/2/1/20]. As in fetal ovaries, the differentiating daughters undergo a symmetric division (white arrow, Fig. 5F) for chromosomal "crossing over" with persisting

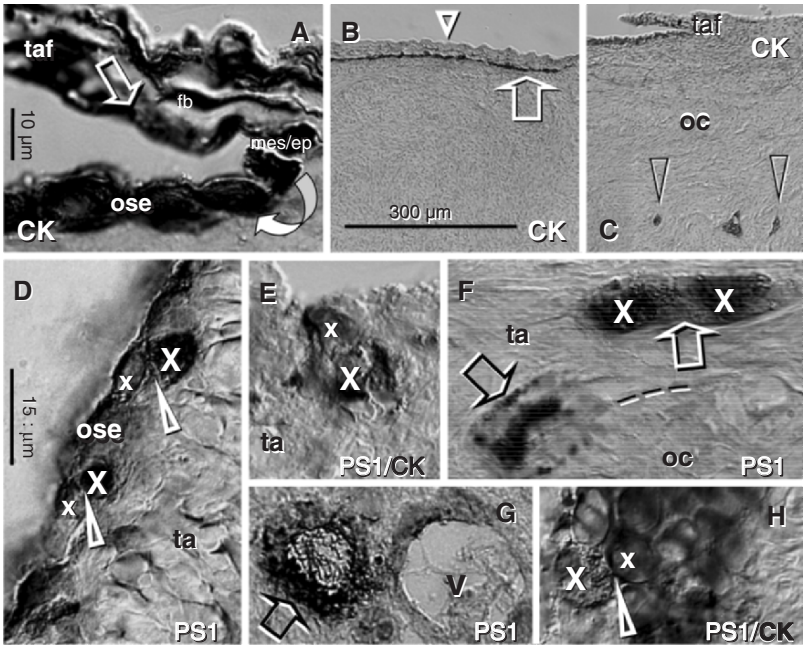

Fig. 5. A–H Initiation of follicular renewal in adult human ovaries. CK18 (A-C), PS1 (D, F and G), and CK/PS1 staining (E and H); **A** transition (*arrow*) of CK+ mesenchymal cells of fibroblasts (fb) type in TA to the OSE cells (ose) and TA flap (taf) evagination. Note mesenchymal/epithelial (mes/ep) intermediate stages; **B** bilaminar OSE cord (*arrow*) descending into ovarian cortex. Note lack of OSE at the surface (*arrowhead*); **C** Wide TA with CK+ mesenchymal cells and flap (taf) extending from ovarian surface. In the upper ovarian cortex (oc) the epithelial cords fragment into epithelial nests (*arrowheads*); **D** segments of OSE show cytoplasmic PS1expression (*small X*) and give rise to cells exhibiting nuclear PS1 (*large X*) by asymmetric division; **E** asymmetrically dividing OSE cell (note perpendicular orientation toward surface) produces CK+/PS1- progenitor daughter (*small X*) and CK–/PS1+ differentiating daughter (*large X*), which descends into the TA; **F** in TA, the putative germ cells show symmetric division (*black arrow*) and exhibit also development of cytoplasmic PS1 when entering (*white arrow*) the upper ovarian cortex (oc); **G** In the cortex, the cells show diminution of nuclear and increase of cytoplasmic PS1 staining (*arrow*), particularly when attached to the cortical vessels (*v*); **H** The asymmetric division (*arrowhead*) giving rise to the putative PS1+ (*large X*) germ cells from CK+ OSE stem cells (*small x*) could be observed at the periphery of cortical epithelial crypts. *Bar* in (B) for (B) and (C), *bar* in (D) for (D–H). For details see text and for color http://www.rbej.com/content/2/1/20. Adapted from Bukovsky et al. (2004) – A.B. copyright

nuclear PS1 expression (X and X, Fig. 5F), and enter the ovarian cortex (black arrow).

From this point on, however, there were some differences compared to fetal ovaries. The germ cells should somehow reach granulosa cell nests in the deep ovarian cortex, close to the medulla, where new primary follicles are formed. Hence, to assemble with granulosa cells, the germ cells have to pass a distance much larger (900–1200 μm) than in small fetal ovaries, where granulosa cells are available closer (100–150 μm) (Bukovsky 2006a), and this transition has to be relatively fast due to the limited life of germ cells and unassembled oocytes in adult human ovaries. For such transit, the germ cells utilize a blood stream, after entering cortical vessels under the TA. The cells associating with vasculature show diminution of nuclear and appearance of cytoplasmic PS1 expression (arrow, Fig. 5G).

An alternative origin of germ cells in adult human ovaries by asymmetric division (arrowhead, Fig. 5H) are OSE cells in epithelial crypts located deeply in the ovarian cortex. Such germ cells attain the tadpole shape and are able to reach and assemble with neighboring epithelial nests lying in about 150 μm distance, or enter vasculature and saturate distant nests associated with vessels (Bukovsky et al. 2004).

3.2.3 Monocyte-derived Cells and T Lymphocytes Accompany Asymmetric Division of OSE Stem Cells and Migration of Germ Cells in Adult Human Ovaries

Similar to fetal ovaries, we also detected association of MDC and T cells with asymmetric division of OSE stem cells during the commitment of differentiating daughters to germ cells and their migration in adult human ovaries (Bukovsky et al. 1995). We used single color immunohistochemistry with antibodies recognizing CD14 of primitive MDC, CD8 of T lymphocytes, HLA-DR of activated MDC, and Thy-1 DP of vascular pericytes. Asymmetric division of OSE cells and development of putative germ cells were visualized by differential interference contrast and digital camera with detail enhancement.

Association of CD14 primitive MDC with OSE and secretion of CD14 among OSE cells (arrows, Fig. 6A), accompanies asymmetric division of OSE cells. Characteristically, the progenitor daughter persisting in the OSE is smaller (small white x) as compared to the differentiating daughter (large black X, Fig. 6A). This resembles asymmetric divisions of OSE demonstrated above by MHC class I immunohistochemistry in the fetal ovaries (arrowhead, Fig. 3C) and by single and double color immunohistochemistry in adult human ovaries (Fig. 5D,E).

The asymmetric division of OSE cells with emergence of germ cells is also accompanied by CD8 T lymphocytes, which show extensions

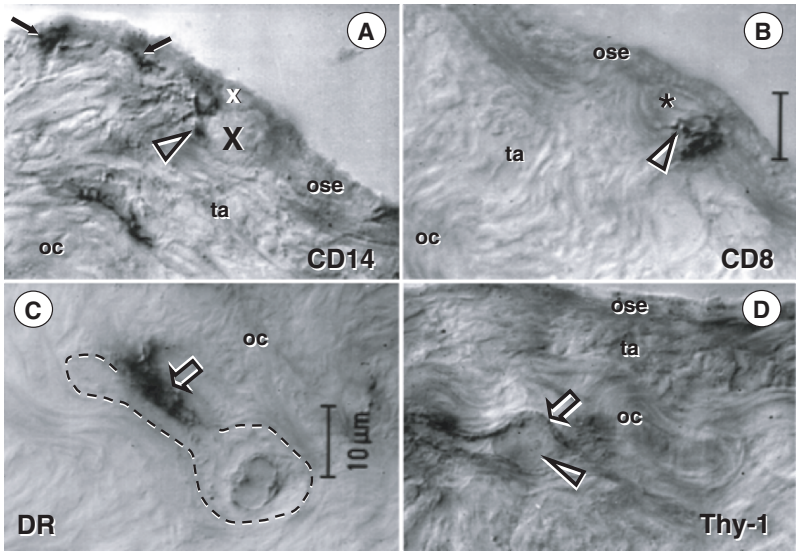

Fig. 6. A–D Staining of the adult human ovarian OSE (ose), tunica albuginea (ta), and adjacent ovarian cortex (oc) for CD14 of primitive and HLA-DR of activated MDC, CD8 of T lymphocytes, and Thy-1 DP of vascular pericytes, as indicated in panels: **A** small CD14+ primitive MDC show secretion of CD14 among OSE cells (*arrows*). *Arrowhead* indicates MDC extensions toward OSE cell exhibiting asymmetric division (*small and large X*); **B** emergence of putative germ cell (*asterisk*) under the OSE is also accompanied by CD8 T cells (*arrowhead*); **C** within the ovarian cortex, the putative germ cell exhibit a tadpole shape (*dashed line*), and is accompanied by an activated MDC (*arrow*); **D** intravascular putative germ cell (*arrowhead*); the *arrow* indicates Thy-1 DP+ pericytes of cortical microvasculature. Note that the vessel with putative germ cell is in the ovarian cortex adjacent to the TA. *Bars* in (B) and (C) for (A–D). Adapted from Bukovsky et al. (1995), with permission of Munksgaard International Publishers Ltd. Copenhagen, Denmark

(arrowhead, Fig. 6B) toward putative germ cells (asterisks). This resembles a similar process in fetal ovaries (Fig. 4C). However, as pointed out above (see Sect. 3.2.2), the germ cells in adult human ovaries should pass a long distance to form primary follicles by assembly with the nests of primitive granulosa cells. They attain a tadpole-like shape (dashed line, Fig. 6C) and migrate with the assistance of activated MDC (arrow) from TA to adjacent upper cortex. Within the upper cortex, the tadpole-like cells lacking MHC class I expression intimately associate with MHC class I^+ endothelial cells of the cortical microvasculature (Bukovsky et al. 1995) and intravascular putative germ cells are apparent in the ovarian cortex (arrowhead, Fig. 6D).

Our observations and views on oogenesis in fetal and adult human ovaries are summarized in Fig. 7. Midpregnancy human fetal OSE is a source of primitive granulosa cells [pg, Fig. 7A; (Bukovsky et al. 2005a)]

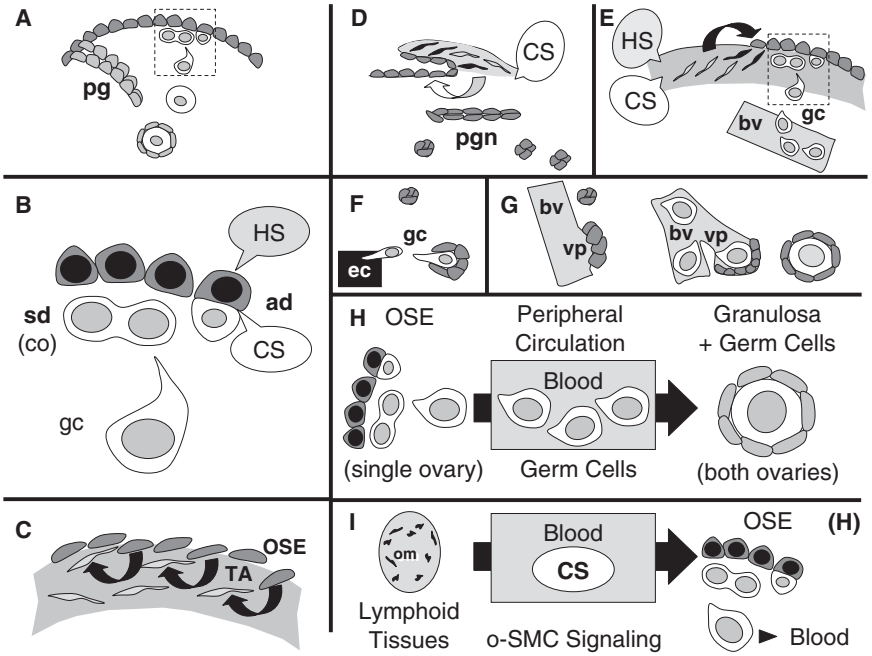

Figure 7. A–I Survey of oogenesis and formation of ovarian follicles in human ovaries: **A** OSE in midpregnancy fetal ovaries is a source of primitive granulosa (pg) and germ cells (*dashed box – see B*); **B** asymmetric division (ad) of OSE stem cells and symmetric division (sd) of germ cells (gc) entering the cortex; HS, hormonal signaling (E2, hCG), CS, cellular signaling of o-SMC; **C** perinatal period is associated with development of subepithelial TA layer by a transition of OSE cells into mesenchymal type cells (*arched arrows*). Around menarche and during the prime reproductive period, the TA mesenchymal cells differentiate back into OSE cells forming: **D** primitive granulosa cell nests (pgn) or: **E** germ cells entering blood vessels; **F** the deep cortical epithelial crypts (ec) are an alternative source of germ cells, which either associate with adjacent nests, or also enter blood vessels; **G** other nests associate with deep cortical vessels and form vascular pockets (vp) to catch the germ cells from the blood stream and form primary follicles; **H** the OSE-Blood-Follicle pathway; **I** the Lymphoid/bone marrow–blood-OSE pathway, continuing as in (H). Reprinted from Bukovsky et al. (2006), with permission of Informa Healthcare, Informa UK Ltd

and germ cells (dashed box; see also Figs. 3C and 7B). The germ cells differentiate into oocytes which associate with available granulosa cells to form fetal follicles. Primitive germ cells originate by asymmetric division [ad, Fig. 7B; (Bukovsky et al. 2005a)] from OSE cells influenced by hormonal (HS; elevated hCG and E2 levels) and cellular signaling [CS; i.e., ovary-specific mesenchymal cells (o-SMC) – see Table 1 and below].

Table 1. Working model on age-associated changes of ovary-specific mesenchymal cells (o-SMC) and hormonal signals [LH/hCG and estradiol (E_2)] required for the initiation and resumption of oogenesis in human ovaries. From Bukovsky et al. (2005a), with permission of Humana Press

Period of life	o-SMC[c]	LH/hCG[d]	E_2[e]	Oogenesis
First trimester-midpregnancy	Yes	Yes	Yes	Yes[f]
Last trimester-newborn	Yes	**No**	Yes	**No**[f]
Postnatal-menarche	Yes	**No**	**No**	**No**[g]
Reproductive period[a]	Yes	Yes	Yes	Yes[f]
Premenopause[b]	**No**	Yes	Yes	**No**[g]
Postmenopause	**No**	Yes	**No**	**No**[f]

[a]From menarche till 38±2 years of age
[b]From 38±2 years till menopause
[c]Specialized mesenchymal cells (tissue macrophages and T cells) with commitment for stimulation of OSE stem cells to produce germ cells by asymmetric division
[d]Levels corresponding to the mid cycle LH peak, or more [hCG levels should be 10x more, since it has a 10% affinity to the LH receptor compared to that of LH (Bousfield et al. 1996)]
[e]Levels corresponding to the preovulatory E_2 peak, or more
[f]Confirmed
[g]Predicted

The primitive germ cells undergo symmetric division (sd), which may be associated with chromosomal crossing over (cro), and tadpole-like germ cells (gc) migrate into the cortex, where they differentiate into oocytes and form ovarian follicles (see Fig. 7A). In perinatal ovaries, the OSE cells form a loose subepithelial layer of TA by epithelial-mesenchymal transition (arched arrows; Fig. 7C).

Around menarche and during the prime reproductive period, the TA mesenchymal cells are influenced by blood delivered CS, such as o-SMC, i.e. MDC and T lymphocytes, and differentiate back into segments of OSE cells (arched arrow, Fig. 7D), forming cords of primitive granulosa cells, which descend into the cortex and fragment into the primitive granulosa cell nests (pgn, Fig. 7D; see also Fig. 5A,C). When hormonal signaling (HS, Fig. 7E) is also present, the TA mesenchymal cells form segments above the TA (arched arrow) and produce germ cells (see Fig. 5D,E), as in fetal ovaries (dashed box). Next the tadpole-like germ cells migrate to enter adjacent blood vessels (bv). The OSE-derived epithelial crypts (ec, panel 7F) in the deep cortex are an alternative source of germ cells (gc; see Fig. 5H), which may assemble with adjacent nests to form primary follicles.

If epithelial crypts are absent, the nests associate with vessels and form vascular pockets (vp, Fig. 7G) to catch circulating germ cells and form primary follicles.

Hence, once OSE-derived germ cells are stimulated to emerge by cellular and hormonal signaling during the midcycle and enter peripheral circulation, they are ready to form primary follicles without delay, assuming the nests of primitive granulosa cells are available within the ovaries (Fig. 7H). Even if oogenesis is initiated in only one of the ovaries (single ovary, Fig. 7H), the germ cells entering peripheral blood are capable of saturation of the epithelial nests in both of them (both ovaries). Due to the apparent affinity of germ cells to settle in ovarian vessels, supernumerary germ cells often differentiate into oocytes accumulating and degenerating in ovarian medullary vessels (Bukovsky et al. 2004). Prior to the production of germ cells from OSE, the committed o-SMC, generated in lymphoid tissues and bone marrow and carrying ovarian memory (om), are available in the circulation to reach the OSE and provide "cellular signaling" (CS) for initiation of oogenesis if "hormonal signaling" is also present (Fig. 7I). Resulting germ cells enter the peripheral circulation and the process continues as indicated in Fig. 7H.

The bone marrow, which was proposed to be an extra-ovarian source of germ cells (Johnson et al. 2005), represents a tissue highly supplied by the blood. Therefore, it may show the presence of many OSE-derived germ cells contaminating the peripheral blood after midcycle, when the ovarian germ cells enter circulation. The idea on the ovarian origin of germ cells detected in peripheral blood and bone marrow (Bukovsky 2005) is supported by the observations that the bone marrow shows no "oogenetic" properties during other periods of the ovarian cycle or in ovariectomized animals (Johnson et al. 2005).

Altogether, we always believed that the current dogma on the fetal origin of oocytes in adult mammalian ovaries does not fit with the Darwinian evolutionary theory (Bukovsky and Presl 1977), and provided first immunohistochemical evidence on the oogenesis from OSE cells and follicular renewal in adult human ovaries in 1995 (Bukovsky et al. 1995) and on the origin of human germ cells by asymmetric division of OSE cells (Bukovsky et al. 2004, 2005a).

In human females, the new germ cells and primary follicles appear to differentiate from the OSE during two time periods only, the fetal and prime reproductive life spans (Table 1). An important question is why would human female gametes and primary follicles differentiate during the fetal period, if they are not needed until menarche several years later? Development of immune tolerance toward self tissues during immune adaptation (Klein 1982) might explain this requirement for fetal differentiation of oocytes and primary ovarian follicles, as we have earlier suggested (Bukovsky and Presl 1977). If this does not occur, the oocytes and primary follicles would face the fate of the human corpus luteum, which is absent in

fetal ovaries and whose functional life in the adult ovary, except during the immunologically unique situation of pregnancy, lasts for only several days. In addition, a shorter than normal period of fetal oogenesis during immune adaptation could cause shorter period of follicular renewal during adulthood and premature ovarian failure (Bukovsky et al. 2006; Bukovsky 2006b).

3.2.4 Thymus and Reproduction

The thymus plays an important role in the immune system, and it has been suggested that thymic cells and peptides play a role in determining reproductive lifespan in females (Bukovsky and Presl 1979; Rebar 1982; Suh et al. 1985). The relationship of age-associated thymic involution with diminution of ovarian function is supported by the alteration of ovarian function in neonatally thymectomized mice (Nishizuka and Sakakura 1969). In addition, in congenitally athymic (nude) mice, follicular loss is first evident at two months of age and this is specifically due to a reduction in the numbers of primary follicles. The first ovulation is delayed until two and half months of age, compared to the first ovulation in the one and half month old normal mouse females. By four months, an overall reduction in all fractions of the follicle population occurs in nude mice, and ovulation ceases (Lintern Moore and Pantelouris 1975). Interestingly, the absence of the thymus might also be responsible for the lack of hair in nude mice, due to the lack of thymus-derived T cells, which might be required for hair development. Similarly, a baldness more likely develops in aging men, but is less likely in women, probably since the immune system in females works more efficiently and effectively longer than in males (Aspinall 2000).

3.2.5 Incomplete Asymmetric Division and Nuclear Endoreplication of Germ Cells

The germ cell committed to becoming an oocyte needs more organelles than it is able to generate per se. In adult human ovaries, additional organelles are provided by granulosa cells during formation of new primary follicles. The extensions of granulosa cells penetrate the ooplasm and contribute to the formation of a paranuclear Balbiani body, a source of additional mitochondria for the oocyte development. The Balbiani body persists in oocytes of resting primary follicles and the mitochondria are released with the initiation of follicular growth [see Bukovsky et al. (2004) for data and review].

The ovaries of invertebrates exhibit incomplete asymmetric division of oocytes, which results in nuclear endoreplication and production of

"chains" of nurse cells connected between themselves and to the oocyte by cytoplasmic bridges (Alberts et al. 2002). Cell "chains" connected by intercellular bridges were described in Drosophila ovaries (Cox and Spradling 2003) and fetal mouse germ cell cysts (Pepling and Spradling 1998). These bridges are utilized for the supply of organelles from nurse cells to the oocyte (Alberts et al. 2002; Cox and Spradling 2003). Hence, in some instances, a primitive germ cell endoreplication mechanism results in a syncytial "chain" of sister cells, which are exhaustively exploited as a source of organelles the oocyte committed cell needs to mature into a functional egg. The formation of syncytial type cell "chains" is also characteristic for the development of spermatids (Alberts et al. 2002).

3.2.6 Conclusion on the Role of Ovarian Surface Epithelium in Adult Human Ovaries

Observations from adult human ovaries indicate that TA mesenchymal cells are precursors of OSE stem cells, which have a potential to differentiate into germ and granulosa cells. In contrast to fetal ovaries entirely covered by OSE stem cells, in functional human ovaries the OSE is found in certain areas only (Motta et al. 1980; Bukovsky et al. 2004) – see Fig. 5B,C. However, in women with anovulatory cycles, or patients with polycystic or sclerotic ovaries, the ovarian surface is completely covered with OSE (Makabe et al. 1980). This is despite similar handling during surgical retrieval, suggested to cause ovarian denudation (Gillett 1991).

The origin of OSE stem cells from TA precursors raises an important question on why the functional adult ovaries do not preserve OSE cells in contrast to the fetal or anovulatory ovaries. The TA mesenchymal cells resembling fibroblasts are certainly more resistant to environmental influences and, therefore, exhibit better condition to be preserved unaffected. This supports the concept of germ cell and oocyte renewal in functional ovaries by asymmetric division of OSE stem cells freshly differentiated by mesenchymal-epithelial transition from TA progenitor cells. Hence, the ovarian TA can be viewed as a can, which preserves environmentally resistant progenitors of environmentally sensitive germ cells.

In human ovaries, the formation of new germ cells and follicles occurs at two occasions, during the second trimester of intrauterine life (fetal primordial and primary follicles) and during the prime reproductive period (follicular renewal). During all other periods of life (perinatal, childhood, and premenopausal), primary follicles with aging oocytes are preserved in the ovaries. While the preservation of primary follicles with aging oocytes until menarche does not represent a threat for the progeny due to the lack of ovulation, the premenopausal ovaries lacking follicular renewal may ovulate chromosomally aberrant oocytes, a source of chromosomal abnormalities in the progeny of mothers in advanced maternal age. Importantly,

formation of new germ cells and primary follicles may require not only involvement of immune system related cells, but also certain hormonal milieu, namely high levels of LH/hCG and estrogens (Table 1). The fetal primary follicles may be able to persist for up to 12–15 years, until replaced by follicular renewal prior to or at menarche.

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References

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular biology of the cell. Garland Science, New York

Arnholdt H, Meisel F, Fandrey K, Lohrs U (1991) Proliferation of villous trophoblast of the human placenta in normal and abnormal pregnancies. Virchows Arch B Cell Pathol Incl Mol Pathol 60:365–372

Aspinall R (2000) Longevity and the immune response. Biogerontology 1:273–278

Bousfield GR, Butnev VY, Gotschall RR, Baker VL, Moore WT (1996) Structural features of mammalian gonadotropins. Mol Cell Endocrinol 125:3–19

Buchanan DL, Kurita T, Taylor JA, Lubahn DB, Cunha GR, Cooke PS (1998) Role of stromal and epithelial estrogen receptors in vaginal epithelial proliferation, stratification, and cornification. Endocrinology 139:4345–4352

Bukovsky A (2005) Can ovarian infertility be treated with bone marrow- or ovary-derived germ cells? Reprod Biol Endocrinol 3:36-http://www.rbej.com/content/3/1/36

Bukovsky A (2006a) Immune system involvement in the regulation of ovarian function and augmentation of cancer. Microsc Res Tech 69:482–500

Bukovsky A (2006b) Oogenesis from human somatic stem cells and a role of immune adaptation in premature ovarian failure. Curr Stem Cell Res Ther 1:289–303

Bukovsky A, Presl J (1977) Origin of "definitive" oocytes in the mammal ovary. Cesk Gynekol 42:285–294

Bukovsky A, Presl J (1979) Ovarian function and the immune system. Med Hypotheses 5:415–436

Bukovsky A, Keenan JA, Caudle MR, Wimalasena J, Upadhyaya NB, Van Meter SE (1995) Immunohistochemical studies of the adult human ovary: possible contribution of immune and epithelial factors to folliculogenesis. Am J Reprod Immunol 33:323–340

Bukovsky A, Caudle MR, Keenan JA, Upadhyaya NB, Van Meter S, Wimalasena J, Elder RF (2001) Association of mesenchymal cells and immunoglobulins with differentiating epithelial cells. BMC Dev Biol 1:11-http://www.biomedcentral.com/1471-213X/1/11

Bukovsky A, Caudle MR, Cekanova M, Fernando RI, Wimalasena J, Foster JS, Henley DC, Elder RF (2003a) Placental expression of estrogen receptor beta

and its hormone binding variant – comparison with estrogen receptor alpha and a role for estrogen receptors in asymmetric division and differentiation of estrogen-dependent cells. Reprod Biol Endocrinol 1:36-http://www.rbej.com/content/1/1/36

Bukovsky A, Cekanova M, Caudle MR, Wimalasena J, Foster JS, Henley DC, Elder RF (2003b) Expression and localization of estrogen receptor-alpha protein in normal and abnormal term placentae and stimulation of trophoblast differentiation by estradiol. Reprod Biol Endocrinol 1:13-http://www.rbej.com/content/1/1/13

Bukovsky A, Caudle MR, Svetlikova M, Upadhyaya NB (2004) Origin of germ cells and formation of new primary follicles in adult human ovaries. Reprod Biol Endocrinol 2:20-http://www.rbej.com/content/2/1/20

Bukovsky A, Caudle MR, Svetlikova M, Wimalasena J, Ayala ME, Dominguez R (2005a) Oogenesis in adult mammals, including humans: a review. Endocrine 26:301–316

Bukovsky A, Svetlikova M, Caudle MR (2005b) Oogenesis in cultures derived from adult human ovaries. Reprod Biol Endocrinol 3:17-http://www.rbej.com/content/3/1/17

Bukovsky A, Copas P, Virant-Klun I (2006) Potential new strategies for the treatment of ovarian infertility and degenerative diseases with autologous ovarian stem cells. Expert Opin Biol Ther 6(4):341–365

Byskov AG, Skakkebaek NE, Stafanger G, Peters H (1977) Influence of ovarian surface epithelium and rete ovarii on follicle formation. J Anat 123:77–86

Castellucci M, Kaufmann P (1995) Basic structure of the villous trees. In: Benirschke K, Kaufmann P (eds) Pathology of the human placenta, 3rd edn. Springer, Berling Heidelberg New York, pp 57–115

Castracane VD, Goldzieher JW (1986) The relationship of estrogen to placental steroidogenesis in the baboon. J Clin Endocrinol Metab 62:1163–1166

Cayouette M, Raff M (2002) Asymmetric segregation of Numb: a mechanism for neural specification from Drosophila to mammals. Nat Neurosci 5:1265–1269

Conboy IM, Rando TA (2002) The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3:397–409

Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB, Cunha GR (1997) Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci USA 94: 6535–6540

Cox RT, Spradling AC (2003) A Balbiani body and the fusome mediate mitochondrial inheritance during Drosophila oogenesis. Development 130:1579–1590

Diel P (2002) Tissue-specific estrogenic response and molecular mechanisms. Toxicol Lett 127:217–224

Esterman A, Greco MA, Mitani Y, Finlay TH, Ismail-Beigi F, Dancis J (1997) The effect of hypoxia on human trophoblast in culture: morphology, glucose transport and metabolism. Placenta 18:129–136

French MB, Koch U, Shaye RE, McGill MA, Dho SE, Guidos CJ, McGlade CJ (2002) Transgenic expression of numb inhibits notch signaling in immature thymocytes but does not alter T cell fate specification. J Immunol 168:3173–3180

Gillett WR (1991) Artefactual loss of human ovarian surface epithelium: potential clinical significance. Reprod Fertil Dev 3:93–98

Huttner WB, Kosodo Y (2005) Symmetric versus asymmetric cell division during neurogenesis in the developing vertebrate central nervous system. Curr Opin Cell Biol 17:648–657

Jan YN, Jan LY (1998) Asymmetric cell division. Nature 392:775–778

Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL (2005) Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 122:303–315

Kanai M, Shiozawa T, Xin L, Nikaido T, Fujii S (1998) Immunohistochemical detection of sex steroid receptors, cyclins, and cyclin-dependent kinases in the normal and neoplastic squamous epithelia of the uterine cervix. Cancer 82:1709–1719

Klein J (1982) Immunology: the science of self-nonself discrimination. Wiley, New York

Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF (1986) Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 118:1567–1582

Klotz DM, Hewitt SC, Ciana P, Raviscioni M, Lindzey JK, Foley J, Maggi A, DiAugustine RP, Korach KS (2002) Requirement of estrogen receptor-alpha in insulin-like growth factor-1 (IGF-1)-induced uterine responses and in vivo evidence for IGF-1/estrogen receptor cross-talk. J Biol Chem 277:8531–8537

Kneussl ES, Ances IG, Albrecht ED (1982) A specific cytosolic estrogen receptor in human term placenta. Am J Obstet Gynecol 144:803–809

Kraut R, Chia W, Jan LY, Jan YN, Knoblich JA (1996) Role of inscuteable in orienting asymmetric cell divisions in Drosophila. Nature 383:50–55

Lintern Moore S, Pantelouris EM (1975) Ovarian development in athymic nude mice. The size and composition of the follicle population. Mech Ageing Dev 4:385–390

Lobo RA (1997) Endocrinology of pregnancy. In: Lobo RA, Mishell DR Jr, Paulson RJ, Shoupe D (eds) Mishell's textbook of infertility, contraception, and reproductive endocrinology. Blackwell Science, Malden, Massachusetts, pp 183–206

Makabe S, Iwaki A, Hafez ESE, Motta PM (1980) Physiomorphology of fertile and infertile human ovaries. In: Motta PM, Hafez ESE (eds) Biology of the ovary. Martinus Nijhoff Publishers, The Hague, pp 279–290

McKenzie PP, Foster JS, House S, Bukovsky A, Caudle MR, Wimalasena J (1998) Expression of G_1 cyclins and cyclin-dependent kinase-2 activity during terminal differentiation of cultured human trophoblast. Biol Reprod 58:1283–1289

Motta PM, Makabe S (1982) Development of the ovarian surface and associated germ cells in the human fetus. Cell Tissue Res 226:493–510

Motta PM, Makabe S (1986) Germ cells in the ovarian surface during fetal development in humans. A three-dimensional microanatomical study by scanning and transmission electron microscopy. J Submicrosc Cytol 18:271–290

Motta PM, Van Blerkom J, Makabe S (1980) Changes in the surface morphology of ovarian 'germinal' epithelium during the reproductive cycle and in some pathological conditions. J Submicrosc Cytol 12:407–425

Nishizuka Y, Sakakura T (1969) Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. Science 166:753–755

Pepe GJ, Albrecht ED (1999) Regulation of functional differentiation of the placental villous syncytiotrophoblast by estrogen during primate pregnancy. Steroids 64:624–627

Pepling ME, Spradling AC (1998) Female mouse germ cells form synchronously dividing cysts. Development 125:3323–3328

Peters H, McNatty KP (1980) The ovary. A correlation of structure and function in mammals. University of California Press, Berkeley and Los Angeles, California

Petersen PH, Zou K, Hwang JK, Jan YN, Zhong W (2002) Progenitor cell maintenance requires numb and numblike during mouse neurogenesis. Nature 419:929–934

Petraglia F, de Micheroux AA, Florio P, Salvatori M, Gallinelli A, Cela V, Palumbo MA, Genazzani AR (1995) Steroid-protein interaction in human placenta. J Steroid Biochem Mol Biol 53:227–231

Rebar RW (1982) The thymus gland and reproduction: do thymic peptides influence the reproductive lifespan in females? J Am Geriatr Soc 30:603–606

Sachs ES, Jahoda MG, Niermeijer MF, Galjaard H (1977) An unexpected high frequency of trisomic fetuses in 229 pregnancies monitored for advanced maternal age. Hum Genet 36:43–46

Shanker YG, Rao AJ (1997) Regulation of progesterone biosynthesis in the human placenta by estradiol 17 beta and progesterone. Biochem Mol Biol Int 43: 591–599

Shen Q, Temple S (2002) Creating asymmetric cell divisions by skewing endocytosis. Sci STKE 2002:E52

Shen Q, Zhong W, Jan YN, Temple S (2002) Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. Development 129:4843–4853

Shih IM, Kurman RJ (1998) Ki-67 labeling index in the differential diagnosis of exaggerated placental site, placental site trophoblastic tumor, and choriocarcinoma: a double immunohistochemical staining technique using Ki-67 and MelCAM antibodies. Hum Pathol 29:27–33

Simkins CS (1928) Origin of the sex cells in man. Am J Anat 41:249–253

Simkins CS (1932) Development of the human ovary from birth to sexual maturity. J Anat 51:465–505

Skinner SM, Dunbar BS (1992) Localization of a carbohydrate antigen associated with growing oocytes and ovarian surface epithelium. J Histochem Cytochem 40:1031–1036

Sugiyama-Nakagiri Y, Akiyama M, Shibata S, Okano H, Shimizu H (2006) Expression of RNA-binding protein Musashi in hair follicle development and hair cycle progression. Am J Pathol 168:80-92

Suh BY, Naylor PH, Goldstein AL, Rebar RW (1985) Modulation of thymosin beta 4 by estrogen. Am J Obstet Gynecol 151:544-549

Takano H, Ema H, Sudo K, Nakauchi H (2004) Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med 199: 295–302

Van Wagenen G, Simpson ME (1965) Embryology of the ovary and testis Homo sapiens and Macaca mulatta. Yale University Press, New Haven

Yamada Z, Kitagawa M, Takemura T, Hirokawa K (2001) Effect of maternal age on incidences of apoptotic and proliferative cells in trophoblasts of full-term human placenta. Mol Hum Reprod 7:1179–1185

Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S (2000) Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 408:92–96

Younes MA, Besch NF, Besch PK (1981) Estradiol and progesterone binding in human term placental cytosol. Am J Obstet Gynecol 141:170–174

Asymmetric Stem Cell Division in Development and Cancer

Emmanuel Caussinus[1] and Frank Hirth[2]

List of Abbreviations

Baz	Bazooka
Brat	Brain tumor
DaPKC	*Drosophila* atypical Protein Kinase C
Dlg	Disc Large
GMC	Ganglion Mother Cell
CNS	Central Nervous System
GSC	Germline Stem Cell
Insc	Inscuteable
Lgl	Lethal (2) giant larvae
Mira	Miranda
Pins	Partner of Inscuteable
Pon	Partner of Numb
Pros	Prospero

Abstract

Asymmetric stem cell division leads to another stem cell via self-renewal, and a second cell type which can be either a differentiating progenitor or a postmitotic cell. The regulation of this balanced process is mainly achieved by polarization of the stem cell along its apical-basal axis and the basal localization and asymmetric segregation of cell fate determinants solely to the differentiating cell. It has long been speculated that disturbance of this process can induce a cancer-like state. Recent molecular genetic evidence in *Drosophila melanogaster* suggests that impaired polarity formation in neuroblast stem cells results in symmetric stem cell divisions, whereas defects in progenitor cell differentiation leads to mutant cells that are unable to differentiate but rather continue to proliferate. In both cases, the net result is unrestrained self-renewal of mutant stem cells, eventually leading to hyperproliferation and malignant neoplastic tissue formation. Thus, deregulated stem cells can play a pivotal role in *Drosophila* tumor formation. Moreover, recent evidence suggests that so-called cancer stem cells may drive the growth and metastasis

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of human tumors too. Indeed, cancer stem cells have already been identified in leukemia, and in solid tumors of the breast and brain. In addition, inappropriate activation of pathways promoting the self-renewal of somatic stem cells including defects in asymmetric cell division has been shown to cause neoplastic proliferation and cancer formation. Taken together, these data indicate that evolutionary conserved mechanisms regulate stem and progenitor cell self-renewal and tumor suppression via asymmetric cell division control.

1 Introduction

Stem cells are defined by two characteristic features: their ability to self-renew and to generate mature cells through differentiation. The majority of stem cells are found in the developing organism, starting with the totipotent zygote and subsequently generating pluripotent stem cells of the early embryo. Later during germ layer formation and organogenesis, stem cells become increasingly restricted in their lineage potential and give rise to progeny that comprise specific, mature tissue (Eckfeldt et al. 2005). Accordingly, development is driven by stem cell self-renewal and differentiation, a process which is strictly regulated in order to keep a balance between the maintenance of stem cells and the required supply of fully differentiated cells. This intricate balance is achieved by regulating the number and the mode of stem cell divisions, which can be either symmetric or asymmetric (Fig. 1A).

2 Stem Cells in Development

Symmetric stem cell divisions are common during development of both invertebrates and vertebrates, but they can also be observed in adults as exemplified by wound healing and tissue regeneration processes (Eckfeldt et al. 2005). Symmetric stem cell divisions generate two daughter cells with the same cell fate, thereby expanding the stem cell pool required for extended proliferation phases or generating two differentiating, postmitotic cells (Fig. 1A) (see, for instance, Huttner and Kosodo 2005).

Asymmetric stem cell division is a common strategy to accomplish cellular diversity during development by generating two daughter cells with different fates (Fig. 1A). This mode of division generates another stem cell by self-renewal and a second different cell type which can be either a progenitor cell or a terminally differentiated postmitotic cell. Asymmetric stem cell division can be achieved by either intrinsic or extrinsic mechanisms (Fig. 1B). Intrinsic mechanisms rely on the asymmetric localization of cell

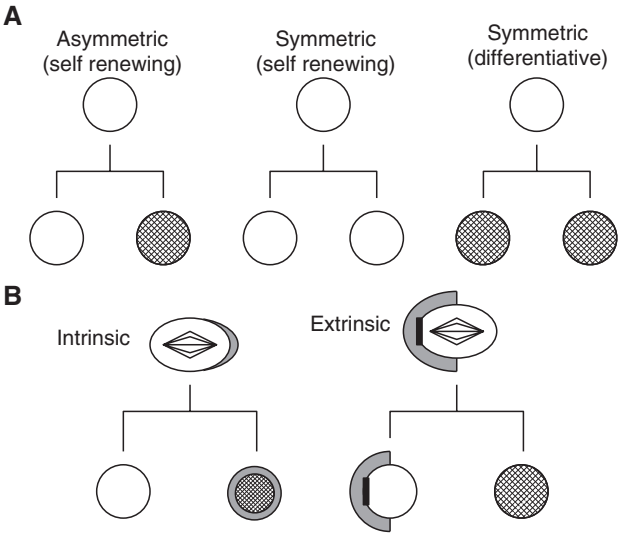

Fig. 1. A,B Regulation of stem cell division: **A** strategies for self-renewal and differentiation. Stem cells (*open circles*) can maintain a balance between self-renewal and differentiation (*squared circles*) by dividing asymmetrically. Each stem cell generates another stem cell and a sibling daughter cell destined to differentiate. Alternatively, each stem cell divides symmetrically either generating two daughter stem cells in order to expand the stem cell pool (proliferative) or two differentiating, postmitotic daughter cells (differentiative); **B** intrinsic vs extrinsic regulation of asymmetric stem cell division. Intrinsic regulation relies on the asymmetric segregation of intracellular cell fate determinants (*grey crescent and circle*) to only one daughter cell. Extrinsic mechanisms involve intercellular communications (*black rectangles*) between a niche (*grey horseshoe*) and the stem cell itself. The niche provides support and stimuli necessary for self-renewal and to prevent differentiation; hence, the daughter cell becoming located adjacent to the niche will maintain a stem cell status, whereas its sibling lacking contact to the niche is forced into cell cycle exit and differentiation

fate determinants and the orientation of the mitotic spindle to allow asymmetric segregation of cell fate determinants to just one daughter cell (Hawkins and Garriga 1998; Knoblich 2001). Extrinsic mechanisms involve cell-cell communication and the asymmetric placement of daughter cells relative to external cues. This social context is often called the "stem cell niche", a cellular microenvironment which provides support and stimuli necessary for a stem cell to maintain self-renewal capacities and to prevent differentiation. Correspondingly, daughter cells lacking contact to the niche are deprived of supportive stimuli and hence are forced into cell cycle exit and differentiation.

Insights into the stem cell niche come from studies on *Drosophila* germline stem cells (GSCs), which interact with specialized somatic cells in

the niche – known as cap cells (during oogenesis in the ovary) or hub cells (during spermatogenesis in the testes). This physical interaction maintains the undifferentiated state of the stem cell and is mediated through a cadherin-catenin pathway. The GSC-cap/hub-cell interaction also regulates symmetric versus asymmetric GSC divisions by polarizing the stem cell, affecting the orientation of the mitotic spindle, and the partition of cell fate determinants in daughter cells (Li L and Xie 2005). However, detailed insights into the genetic mechanisms regulating niche-dependent stem cell self-renewal are only starting to emerge. Most of our current knowledge on the regulation of stem cell proliferation and differentiation comes from studies on the intrinsic regulation of asymmetric stem cell division in the developing central nervous system (CNS) of *Drosophila*.

2.1 Stem and Progenitor Cells in Drosophila Neurogenesis

The *Drosophila* CNS derives from neural stem cells called neuroblasts which proliferate during two neurogenic periods, one in the embryo and another during larval life (Campos-Ortega and Hartenstein 1997). In the embryonic neurogenic period, individual neuroblasts delaminate in a stereotyped pattern from the neuroectoderm and divide repeatedly and asymmetrically to generate a new neuroblast and a smaller daughter cell, called a ganglion mother cell (GMC) (Skeath and Thor 2003). Each GMC is a transient intermediate progenitor cell that generally divides once to produce a pair of lineage-specific postmitotic ganglion cells (neurons or glia), which subsequently initiate their differentiation processes. Thus, embryonic neuroblasts are stem cells intrinsically polarized along their apicobasal axis that use asymmetric cell divisions in order to generate neurons and glial cells (Fig. 2A). They divide no more than 12 times (Bossing et al. 1996) and shrink with each division, possibly causing cell cycle exit simply because they become too small (Fuse et al. 2003). Towards the end of embryogenesis, most neuroblasts stop proliferating and enter a period of quiescence. During the second, larval neurogenic period, most neuroblasts resume proliferation and generate the majority of the cells that comprise the central brain and ventral ganglia of the adult (Maurange and Gould 2005).

Drosophila neuroblasts and GMCs differ in many aspects: neuroblasts are usually much bigger than GMCs; neuroblasts are located apically and remain associated with the neuroepithelial layer (during embryogenesis) and the cortical layer (during larval life), whereas GMCs and their progeny migrate basally to the interior of the developing CNS; and neuroblasts are mitotically more active than GMCs. In addition, neuroblast and GMC have different gene expression profiles, reflecting differential cell cycle activity and progression from proliferative activity to terminal differentiation (Ceron et al. 2001; Lee et al. 2006a; Bello et al. 2006). Thus, wildtype neural

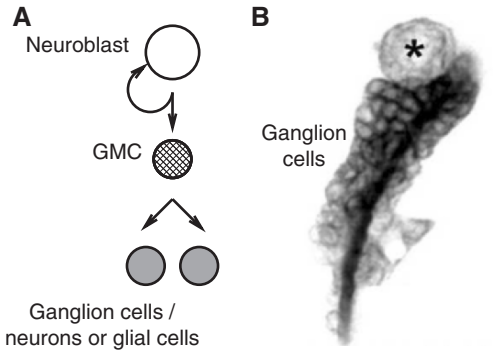

Fig. 2. A,B Asymmetric stem cell division in *Drosophila* neurogenesis: **A** neural lineage tree. Neuroblast stem cells divide asymmetrically giving rise to another neuroblast and a ganglion mother cell (GMC), which generally divides once to produce two postmitotic cells (neurons or glia in the embryo, or larval ganglion cells which subsequently differentiate into neurons or glial cells); **B** larval brain MARCM clone. Green fluorescent protein labeling shows a single large neuroblast (*asterisk*) and its associated progeny (GMCs and ganglion cells) (modified and redrawn, with permission, from Bello et al. (2006), *Development*. © 2006 Company of Biologists)

lineages of the developing CNS of *Drosophila* comprise three different cell types that differ in size, gene expression profile, cell cycle activity, and their potency to generate neural progeny: asymmetrically dividing stem cells (neuroblasts), symmetrically dividing intermediate progenitor cells (GMCs) and differentiating, postmitotic neurons or glial cells (Fig. 2A). As is the case with other stem cell lineages, neuroblast lineages of the *Drosophila* CNS are clonally related and can be visualized in the larva using mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo 2001), allowing the positive labeling of an entire neuroblast lineage with green fluorescent protein (Fig. 2B). The number of times a neuroblast and GMC divide as well as the mode of their divisions are tightly regulated and the underlying mechanisms are best understood in the embryonic CNS.

2.2 Asymmetric Stem Cell Division in the Embryonic CNS of Drosophila

In the developing embryonic CNS of *Drosophila*, asymmetric neuroblast division is regulated by a complex machinery (Fig. 3) that includes several components involved in (1) neuroblast polarization along an apical-basal axis, (2) orientation of the mitotic spindle along the apical-basal axis, and during cytokinesis (3) the basal localization and asymmetric segregation of cell fate determinants solely to the GMC.

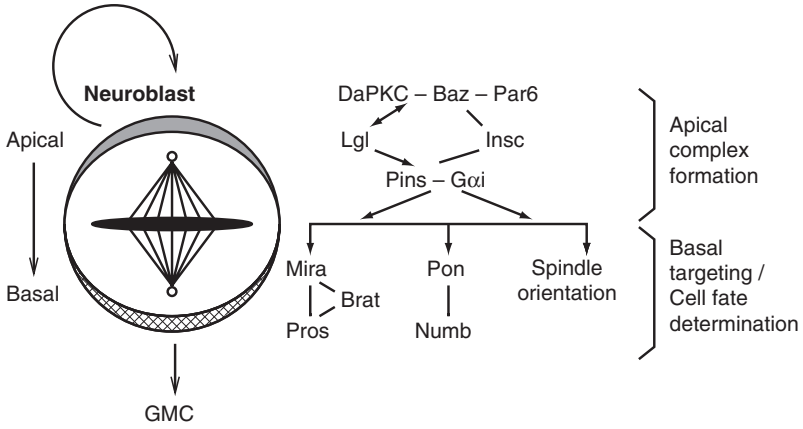

Fig. 3. Molecular machinery involved in asymmetric neuroblast division. Dividing neuroblast stem cells are polarized along the apical-basal axis, with Lgl, DaPKC, Baz, Par6, Insc, Pins, and Gαi localized to the apical cortex (*grey crescent*) where they form a functional apical complex: Lgl and Pins interact with DaPKC, DaPKC and Par6 bind to Baz, and Baz binds to Insc; Pins also physically interacts with Insc and Gαi. Apical complex formation in turn enables asymmetric segregation of the basal components Mira/Brat/Pros and Pon/Numb (*squared crescent*) and mitotic spindle orientation, resulting in progenitor cell fate determination. Thus, during asymmetric neuroblast division, apical complex formation and basal targeting simultaneously ensure stem cell self-renewal (apical) and the formation of a differentiating GMC (basal) (modified after Chia and Wang 2002)

For neuroblasts, both the orientation of their mitotic spindles along their apical–basal axis, as well as the asymmetric localization of the cell fate determinants to the basal cortex, depend on the formation and maintenance of an evolutionary conserved protein complex known as the Par complex. This protein complex consists of *Drosophila* atypical protein kinase C, (DaPKC) and two PDZ domain-containing proteins, Bazooka (Baz, a *Drosophila* homologue of the nematode Par-3), and DmPar6 (Wodarz and Huttner 2003). The Par complex co-localizes with the neuroblast-specific protein Inscuteable (Insc), thereby establishing and preserving an apical-basal polarity in delaminating neuroblasts. During mitosis, the Insc/Par complex localizes to the apical cortex as a crescent and recruits another evolutionarily conserved protein complex, which comprises a protein with multiple tetratricopeptide repeats, 'Partner of Inscuteable' (Pins) and a subunit of the heterotrimeric G protein complex Gαi. Mutations affecting apical complex components lead to defects in mitotic spindle orientation and mislocalization of basal components in dividing neuroblasts (for review see Wang and Chia 2005). These apical complex components are therefore key

molecules, which act to facilitate apical–basal spindle orientation as well as the asymmetric localization and segregation of cell fate determinants.

In addition to the apical components, three other classes of molecules have been identified which act to facilitate the basal localization of cell fate determinants but are not required for apical complex formation; these molecules include the so called 'adaptor molecules', two tumour suppressors as well as two myosins. Thus, the apical complex directs the basal localization and segregation of cell-fate determinants, such as Prospero (Pros) and Numb and their adaptor proteins, Miranda (Mira) and Partner of Numb (Pon) through two cortically localized tumor suppressors, Discs large (Dlg) and Lethal (2) giant larvae (Lgl). Phosphorylation of Lgl by apically localized DaPKC leads to Lgl inactivation, while Lgl activity restricts myosin II activity to the apical cortex, resulting in the "push" of cell-fate determinants to the basal cortex. In contrast to the function of Myosin II in excluding cell-fate determinants from the apical cortex, Myosin VI (also termed Jaguar) positively regulates basal localization and segregation of Mira/Pros via vesicle transport (for review see Wang and Chia 2005).

The ultimate goal of this machinery is the segregation of cell fate determinants like Pros into only one daughter cell that will become an intermediate progenitor, the GMC, which is thereby destined to differentiate into neurons or glial cells. Pros is a homeodomain-containing transcription factor that is transcribed and translated in dividing neuroblasts where it localizes as crescent to the basal cell cortex. With the help of the adaptor protein Mira as well as the tripartite motif protein Brain tumor (Brat) (Arama et al. 2000; Sonoda and Wharton 2001; Frank et al. 2002; Loop et al. 2004), Pros segregates preferentially to the basal GMC progeny. Once segregated to the GMC daughter cell, Mira is rapidly degraded from the cell cortex and Pros translocates to the GMC nucleus, where it is required for the repression of stem cell-specific genes and the simultaneous transcriptional activation of a cellular differentiation program (Li L and Vaessin 2000). Thus, the net result of an asymmetrically segregated cell fate determinant like Pros is the restriction of GMC proliferation to one terminal division, hence to ensure cell cycle exit and its ultimate differentiation into neurons or glial cells. Correspondingly, the exclusive segregation of Pros to only the GMC allows the concurrent limitation of self-renewing capacities solely to the stem cell neuroblast.

2.3 Cell Polarity During Postembryonic Stem Cell Division in the Drosophila CNS

The asymmetrically dividing embryonic neuroblasts of *Drosophila* arrest their cell cycle after the completion of embryogenesis and remain quiescent until after larval hatching, when they enlarge before the initiation of their

first S-phase and re-enter the cell cycle (Maurrange and Gould 2005). Larval neuroblasts can divide symmetrically or asymmetrically. In early third-instar larvae, neuroblasts preferentially divide symmetrically whereas in late third-instar larvae, they divide asymmetrically to generate two daughter cells with different cell size and cell fate, a neuroblast and a GMC, similar to embryonic neuroblasts.

Larval neuroblast asymmetric divisions are far less well studied but they share a number of similarities with embryonic neuroblasts; they express genes such as *deadpan*, *worniu*, and cortical *Miranda*, whereas GMCs show only transient expression of *worniu* and cortical *Miranda*. BrdU incorporation and immunolabelling with antibodies against mitotic markers such as phosphorylated Histone H3 reveal that only neuroblasts and GMCs are actively engaged in the cell cycle, whereas GMC progeny has exit the cycle and undergoes differentiation. These postmitotic ganglion cells express specific differentiation markers, such as Elav or Pros, which are specifically found in the nuclei of ganglion cells. However, in contrast to embryonic neuroblasts, larval neuroblasts do not possess a clear apical–basal orientation to the surface of the brain, but remain polarized in different orientations (Ceron et al. 2001; Akong et al. 2002; Lee et al. 2006a; Bello et al. 2006).

A number of molecules that play a role in asymmetric division of embryonic neuroblasts are also involved in larval neuroblast divisions. These include components known to act in the establishment of apical-basal polarity, namely DaPKC (Rolls et al. 2003; Lee et al. 2006a), Insc (Ceron et al. 2001), and Pins (Lee et al. 2006a). Mira and Numb localize asymmetrically to form cortical crescents during larval neuroblast divisions and segregate solely to the GMC following cytokinesis (Ceron et al. 2001; Bello et al. 2006). However, Pros protein and mRNA are not consistently observed in dividing larval neuroblasts, whereas Pros protein is transiently detectable in the nuclei of terminally dividing GMCs and is persistently detectable in differentiating, postmitotic ganglion cells of the larval CNS (Ceron et al. 2001; Akong et al. 2002; Bello et al. 2006).

Although it is not clear yet whether similar mechanisms regulate asymmetric cell division in embryonic and larval neuroblasts, several studies indicate that neuroblast polarization, mitotic spindle orientation as well as basal localization and asymmetric segregation of cell fate determinants also characterize dividing larval neuroblasts (Ceron et al. 2001; Rolls et al 2003; Albertson and Doe 2003). Strikingly, though, mutational inactivation of molecules involved in asymmetric division of larval neuroblasts not only affect mitotic spindle orientation, the proper segregation of cell fate determinants, and daughter cell size (Rolls et al 2003; Albertson and Doe 2003), but they also cause phenotypes displaying characteristic cancer-like features typical for malignant neoplasm which had already been described in *Drosophila* more than three decades ago.

3
Malignant Neoplasm of Genetic Origin in *Drosophila*

In 1967, Gateff and Schneidermann reported on the occurrence of a spontaneous, recessive-lethal mutation in *Drosophila* which resulted in a tumor-like phenotype in the larva. Developmental analysis revealed that the primordia of the adult integument, the imaginal discs, represented lethal, transplantable, and noninvasive neoplasm, and that the presumptive adult optic centers of the larval brain developed into a malignant neuroblastoma (Gateff and Schneiderman 1967). The subsequent mendelian analysis and molecular cloning of the genetic locus demonstrated that the mutation was a hypomorphic allele of Lgl (Gateff 1978a; Mechler et al. 1985). Thus, *Drosophila* provided the first example of a tumor suppressor gene, and since then more than 80 genes have been identified whose inactivation produce heritable tumors in various larval tissues of *Drosophila* (Potter et al. 2000). From the analysis of the *Drosophila* and human genomic sequences, it has become apparent that homologues of many cancer-causing genes exist in *Drosophila* including oncogenes and tumor suppressors, as well as members of signaling pathways involved in cancer formation (Fortini et al. 2000; Brumby and Richardson 2005).

Drosophila tumors have been described in the larva and the adult, affecting cells and tissues such as the blood cells, epithelial cells of the imaginal discs, larval CNS tissue and the male and female adult germ cells (Watson et al. 1994; Potter et al. 2000). Mutations resulting in tumor formation can be classified as hyperplastic or neoplastic, depending on whether tissue architecture is maintained or not (Brumby and Richardson 2005). Hyperplastic mutations cause overproliferation without affecting tissue architecture, whereas neoplastic mutations such as Lgl or Dlg cause overproliferation together with loss of tissue architecture and differentiation defects. In addition, *Drosophila* neoplasm are characterized by their rapid growth in situ as well as after transplantation. These transplantation assays showed that larval tumor tissue implanted into adult wildtype host abdomen was able to actively degrade protecting basement membranes and invaded to distant sites within the host's body, including the gut, the thoracic muscles and the ovary (Gateff et al. 1993; Woodhouse et al. 1994, 1998, 2003). Clonal analyses, by which patches of mutant tissue are generated in a surrounding of essentially wild-type cells, finally demonstrated in vivo the ability of neoplastic mutations to invade and metastasize into second side tumors (Pagliarini and Xu 2003; Brumby and Richardson 2003).

Thus, *Drosophila* tumors display several characteristic features of cancer cell phenotypes that are manifestations of essential alterations in cell physiology which collectively dictate malignant growth (Hanahan and Weinberg 2000). These include impaired differentiation, self-sufficiency in growth and

proliferation, insensitivity to anti-proliferative signals, the ability to evade programmed cell death, and invasion/metastasis. In this sense, *Drosophila* tumor cells resemble several of the features that also characterize human cancer cells (Hanahan and Weinberg 2000; Brumby and Richardson 2005). Indeed, as is the case for human cancer cells, aberrant karyotypes have been described for example for Lgl-mutant tumors, including increased polyploidy and aneuploidy (Gateff 1978b). Moreover, neoplastic tumor suppressor mutants like Lgl, Dlg, and Brat are characterized by disrupted cell polarity and the adoption of migratory, invasive behavior (Woodhouse et al. 1998; Arama et al. 2000; Humbert et al. 2003; Loop et al. 2004).

3.1 Induction of Tumor Growth by Altered Stem Cell Division in Drosophila

Tumor suppressor proteins like Lgl and Dlg have been shown to act in asymmetric stem cell division (reviewed in Chia and Yang 2002; Wodarz and Huttner 2003), suggesting that impaired cell fate determination can cause tumor growth and cancer formation in the developing CNS of *Drosophila*. Recent studies using clonal analysis in larval brains as well as transplantation assays have shown that this is indeed the case. Thus, ectopic cortical localization of DaPKC or mutational inactivation of Lgl, Pins, Numb, Mira, Brat, or Pros cause neoplastic tissue formation and tumor growth in the larval CNS of *Drosophila,* showing several hallmarks of cancer. The resulting tumor tissue are characterized by pleiomorphic cells, unrestrained growth and proliferation as well as genome instability, as evidenced by a variety of karyotypic abnormalities (Caussinus and Gonzales 2005; Lee et al. 2006a, b; Betschinger et al. 2006; Bello et al. 2006).

These studies also suggest, however, that substantial differences exist between the respective tumor tissues as to which cell type is affected by the mutations and how neoplastic tissue formation takes place. Although still tentative, the available data suggest that altered function of apical complex mediators such as DaPKC, Lgl, and Pins affect neuroblast stem cell self-renewal (Lee et al. 2006a), whereas defects in members of the basal targeting machinery (Mira, Brat) or in cell fate determination (Pros, Numb) impair terminal differentiation of GMCs (Caussinus and Gonzales 2005; Lee et al. 2006b; Betschinger et al. 2006; Bello et al. 2006). Yet in both cases, the otherwise tight regulation of self-renewing capacities of either stem or progenitor cells appears to fail, resulting in malignant neoplasm and hence clonally related brain tumor formation, and upon transplantation, into invasion and metastasis.

Thus, clonal analysis in *Drosophila* larval brains showed that Pins mutant neuroblasts rapidly fail to self-renew, resulting in a marked decrease of neuroblast numbers that is not due to neuroblast cell death but to the occurrence of GMC/GMC siblings and in turn to the premature

termination of neuroblast lineage formation (Lee et al. 2006a). In contrast, however, when transplanted into wildtype host, trans-heterozygous Pins mutant tissue results in one-fifth of the cases in tumor formation due to the uncontrolled division of neuroblast stem cells (Caussinus and Gonzalez 2005). Lgl mutant neural lineages result in multiple neuroblasts due to occasional ectopic self-renewal (Rolls et al. 2003; Lee et al. 2006a), whereas Lgl Pins double mutant neuroblasts all divide symmetrically to self-renew, filling the brain with neuroblasts at the expense of differentiating ganglion cells (Lee et al. 2006a). These data suggest that Lgl inhibits stem cell self-renewal, whereas Pins has dual functions in promoting and inhibiting stem cell self-renewal (Fig. 4A).

The key factor to understand Lgl and Pins activity appears to be DaPKC (Rolls et al. 2003) whose sub-cellular localization is not only maintained in Lgl Pins double mutants but shows ectopic uniform cortical distribution, suggesting that both Lgl and Pins are required to restrict DaPKC to the apical neuroblast cortex. Indeed, overexpression of a membrane-targeted DaPKC but not a kinase-dead mutant isoform results in increased numbers of larval brain neuroblasts, whereas a decrease in DaPKC expression reduces neuroblast stem cell numbers. Genetic interaction experiments finally show that Lgl DaPKC double mutants have normal numbers of neuroblasts and that DaPKC is fully epistatic to Lgl, suggesting that DaPKC directly promotes neuroblast self-renewal (Fig. 4A) (Lee et al. 2006a).

3.2 Drosophila Stem Cell Self-Renewal and Tumor Suppression

The results by Lee et al. (2006a) demonstrate that asymmetric cell division provides a mechanism to regulate the balance between stem cell maintenance and the required supply of fully differentiated cells during larval neurogenesis of *Drosophila*. Moreover, these data together with earlier results on the tumor suppressor activity of Lgl (Gateff 1978a; Woodhouse et al. 1998; Rolls et al. 2003) indicate that a breakdown of neuroblast asymmetry leads to symmetric, proliferative stem cell divisions and hence impaired differentiation. This provides compelling evidence that unrestrained stem cell self-renewal can cause overproliferation phenotypes and neoplastic tissue formation in *Drosophila*. In this sense, impaired asymmetric cell division and in turn errors in the process of normal differentiation can be regarded as initiating events in the formation of malignant tumors (Harris 2005). It is therefore conceivable that mutations affecting other components of the asymmetric cell division machinery may also cause comparable cancer-like phenotypes. Indeed, mutational inactivation of basal components involved in asymmetric neuroblast division also result in hyper-proliferation and malignant neoplasm of the larval CNS in *Drosophila*.

Using transplantation assays, Caussinus and Gonzalez (2005) showed that pieces of brains from larvae carrying homozygous Numb, Mira, or

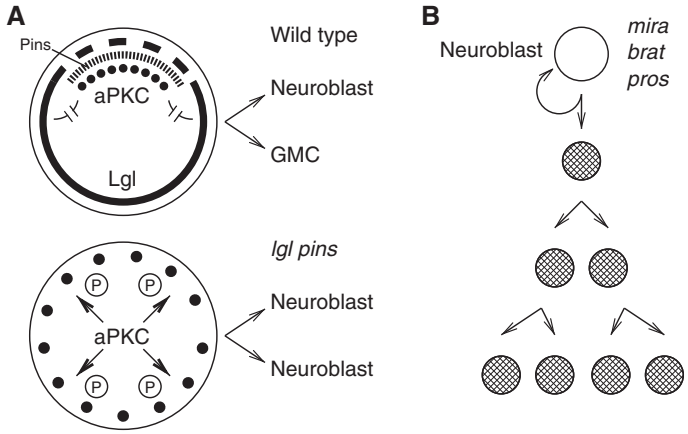

Fig. 4. A,B Asymmetric cell division regulates stem/progenitor cell self-renewal and tumor suppression in Drosophila: **A** DaPKC, Lgl, Pins regulate neural stem cell self-renewal and tumor suppression. In this model, Pins anchors DaPKC apically and Lgl inhibits DaPKC localization basally, whereas DaPKC can phosphorylate and directly inhibit Lgl function, suggesting a mutual inhibition between Lgl and DaPKC that results in stabilization of apical DaPKC localization. During asymmetric neuroblast division, the net result of this mutual inhibition is the reliable partitioning of DaPKC to only the resulting neuroblast (and not the GMC sibling), and hence the inheritance of self-renewing capacities solely to the stem cell. Defects in apical complex formation (Lgl mutant, Lgl Pins double mutant, or ectopic cortical DaPKC accumulation) lead to symmetric neuroblast stem cell division and impaired differentiation, eventually causing tumor formation (modified after Lee et al. 2006a); **B** Mira, Brat, and Pros regulate neural progenitor cell self-renewal and tumor suppression. In asymmetrically dividing neuroblast stem cells, Mira and Brat regulate basal targeting of the GMC cell fate determinant Pros, thereby restricting GMC proliferation to one terminal division, hence to ensure cell cycle exit and its ultimate differentiation into neurons or glial cells. Impaired basal targeting and defective cell fate determination lead to excessive numbers of over-proliferating mutant progenitor cells (*squared circles*) which display limitless stem cell-like self-renewal, eventually resulting in clonally derived malignant tumors (modified after Bello et al. 2006)

Pros mutant tissue can grow to more than 100 times their original size. The resulting neoplastic tissue and second side tumors severely damaged and displaced organs in the abdomen of the host, who rarely survived beyond three weeks after transplantation. Nevertheless, the tumors could be iteratively re-transplanted into new hosts, showing that the mutant cells became immortal and proliferated endlessly. Moreover, the resulting tumor cells displayed genome instability as evidenced by a variety of karyotypic abnormalities and impaired centrosome cycles. These results indicated that similar to altered function of apical complex mediators such as DaPKC, Lgl,

and Pins, defects in the basal targeting machinery affecting Mira, or the cell fate determinants Pros or Numb, also result in de-regulated neuroblast stem cell self-renewal and malignant neoplastic tissue formation. However, based on their expression profile of cell type-specific markers, no obvious differences between any of the tumor lines were observable, and the cell types found in the tumor masses resembled neuroblasts, GMCs, and ganglion cells (Caussinus and Gonzalez 2005). Which cell type, then, leads to overproliferation and eventually malignant tumor formation due to improper cell-fate determination?

In the typical neuroblast lineage proliferation paradigm, an asymmetrically dividing neuroblast gives rise to another neuroblast and a GMC, which in turn terminally divides to generate differentiating, postmitotic ganglion cells (Fig. 2A). It is therefore conceivable, that neuroblasts mutant for Numb, Mira, or Pros divide symmetrically generating two daughter neuroblasts as is the case for impaired apical complex formation, or that neuroblasts carry on dividing asymmetrically but limitless beyond the normal number of times they divide. Yet, in wildtype, the total number of times a neuroblast stem cell divides is limited since cessation of proliferation in the developing *Drosophila* CNS occurs during puparium formation and metamorphosis (Ito and Hotta, 1992; F. Hirth, unpublished observation). The underlying genetic mechanisms are currently unknown, although for a subset of actively dividing larval neuroblasts, it has recently been shown that they are eliminated by programmed cell death, thereby limiting the number of progeny produced (Peterson et al. 2002; Bello et al. 2003). Numb, Mira, and Pros mutant transplant tissue, however, continue to proliferate in adults after many passages from host to host, suggesting that these cells are either able to escape, or that they lack cell cycle termination signals. Moreover, although neuroblasts account for most of the mitotic activity observed in these tumors, the detectable neuroblasts apparently divide asymmetrically. In addition, both neuroblasts and ganglion cells were not markedly diluted or over-represented as the tumors aged or after iterative re-transplantation from host to host (Caussinus and Gonzalez 2005). This suggests that neither symmetric nor limitless asymmetric neuroblast divisions can account for the resulting Numb, Mira, or Pros mutant neuroblastoma, since the tumors would contain either little more than neuroblasts or would retain a constant number of neuroblasts which would be diluted over time among their offspring.

A solution to this apparent paradox comes from a scenario that calls on a low frequency of neuroblast divisions resulting in two twin cells resembling stem cell-like features -be it because the preceding division is symmetric in terms of cell fate, leading to a self-renewed neuroblast and another asymmetrically dividing neuroblast-like cell, or because a neuroblast not only self-renews but also leads to a GMC that is unable to differentiate via a single terminal division but rather becomes reverted into another stem cell that continues to proliferate too. Although it is not clear yet whether one or both of these possibilities are the causing event, recent studies using mosaic

analysis of Brat, Mira, and Pros mutant clones in larval brains of *Drosophila* suggest that impaired basal targeting and defective cell fate determination during asymmetric neuroblast division lead to the formation of malignant neoplasm due to excessive numbers of overproliferating neural progenitor cells (Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b).

3.3 Induction of Tumor Growth by Impaired Progenitor Cell Differentiation in Drosophila

Clonal analysis and genetic interaction studies show that the tumor suppressor Brat and the adaptor molecule Mira are required for asymmetric segregation of the cell fate determinant Pros and hence the restriction of GMC proliferation to one terminal division. Moreover, impaired Brat, Mira, or Pros activity results in impaired GMC cell fate determination eventually leading to neoplastic tissue formation and the concomitant loss of differentiated ganglion cells in the larval brain of *Drosophila* (Fig. 4B) (Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b). Thus, mosaic analysis of Brat revealed that the majority of mutant clones were up to 10 times larger than wildtype control clones, comprising an excessive number of pleiomorphic cells that are unable to exit the cell cycle. A large number of Brat mutant cells continued to proliferate into adulthood and displayed mitotic activity even in three week-old adult flies (Bello et al. 2006). Detailed expression analysis of markers specific for neural lineage cell types demonstrated that Brat mutant clones comprise a wildtype-like number of asymmetrically dividing neuroblasts; yet the majority of mutant cells displayed sustained expression of neural progenitor cell markers, and simultaneously lacked marker gene expression specific for differentiating ganglion cells, indicating that Brat mutation impairs proliferation control of neural progenitors and subsequent differentiation into ganglion cells (Bello et al. 2006; Betschinger et al. 2006). Similar observations have been made for Pros and Mira mutant clones. Thus, Pros mutant clones phenocopy Brat mutant clones in that they are dramatically enlarged and essentially devoid of differentiating, postmitotic ganglion cells. Rather these clones comprise cells that display sustained expression of stem cell markers as well as increased mitotic activity (Bello et al. 2006).

Genetic rescue experiments provided evidence that Pros acts downstream of Brat in progenitor cell differentiation and brain tumor suppression (Bello et al. 2006). Using mosaic analysis, Brat mutant larval brain clones were generated that simultaneously over-expressed wildtype Brat or Pros. In both cases, the resulting clones were comparable in size, cell number, and marker gene expression to wildtype control clones (Bello et al. 2006), suggesting that loss of nuclear Pros expression in Brat mutant clones is causally related to their overproliferation phenotype. Moreover, consistent with a possible role of Mira and Brat for Pros cargo, individual Mira mutant clones generated in larval neuroblast lineages (F. Hirth,

unpublished) or depletion of functional Mira by transgenic RNAi (Betschinger et al. 2006) cause hyper-proliferation and malignant neoplastic tumor formation in the larval central brain of *Drosophila* (Fig. 4B).

A direct link between compromised inheritance of GMC cell-fate determination and unrestrained growth of larval brain neural lineages was further indicated by BrdU pulse-chase experiments (Lee et al. 2006b). BrdU incorporation is a marker of DNA replication and was used to distinguish between cells that continue to proliferate (as they initially incorporate BrdU but rapidly dilute it out during the chase period) and cells that are differentiating (as they incorporate BrdU during their terminal division and maintain BrdU during the chase period). BrdU pulse-chase experiments performed in wild-type larvae revealed that proliferative neuroblasts dilute out BrdU incorporation, whereas postmitotic ganglion cells retained BrdU incorporation. Similar experiments performed in Brat mutant larvae revealed different results depending on chase length. Thus, after a 24-h chase, large neuroblasts were surrounded by many small cells maintaining neuroblast marker gene expression and BrdU incorporation. After a 72-h chase, a decrease in BrdU+ cells were observed, with the remaining BrdU-positive cells displaying an expression profile of ganglion cells. Based on this steep decline in the number of BrdU+ cells during the 24- to 72-h chase period, and a corresponding increase in neuroblast numbers during this time, Lee et al. (2006b) proposed that Brat mutant larval neuroblast lineages generate GMC-sized progeny that are cell cycle delayed and continue to express stem cell-specific genes. Accordingly, a minority of these cells is able to acquire a GMC fate and eventually differentiate into postmitotic ganglion cells, but the majority of mutant cells appear to be transformed into neuroblast-like stem cells that are unable to exit the cell cycle (Lee et al. 2006b; Bello et al. 2006; Betschinger et al. 2006).

Finally, genetic interaction studies showed that Brat binds to the cargo binding domain of Mira and co-localizes together with Pros at the basal cortex of dividing neuroblasts (Betschinger et al. 2006; Lee et al. 2006b). Localization analysis in Mira mutants revealed that the usual asymmetric segregation of Pros and Brat into the GMC is perturbed. Conversely, in Pros or Brat mutants, Mira expression is seen in both daughter cells after neuroblast division (Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b). Furthermore, in Pros mutants, Brat expression and localization are unaffected (Lee et al. 2006b; Betschinger et al. 2006; Bello et al. 2006), whereas in Brat mutants, Pros is hardly detected in any of the cells derived from Brat mutant larval lineages (Bello et al. 2006). These results indicate that consistent with Brat being a cargo of Mira, Mira is required for Brat localization, whereas Brat together with Mira promote asymmetric segregation of Pros into the GMC where they are required for neural progenitor cell differentiation and tumor suppression in the larval brain of *Drosophila*. (Betschinger et al. 2006; Lee et al. 2006b; Bello et al. 2006). How could Brat and Pros exert such an activity in the GMC?

As has been shown in the embryonic CNS, the transcription factor Pros acts either as an activator or as a repressor of target-gene transcription, depending on the context (Li L and Vaessin 2000; Prokopenko and Chia 2005). Consistent with the occurrence of supernumerary cell divisions in Pros mutants, key cell cycle regulators like Cyclin A, Cyclin B, Cyclin E, and String (Cdc25) have been identified among the target genes that are de-repressed in Pros mutant tissue (Li L and Vaessin 2000; Bello et al. 2006). In contrast, Brat has been shown to interact with RNA binding proteins by direct protein-protein interactions and to repress translation of specific mRNAs (Sonoda and Wharton 2001). Moreover, genetic evidence suggests that one of the proteins translational repressed by Brat in wildtype neuroblasts is the transcription factor Myc (Betschinger et al. 2006). Among the targets of Myc are many genes that are involved in the RNA-polymerase I-dependent transcription of rRNA and ribosome biosynthesis, and overexpression of Myc leads to enlarged nucleoli and increased protein synthesis, which causes increase in cell size (Grewal et al. 2005). Consistent with these findings, Brat mutant cells also have enlarged nucleoli (Frank et al. 2002; Betschinger et al. 2006) and some evidence indicates that Brat mutant GMCs increase in size and eventually convert to ectopic neuroblast-like stem cells (Lee et al. 2006b).

Taken together, the available molecular genetic data in *Drosophila* provide compelling evidence that stem and progenitor cell self-renewal and tumor suppression are interconnected via asymmetric cell division control. The available data demonstrate that impaired apical complex formation in neuroblasts leads to symmetric stem cell divisions (Rolls et al. 2003; Caussinus and Gonzalez 2005; Lee et al. 2006a), whereas defects in basal targeting and progenitor cell fate determination lead to mutant cells that are unable to terminally differentiate but rather continue to proliferate (Caussinus and Gonzalez 2005; Betschinger et al. 2006; Lee et al. 2006b; Bello et al. 2006). In both cases, the net result is unrestrained self-renewal of mutant cells which display characteristic features of deregulated stem cells, eventually leading to hyperproliferation and malignant neoplastic tissue formation in *Drosophila* (Fig. 4). These data therefore demonstrate that deregulated stem cells can play a pivotal role in *Drosophila* tumor formation, and recent evidence suggests that so-called cancer stem cells may drive the growth and metastasis of solid tumors in human too (Al-Hajj and Clarke 2004).

4
Altered Stem and Progenitor Cell Self-Renewal and Cancer Stem Cells

Epithelial cancers such as cancer of the colon, breast, lung and prostate are the most common cancers in adults. In each of these tissues, the mature cells are thought to be constantly replenished by a minority population of

tissue stem cells. These stem cells give rise to a rapidly dividing population of transit-amplifying progenitor cells which finally give rise to the mature epithelial cells in the tissue (Eckfeldt et al. 2005). In most tissues, the only long-lived cells are stem cells, whereas other cells typically have a lifespan measured in days or weeks. This is in apparent contrast to cancer cells which give rise to tumors that develop over a period of months to years and like normal tissue consist of heterogeneous populations of cells. In previous models of cancer, the up-regulated growth of tumors was attributed to the serial acquisition of genetic events that resulted in the typical hallmarks of cancer (Hanahan and Weinberg 2000; Hahn and Weinberg 2002).

Recent evidence however suggests that cancers can be viewed as an abnormal organ in which tumor growth is driven by a population of cancer stem cells, which can give rise to both more cancer stem cells as well as to non-tumorigenic cancer cells (Al-Hajj and Clarke 2004; Clarke and Fuller 2006). In marked contrast to the cancer stem cells, these latter cells have either no or a markedly diminished capacity to form new tumors – because they lack the ability to self-renew. In such a cancer stem cell model, the key event in tumorigenesis is the disruption of genes involved in the regulation of self-renewal, leading to a clonally derived tumor arising from a cancer stem cell. Accordingly, the tumor cell-of-origin would originate from a stem/progenitor or more differentiated cell via acquisition of mutations that dysregulate or allow reacquisition of self-renewal mechanisms (Reya et al. 2001; Pardal et al. 2003; Fomchenko and Holland 2005). In support of this model, cancer stem cells have already been identified in leukemia, and in solid tumors of the breast and brain (Al-Hajj and Clarke 2004). Moreover, inappropriate activation of the WNT, sonic hedgehog (SHH), Notch, PTEN, and BMI1 pathways have all been shown to promote the self-renewal of somatic stem cells, as well as neoplastic proliferation in the same tissues when dysregulated (Pardal et al. 2003).

Based on these observations, it is conceivable that similar to the situation in *Drosophila*, the machinery promoting asymmetric cell division may play an evolutionary conserved role in mammalian tumor suppression. Indeed, mammalian homologues of Baz, Par6, DaPKC, Lgl, and Numb have been shown to regulate asymmetric cell fate determination and tumor suppression. Thus, mammalian aPKC, Par3, and LGN are involved in asymmetric division of basal epidermal progenitor cells of the skin and their dysregulation can lead to skin cancer (Lechler and Fuchs 2005). Moreover, there is evidence for the asymmetric segregation of vertebrate NUMB homologues (Wodarz and Huttner 2003) that seem to act as asymmetric cell fate determinants. Double knockouts of Numb and Numb-like in the mice dorsal forebrain have been found to lead to impaired neuronal differentiation, hyperproliferation of neural progenitors, and delayed cell-cycle exit (Li HS et al. 2003). In addition, loss of Lgl1/Mlgl/Hugl, one of the two Lgl homologues in mice, results in failure to asymmetrically localize the fate determinant Numb and leads to severe brain dysplasia as neural progenitor cells fail to exit the cell cycle (Klezovitch et al. 2004). Reciprocally, a well-characterized

human tumor suppressor, the kinase Lkb1, whose loss causes the Peutz-Jeghers syndrome, has turned out to be a regulator of cell polarity in worms, flies and humans and might be involved in asymmetric cell division as well (Marignani 2005). Thus, similar to the situation in *Drosophila*, impaired asymmetric cell division and in turn defects in stem or progenitor cell self-renewal appear to be causally related to cancer formation in mammals.

5 Conclusion

Molecular genetic data from *Drosophila* neural stem and progenitor cell proliferation control together with emerging evidence from studies in mammals indicate that evolutionary conserved genetic mechanisms regulate stem and progenitor cell self-renewal and tumor suppression via asymmetric cell division. These studies show that breakdown of asymmetry in dividing *Drosophila* neuroblast stem cells leads to symmetric, proliferative divisions and hence impaired differentiation. Moreover, impaired basal targeting and defective cell fate determination during asymmetric neuroblast division in *Drosophila* lead to the formation of malignant neoplasm due to excessive numbers of over-proliferating mutant progenitor cells. In addition, recent data in human suggest that so-called cancer stem cells may cause leukemia, and solid tumors of the breast and brain. These studies therefore provide evidence that failure of the otherwise tight regulation of self-renewing capacities of either stem or progenitor cells can result in neoplasm, indicating that impaired asymmetric cell division and in turn errors in the process of normal differentiation can be initiating events in the formation of malignant tumors.

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References

Akong K, McCartney BM, Peifer M (2002) *Drosophila* APC2 and APC1 have overlapping roles in the larval brain despite their distinct intracellular localizations. Dev Biol 250:71–90

Albertson R, Doe CQ (2003) Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. Nat Cell Biol 5:166–170

Al-Hajj M, Clarke MF (2004) Self-renewal and solid tumor stem cells. Oncogene 23:7274–7282

Arama E, Dickman D, Kinchie Z, Shearn A, Lev Z (2000) Mutations in the beta-propeller domain of the *Drosophila* brain tumor (brat) protein induce neoplasm in the larval brain. Oncogene 19:3706–3716

Bello BC, Hirth F, Gould AP (2003) A pulse of the *Drosophila* Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. Neuron 37:209–219

Bello B, Reichert H, Hirth F (2006) The brain tumor gene negatively regulates neural progenitor cell proliferation in the larval central brain of *Drosophila*. Development 133:2639–2648

Betschinger J, Mechtler K, Knoblich JA (2006) Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila* neural stem cells. Cell 124:1241–1253

Bossing T, Udolph G, Doe CQ, Technau GM (1996) The embryonic central nervous system lineages of *Drosophila melanogaster*. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev Biol 179:41–64

Brumby AM, Richardson HE (2003) scribble mutants cooperate with oncogenic RAs or Notch to cause neoplastic overgrowth in *Drosophila*. EMBO J 22:5769–5779

Brumby AM, Richardson HE (2005) Using *Drosophila melanogaster* to map human cancer pathways. Nat Rev Cancer 5:626–639

Campos-Ortega JA, Hartenstein V (1997) The embryonic development of *Drosophila melanogaster*. Springer, Berlin Heidelberg New York

Caussinus E, Gonzalez C (2005) Induction of tumour growth by altered stem-cell asymmetric division in *Drosophila melanogaster*. Nat Genet 37:1125–1129

Ceron J, Gonzalez C, Tejedor FJ (2001) Patterns of cell division and expression of asymmetric cell fate determinants in postembryonic neuroblast lineages of *Drosophila*. Dev Biol 230:125–138

Chia W, Yang X (2002) Asymmetric division of *Drosophila* neural progenitors. Curr Opin Genet Dev 12:459–464

Clarke MF, Fuller M (2006) Stem cells and cancer: two faces of eve. Cell 124:1111–1115

Eckfeldt CE, Mendenhall EM, Verfaillie CM (2005) The molecular repertoire of the 'almighty' stem cell. Nat Rev Mol Cell Bio 6:726–737

Fomchenko EI, Holland EC (2005) Stem cells and brain cancer. Exp Cell Res 306:323–329

Fortini ME, Skupski MP, Boguski MS, Hariharan IK (2000) A survey of human disease gene counterparts in the *Drosophila* genome. J Cell Biol 150:F23–F30

Frank DJ, Edgar BA, Roth MB (2002) The *Drosophila melanogaster* gene brain tumor negatively regulates cell growth and ribosomal RNA synthesis. Development 129:399–407

Fuse N, Hisata K, Katzen AL, Matsuzaki F (2003) Heterotrimeric G proteins regulate daughter cell size asymmetry in *Drosophila* neuroblast divisions. Curr Biol 13:947–954

Gateff E (1978a) Malignant neoplasm of genetic origin in *Drosophila melanogaster*. Science 200:1449–1459

Gateff E (1978b) The genetics and epigenetics of neoplasm in *Drosophila*. Biol Rev Camb Philos Soc 53:123–168

Gateff E, Schneidermann HA (1967) Developmental studies of a new mutant of *Drosophila melanogaster* lethal malignant brain tumor l(2)gl4. Am Zool 7:760

Gateff E, Loffler T, Wismar J (1993) A temperature-sensitive brain tumor suppressor mutation of *Drosophila melanogaster*: developmental studies and molecular localization of the gene. Mech Dev 41:15–31

Grewal SS, Li L, Orian A, Eisenmann RN, Edgar BA (2005) Myc-dependent regulation of ribosomal RNA synthesis during *Drosophila* development. Nat Cell Biol 7:295–302

Hahn WC, Weinberg RA (2002) Rules for making human tumor cells. N Engl J Med 347:1593–1603
Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70
Harris H (2005) A long view of fashions in cancer research. BioEssays 27:833–838
Hawkins N, Garriga G (1998) Asymmetric cell division: from A to Z. Genes Dev 12:3625–3638
Humbert P, Russell S, Richardson H (2003) Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. BioEssays 25:542–553
Huttner WB, Kosodo Y (2005) Symmetric versus asymmetric cell division during neurogenesis in the developing vertebrate central nervous system. Curr Opin Cell Biol 17:648–657
Ito K, Hotta Y (1992) Proliferation pattern of postembryonic neuroblasts in the brain of *Drosophila melanogaster*. Dev Biol 149:134–148
Klezovitch O, Fernandez TE, Tapscott SJ, Vasioukhin V (2004) Loss of cell polarity causes severe brain dysplasia in lgl1 knockout mice. Genes Dev 18:559–571
Knoblich JA (2001) Asymmetric cell division during animal development. Nat Rev Mol Cell Biol 2:11–20
Lee T, Luo L (2001) Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. Trends Neurosci 24:251–254
Lee C-Y, Robinson KJ, Doe CQ (2006a) Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature 439:594–598
Lee CY, Wilkinson BD, Siegrist SE, Wharton RP, Doe CQ (2006b) Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. Dev Cell 10:441–449
Lechler T, Fuchs E (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature 437:275–280
Li HS, Wang D, Shen Q, Schonemann MD, Gorski JA, Jones KR, Temple S, Jan LY, Jan YN (2003) Inactivation of numb and numblike in embryonic dorsal forebrain impairs neurogenesis and disrupts cortical morphogenesis. Neuron 40:1105–1118
Li L, Vaessin H (2000) Pan-neural Prospero terminates cell proliferation during *Drosophila* neurogenesis. Genes Dev 14:147–151
Li L, Xie T (2005) Stem cell niche: structure and function. Annu Rev Cell Dev Biol. 21:605–631
Loop T, Leemans R, Stiefel U, Hermida L, Egger B, Xie F, Primig M, Certa U, Fischbach KF, Reichert H, Hirth F (2004) Transcriptional signature of an adult brain tumour in *Drosophila*. BMC Genomics 5:24
Marignani PA (2005) LKB1, the multitasking tumour suppressor kinase. J Clin Pathol 58:15–19
Maurange C, Gould AP (2005) Brainy but not too brainy: starting and stopping neuroblast divisions in *Drosophila*. Trends Neurosci 28:30–36
Mechler BM, McGinnis W, Gehring WJ (1985) Molecular cloning of lethal (2) giant larvae, a recessive oncogene of *Drosophila melanogaster*. EMBO J 4:1551–1557
Pagliarini RA, Xu T (2003) A genetic screen in *Drosophila* for metastatic behaviour. Science 302:1227–1231
Pardal R, Clarke MF, Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3:895–902
Peterson C, Carney GE, Taylor BJ, White K (2002) Reaper is required for neuroblast apoptosis during *Drosophila* development. Development 129:1467–1476

Potter CJ, Turenchalk GS, Xu T (2000) *Drosophila* in cancer research; an expanding role. Trends Genet 16:33–39

Prokopenko SN, Chia W (2005) When timing is everything: role of cell cycle regulation in asymmetric division. Semin Cell Dev Biol 16:423–437

Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111

Rolls MM, Albertson R, Shih HP, Lee CY, Doe CQ (2003) *Drosophila* aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. J Cell Biol 163:1089–1098

Skeath JB, Thor S (2003) Genetic control of *Drosophila* nerve cord development. Curr Opin Neurobiol 13:8–15

Sonoda J, Wharton RP (2001) *Drosophila* brain tumour is a translational repressor. Genes Dev 15:762–773

Wang H, Chia W (2005) *Drosophila* neural progenitor polarity and asymmetric division. Biol Cell 97:63–74

Watson KL, Justice RW, Bryant PJ (1994) *Drosophila* in cancer research: the first fifty tumor suppressor genes. J Cell Sci Suppl 18:19–33

Wodarz A, Huttner WB (2003) Asymmetric cell division during neurogenesis of *Drosophila* and vertebrates. Mech Dev 120:1297–1309

Woodhouse E, Hersperger E, Stetler-Stevenson WG, Liotta LA, Shearn A (1994) Increased type IV collagenase in Lgl-induced invasive tumors of *Drosophila*. Cell Growth Differ 5:151–159

Woodhouse E, Hersperger E, Shearn A (1998) Growth, metastasis, and invasiveness of *Drosophila* tumors caused by mutations in specific tumor suppressor genes. Dev Genes Evol 207:542–550

Woodhouse EC, Fisher A, Bandle RW et al. (2003) *Drosophila* screening model for metastasis: Semaphorin 5c is required for L(2)gl cancer phenotype. Proc Natl Acad Sci USA 100:11463–11468

Asymmetric Distribution of DNA Between Daughter Cells with Final Symmetry Breaking During Aging of Human Fibroblasts

Alvaro Macieira-Coelho

Abstract

Human fibroblasts proliferating in vitro go through functional modifications, lose progressively their capacity to divide, and enter finally a post-mitotic state. These events are supposed to reproduce the developmental steps taking place in vivo during aging of the organism. The gradual changes occurring through proliferation are incompatible with an even distribution of the genetic material during cell division. We measured the amount of DNA on pairs of daughter cells at different population doubling levels of human fibroblasts. It was found that at each doubling in a significant fraction of cells, the distribution of DNA between sister cells is asymmetric. The cell system is in a steady state through the different phases of the fibroblast population life span; then during the last mitoses when the cells enter the terminal phase IV there is symmetry breaking with a phase transition, the cells settling into a new state.

1
Drift of Cell Function Through Serial Divisions

The classical experiments of Meselson and Stahl showing that the synthesis of DNA is semiconservative led to the belief that at each division each daughter cell receives the same kind of genetic information and hence that both sister cells are identical. However, when one follows a cell population during serial proliferation in vitro different parameters show that the cell functions evolve in many respects.

Human fibroblasts proliferating in vitro were extensively analyzed in this respect since they are used for the study of aging at the cellular level. During serial replications they are known to go through three phases (Hayflick and Moorhead 1961): phase I is suggested from the initial rise in the saturation densities and the morphological observations showing the appearance of a homogeneous population, phase II can be defined as the period where the fraction of non-dividing cells is relatively constant, phase

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Asymmetric Cell Division

III is characterized by the slight increase in the number of cells which do not divide during a 24-h period and by the prolongation of the population doubling time. The existence of a final phase IV was later characterized (Macieira-Coelho and Taboury 1982) corresponding to the last three or four doublings with a rapid fall of the maximal cell density and of the fraction of cells synthesizing DNA during a 24-h period; the cells enter a very slow dividing state and eventually become post-mitotic (Fig. 1). Several metabolic events occur during the last three to four doublings, which support the idea of a final phase with distinct characteristics (Macieira-Coelho 1988).

It is obvious from the evolution of the kinetics of proliferation that something must take place during cell division, which progressively changes the cells and finally creates the conditions for the final abrupt steps.

Morphological observations also lead to the conclusion that there is a drift through cell division. There is an increase in cell, nuclear and nucleolar areas (Mitsui and Schneider 1976; Bemiller and Miller 1979); moreover, three types of fibroblasts can be distinguished, I, II and III, with an increasing volume with a shift to the larger volume during the cell population life span (Steinhardt 1985). At the end there is a sudden shift to a large type IV cell (Fig. 2) (Macieira-Coelho 1983) with a significant reorganization of the cytoskeleton and decreased motility (Raes et al. 1983).

Several enzymatic changes have been reported during serial proliferation of human fibroblasts. There is a decline in the ratio between the activities of two enzymes of the purine salvage pathway, hypoxanthine-guanine transferase and adenine-phosphoribosyltransferase (Paz et al. 1981), a decline in ribonucleotide reductase (Dick and Wright 1985), and a progressive increase in the levels of the second enzyme of the pathway for de novo purine biosynthesis, glycinamide ribonucleotide synthetase (Hards and Patterson 1986).

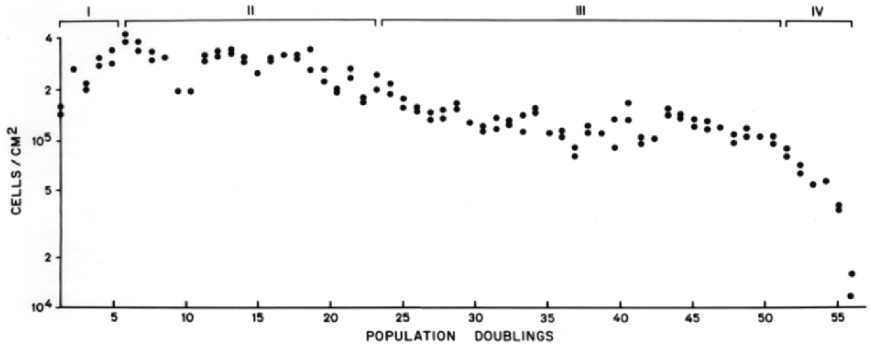

Fig. 1. Maximal cell densities recorded before each cell subcultivation during the entire life span of a human embryonic lung fibroblast line. Each *dot* corresponds to a cell count from a different culture vessel. The population doublings corresponding to Phases I, II, III and IV are indicated

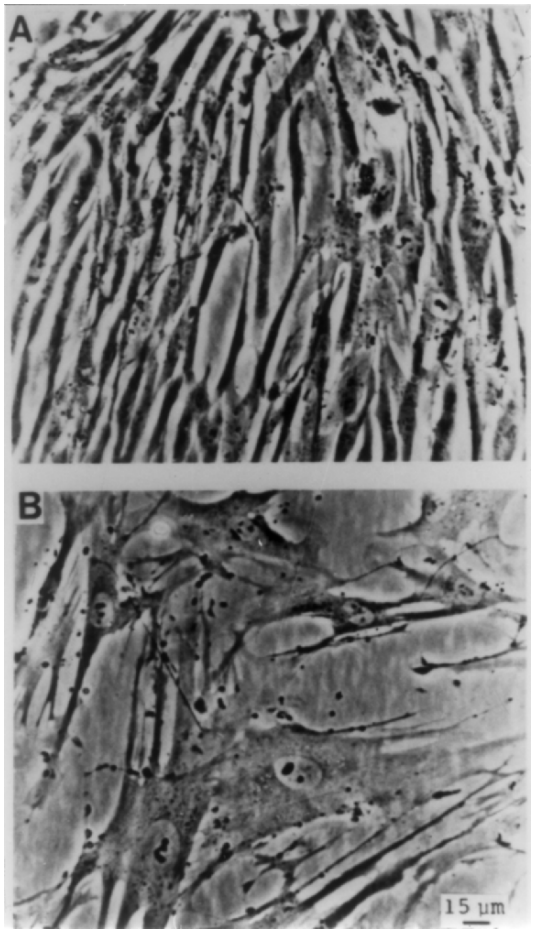

Fig. 2. A,B Morphology of the same cells used for the experiment illustrated in Fig. 3, in: **A** phase II; **B** phase IV

Another interesting enzymatic alteration is the progressive appearance of a new 3′:5′-cyclic AMP-independent histone kinase, raising the possibility that new genes become progressively activated (Kahn et al. 1982).

Changes in glycolysis seem related to the kinetics of proliferation. Between population doubling 20 and 40 glucose uptake and lactate production decrease, thereafter both glucose uptake and lactate production increase (Bittles and Harper 1984); the critical change in the biphasic curve of glucose uptake/lactate production corresponds to the transition from phase II to phase III. The same investigators also observed an increase in the specific activity of piruvate kinase, a regulatory enzyme of the glycolytic pathway.

The relative proportion of cyclin decreases progressively (Celis and Bravo 1984), and during the second half of cell population life span the responsiveness to EGF decreases corresponding to the transition between phases II and III (Kaji and Matsuo 1983).

Progressive modifications of the cell surface became apparent with the reported decline in the negative surface charge and in the activity of a pH 7.8 protease (Bosmann et al. 1976), the increase of albumin transport (Berumen and Macieira-Coelho 1977), and the decrease of the binding, uptake and degradation of low density lipoproteins due to a reduction in the number of receptor sites (Lee et al. 1982).

Several data point to genetic modifications. There is an increased heterogeneity in DNA repeat lengths (Dell'Orco et al. 1986) and decreased hybridization of the probes for the α-globin and β-actin genes (Icard-Liepkalns et al. 1986). The mutation frequencies for diphtheria toxin and thioguanine resistance increase linearly during the first 2/3 of the life span of a human fibroblast population (Gupta 1980). The expression of the EPC-1 (early population doubling cDNA-1) gene declines gradually during the fibroblast proliferation life span (Pignolo et al. 1993). Furthermore, the activity of p53, a positive transactivator of p21 gene expression, was found to increase in a stepwise fashion through the different phases (Bond et al. 1996).

Other modifications occurring during the proliferation life span of human fibroblasts have been reviewed elsewhere (Macieira-Coelho 1988).

2
Structural Reorganization of Chromatin Fibers

Ultrastructural changes accompany the shift in cell morphology; indeed the visualization of the 30-nm solenoid with a loosening procedure (Puvion-Dutilleul and Macieira-Coelho 1982) showed that the density of the 30-nm solenoid chromatin fibers decreases progressively through phases I, II and III mainly at the level of their anchorage to the lamina densa correlating with the decline in the rate of DNA synthesis initiation (Macieira-Coelho 1991).

During phase IV there is decondensation of chromatin (Puvion-Dutilleul and Macieira-Coelho 1982) and a profound reorganization of the fibers with a significant fall of the density coinciding with the dramatic changes in morphology and asymmetric events described below (Fig. 3).

To quantify the changes in the organization of the 30-nm chromatin fibers, pictures like those illustrated in Fig. 3 from cells during phases II, III and IV were screened with an image processor (Macieira-Coelho and Puvion-Dutilleul 1989). Two measurements were made; one expressed the ratio between the dark and light areas and was called the density of the fibers; the other, which was called the spacing, was obtained with a sieve-like procedure that calculated the areas between the fibers. The density of

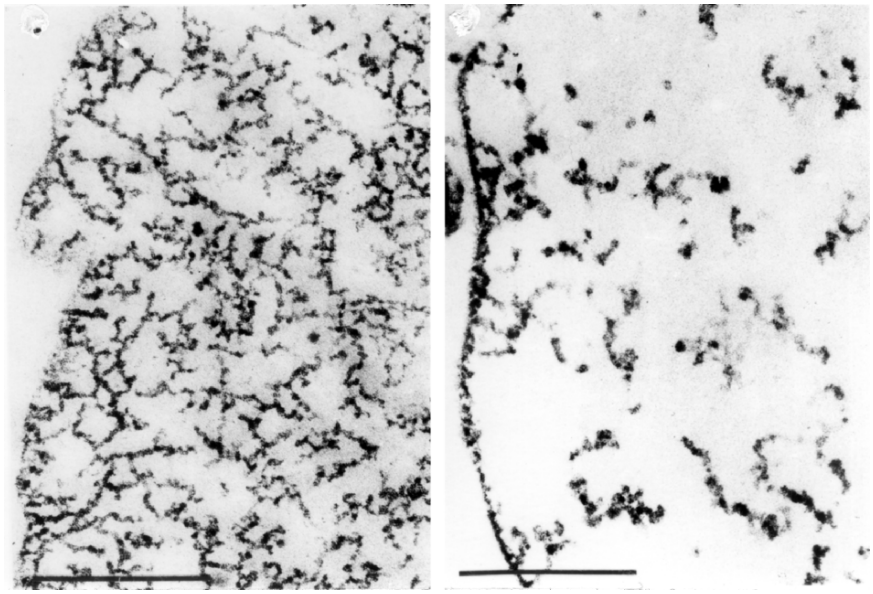

Fig. 3. Distribution of the 30-nm chromatin fibers at the periphery of the nucleus following mild loosening of phase II (*left*) and phase IV (*right*) human embryonic lung fibroblasts

the fibers, mainly at the level of their anchorage to the lamina densa, was found to decrease progressively. On the other hand, the spacing of the fibers was found to follow a two-step pattern; it varied very little during most of the cell population life span and increased abruptly at the end when the cells entered the terminal post-mitotic phase IV.

This reorganization of the 30-nm solenoid fibers has functional implications. Indeed the fall in the density correlates with the decline in the rate of DNA synthesis initiation after cell attachment and spreading of proliferating cells during phases II and III, whereas the evolution of the spacing correlates with the terminal fall in the number of cells capable of initiating DNA synthesis during phase IV (Macieira-Coelho 1991).

3
Asymmetric Distribution of DNA Between Sister Cells

The drift in cell behavior and the reorganization of chromatin fibers through serial proliferation is incompatible with an even genetic transmission through semi-conservative DNA synthesis at each division. Meselson

and Stahl experiments were performed at the level of the cell population, when the phenomenon was analyzed at the level of individual cells it became apparent that deviations from the symmetric distribution of DNA between sister cells occur with a significant frequency during cell division (Macieira-Coelho et al. 1982).

We measured with cytophotometry the DNA content of individual cells in mitosis, a period marked by exact G1 and G2 DNA contents without interference by cells in the S phase. The cells were stained with ethidium bromide or Feulgen-pararosanilin. To ensure that DNA values are the same regardless of the stage of the cell cycle, the values found on interphases were compared with those found for cells in mitosis (Maciera-Coelho et al. 1982). The distribution of the DNA contents of anaphases and telophases, and of metaphases accorded respectively with the G1 and G2 peaks found for interphases. The distribution of DNA of tetraploid anaphases and telophases corresponded to the DNA content of diploid metaphases. The amount of DNA of tetraploid metaphases (14%) corresponded to that of octaploid interphases. Thus the same amount of DNA is found by this method regardless of the geometry of the nucleus and of the packing of DNA. The percentage of cells with a tetraploid value also fitted the data obtained with chromosome counts (Macieira-Coelho et al. 1982). Identical results were found with ethidium bromide and Feulgen-pararosaniline stainings.

The DNA content of each half of anaphase pairs was compared (Fig. 4) to determine the extent of the differences in DNA content between sister cells. Differences were expressed as the percentage of the mean value of each pair. A total of 4000 anaphases were analysed after ethidium bromide staining and 350 after Feulgen staining at different population doubling levels (PDL). With both ethidium bromide and Feulgen-pararosaniline most differences in the DNA content between sister cells were below 5% of the mean of the pairs of sister cells. Repeated measurements were made on the samples stained with Feulgen to evaluate the differences attributable to methodology. The white area in Fig. 4 represents the differences between same measurements with three standard deviations. These data show that in approximately 20% of the cells, at each division, differences between sister cells are significant and not due to methodological errors (Macieira-Coelho et al. 1982). Hence in a large proportion of cells, at each population doubling, the distribution of DNA between sister cells is asymmetric. The distributions of the differences were identical throughout the cell population life span, only during the terminal two to three doublings was an increase found in the number of pairs with large differences (Macieira-Coelho et al. 1982).

If the packing of DNA during the mitotic periods does not interfere with the measurements, the DNA content of anaphases and telophases should be identical and should be the same as that of metaphases when the DNA content of the latter is halved. This was evaluated by plotting on

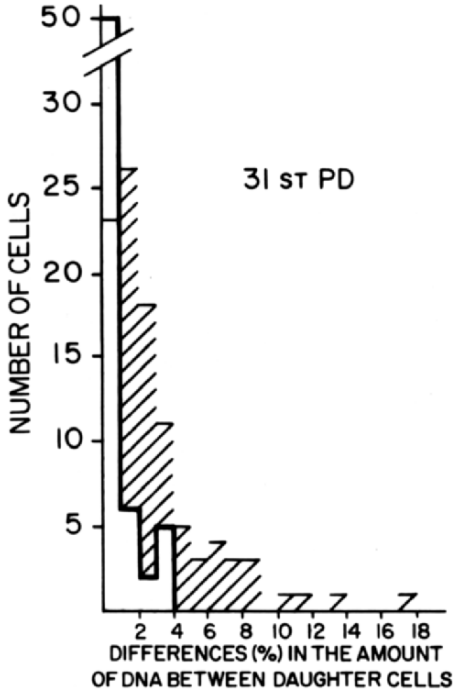

Fig. 4. Distribution of the differences in the amounts of DNA between each half of anaphase pairs after Feulgen-pararosaniline staining expressed as the percentage of the mean of the pair, in human embryonic lung fibroblasts at the 31st population doubling. The *area limited by the continuous line* corresponds to the differences with three standard deviations, found between repeated measurements of the same samples. Each sample was measured twice

probit paper the DNA distributions for these classes of cells (Fig. 5) in such a way that the scale of the abcissa for metaphases is twice that of anaphases and telophases. Indeed the values corresponding to each half of anaphases and telophases, and to that of metaphases overlap.

This type of analysis was performed at different PDL and the DNA values found in the different classes of cells were tested by an analysis of variance, which showed that they were never significantly different ($F=33$ at the 0.05 level). The plot always gave straight lines indicating a Gaussian distribution.

The DNA contents of anaphases and metaphases were measured during the last mitoses when cells reached phase IV and plotted on probit paper (Fig. 6). The plot was not linear as for cells in phases II and III; instead there was a breaking point in the slope suggesting a phase transition. The distribution of DNA between sister cells at higher DNA contents was chaotic.

Microscopical observations showed that the cells were not contaminated by mycoplasma. In addition control DNA measurements were made repeatedly in areas above the cytoplasm to ensure absence of aberrant DNA. With ethidium bromide the total background was below 5% of the mean DNA diploid value and after Feulgen staining below 0.1%.

To determine whether the differences in the DNA content between cells were caused by unequal distribution of the DNA synthesized during the

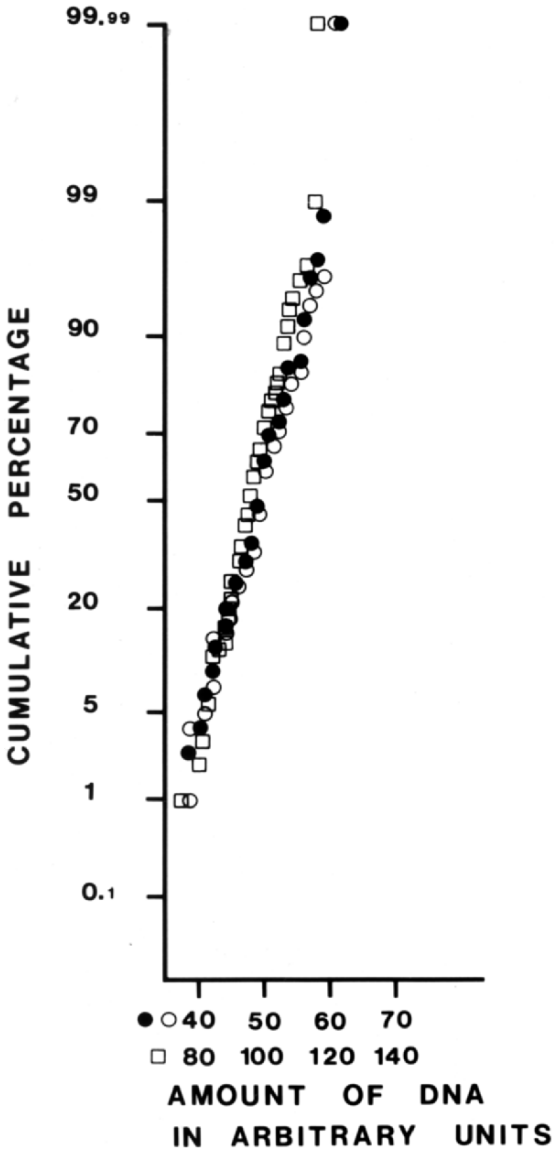

Fig. 5. Plot on probit paper of the distribution of the amount of DNA on metaphases (*squares*) and on each half of anaphases and telophases (*filled and empty circles*) of human embryonic lung fibroblasts in phase II

preceding S period, the number of grains on each half of anaphases and telophases was measured after labeling the DNA with ^{3}H-TdR during a whole S period. To check if ^{3}H-TdR would disturb the distribution of DNA between daughter cells, cultures were labeled with different

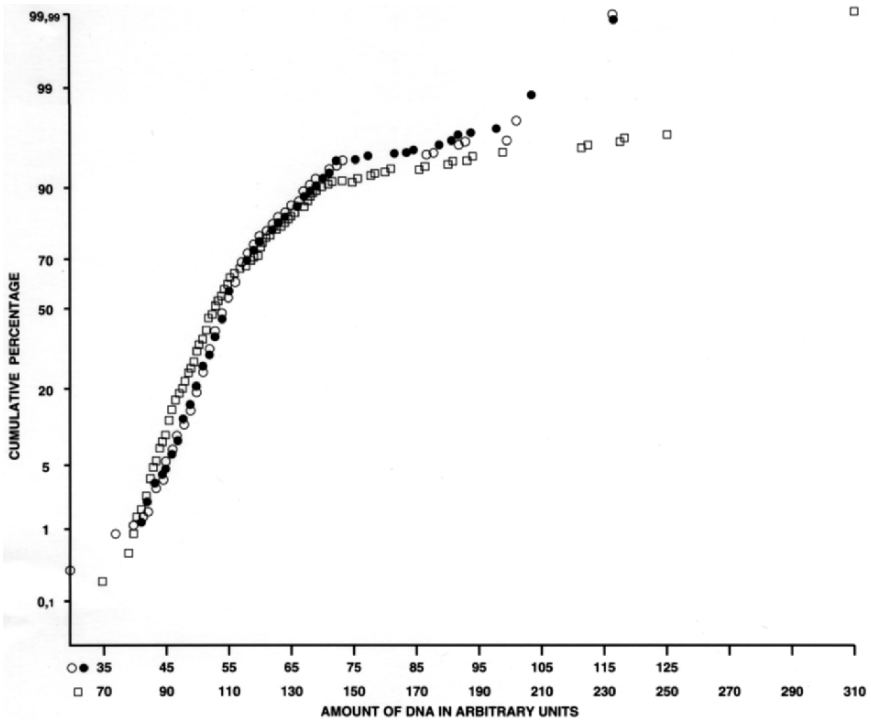

Fig. 6. Plot on probit paper of the distribution of the amount of DNA on metaphases (*squares*) and on each half of anaphases (*filled and empty circles*) of human embryonic lung fibroblasts in phase IV

concentrations of the radioactive precursor and the DNA in each half of anaphases and telophases was measured. Concentrations between 0.01 and 0.2 μCi/ml did not change the distributions of the differences in DNA content between sister cells, beyond those concentrations there was a decline in the number of pairs with less than 5% difference from the mean and an increase in the number of pairs with larger differences. Radioactivity increased the differences between daughter cells in the upper 20% of the distribution, in other words, it increased the fraction of cells with significant differences in DNA content, which reinforces the data showing the asymmetry of DNA distribution.

Since 0.01 μCi/ml ^{3}H-TdR gives a grain spread over the nucleus with a good resolution, this concentration was considered safe for the experiment. The number of grains on daughter cells was measured after labeling the DNA with ^{3}H-TdR during a whole S period (Macieira-Coelho et al. 1982). Ten hours after adding ^{3}H-TdR the peak grain count above anaphases plus telophases and above metaphases reached a plateau around 25 grains with no significant differences between the two classes of cells. There was a

decline in the 16th hour after labeling due to the presence of non-labeled mitoses from the G2-delayed compartment, which can be detected on the percentage metaphase curve. A G2-delayed compartment is known to be present in human fibroblastic populations (Macieira-Coelho and Berumen 1973).

Hence samples from the 10th, 12th, 20th, and 24th hour after labeling were chosen to analyse the differences in the number of grains found on each half of anaphases and telophases. The number of grains found on each daughter cell was plotted and compared with a Monte Carlo simulation. The plot showed a significant fraction of cells where the DNA synthesized during the preceding S period was not distributed evenly between daughter cells. The fraction of cells with significant differences in the number of grains on daughter cells was constant throughout the population life span and increased only during the last mitoses (Macieira-Coelho et al. 1982).

4
Implications for Homeostasis In Vivo

An increased heterogeneity of the amount of DNA per cell during aging of human embryonic diploid fibroblast populations has been previously reported in analyses of the distribution of the DNA content of interphases (Schneider and Fowlkes 1976). Results obtained with lymphocytes from human donors of different ages also showed an increased variance with age in the DNA content between cells (Staiano-Coco et al. 1982). However cytophotometric studies performed on interphases show two peaks corresponding to cells in the G1 and G2 periods and intermediate values corresponding to the cells in the S period. Hence the spread of the G1 and G2 DNA contents of interphases is difficult to analyze because of a lack of clear limits between cells in the different periods of the cycle. Furthermore, an interphase with 4C DNA can be a diploid cell in G2 or a tetraploid in G1. To overcome these difficulties the DNA content was measured in cells in metaphase, anaphase, and telophase (Macieira-Coelho et al. 1982).

The results described above show that the partition of DNA between sister cells deviates from a symmetric distribution in a significant fraction of cells at each population doubling. This fraction is constant through phases I, II and III of the fibroblast population, with DNA contents presenting always a Gaussian distribution; so it seems that the system undergoes a progressive reorganization but is in a steady state. When the cell population completes its potential number of doublings a critical point is reached and the system deviates from equilibrium with a phase transition; a bifurcation seems to take place with the appearance of a new steady state with new cell properties. The final symmetry breaking in cell division coincides with other asymmetric events such as the reorganization of chromatin fibers

(Puvion-Dutilleul and Macieira-Coelho 1982), the presence of extrachromosomal DNA circles (Icard-Liepkalns et al. 1986), the shortening of telomeres (Harley 1991), and the destabilization of nucleosomes (Macieira-Coelho 1991).

The experiments of Meselson and Stahl showing that DNA synthesis is semiconservative were performed on a whole population of cells, however, measurements made on individual cells reveal that in a significant fraction the distribution of DNA between sister cells is asymmetric.

Fibroblasts have an important role in homeostasis through their inductive properties on the differentiation of other cells. Many of the functions of the fibroblast are known. The fibroblast is an ubiquitous cell in the organism and, in this respect, it is responsible for the creation of a microenvironment, either through the synthesis of macromolecules (collagen, proteoglycans, elastin) which act as supporting structures crucial for normal development, or through the synthesis of small soluble diffusible regulators of homeostasis such as growth factors, prostaglandins, heparin-like molecules, $alpha_2$-macroglobulin, albumin, thromboxan A_2, etc.

Fibroblasts differ in their properties, depending on the organ or tissue they come from. An example of this specificity is the finding that human skin fibroblasts synthesize one-tenth as much $alpha_2$-macrogobulin, a plasma protease inhibitor, as lung fibroblasts (Brissenden and Cox 1982). The growth potential of fibroblasts evolves during development according to their location acquiring tissue specificities; the growth potential of embryonic lung fibroblasts is larger than that of embryonic skin fibroblasts (Macieira-Coelho 1988). Moreover, cDNA micro-arrays could ascertain the topographic diversity of fibroblasts based perhaps on the same Hox gene code that determines the development of body parts during embryogenesis (Chang et al. 2002). Thus the fibroblast can be considered as a differentiated cell with a crucial role during development and in homeostasis through interactions with other cell types.

It has been proposed that the evolution of the fibroblast properties through proliferation is a programmed developmental process of terminal differentiation necessary to fulfill a role in homeostasis (Martin et al. 1974). Indeed many results favor this concept. Takahashi and Zeydel (1982) found analogies in enzymatic changes occurring in human embryonic lung fibroblasts in the terminal stage and in 3T3 cells differentiating to adipocytes. In both situations γ-glutamyl transpeptidase, glutathionase, and phosphate-independent glutaminase activities became several-fold higher and the ratio of reduced to oxidized glutathione decreased. Moreover, an increase in the level of the cyclic AMP-dependent protein kinases was also observed during those events in both cell systems and during the differentiation of myoblasts (Liu et al. 1986). The localization of the serum response factor in the nucleus of proliferative fibroblasts and its absence in the terminal stage also occurs during terminal differentiation of some cell lineages (Ding et al. 2001). Matsumura et al. (1979) described an enhanced production of

the colony-stimulating factor for mouse bone marrow cells during the period of growth decline of mouse fibroblasts, a feature interpreted as a differentiation stage.

It is interesting that in plants extra-chromosomal DNA replicates in cells that differentiate from G2. It has been suggested that this is due to the failure of nascent replicons to join when cells reach G2, leaving gaps that serve as recognition sites for the initiation of DNA amplification (Van'T Hof and Bjerknes 1982). The production of extra-chromosomal DNA is also a feature of the terminal phase IV fibroblast (Icard-Liepkalns et al. 1986); hence the same mechanism could be operative in the terminal fibroblast since a prolongation of the G2 period (Macieira-Coelho and Berumen 1973) and a defect in the gap-filling step are present in these cells (Macieira-Coelho 1991). Emergence of extra-chromosomal DNA circles also accompanies cellular differentiation in the early development of mouse embryos (Yamagishi et al. 1983).

In general, in the literature the terminal cells are called senescent. This approach was reinforced by the use of a marker thought to identify post-mitotic cells (Dimri et al. 1995); it consists of the stain for the lysosomal enzyme β-galactosidase, which colors phase IV cells. It was reported that the number of stained cells increase in the skin with age, and although no quantitative analysis was made it was suggested that almost all cells stained meaning that they were all terminally arrested. This is of course incompatible with the data showing that proliferating fibroblast cultures can be obtained from old donors.

It was previously shown that lysosomal enzymes increase during prolonged resting phase in vitro (Macieira-Coelho et al. 1971). These data demonstrated that the accumulation of lysosomal enzymes occurs during a prolonged non-mitotic state, regardless of whether or not it is reversible. Therefore, although lysosomal β-galactosidase expresses a long quiescence, it is not suitable for making a distinction between a reversible and a terminal resting stage. Krishna et al. (1999) analyzed the enzyme at pH 6, supposed to be associated with the non-dividing state, and at pH 4.5. Both forms, which are due to interconversion between the polymeric, dimeric and monomeric forms (Kuo and Wells 1978), were present in a number of tumor cell lines. Krishna et al. concluded that the enzyme determined at pH 6 could hardly be considered as an exclusive marker of the post-mitotic state. Severino et al. (2000) also reexamined the significance of an increase in β-galactosidase activity and concluded that it is found in a variety of conditions hence its interpretation remains unclear.

Other markers have shown that terminally arrested cells are not increased in the tissues of old individuals. Indeed analysis of cells from donors aged 0–93 years showed a non-significant correlation between proliferative ability and donor age and between telomeric DNA and donor age (Allsop et al. 1992). Measurements made on the organization of chromatin fibers showed that the number of terminal post-mitotic cells is not increased in normal old individuals, and the terminal cell obtained in vitro

is not identical to that observed in normal control donors, it is found in vivo only in pathological conditions (Macieira-Coelho 1995). Moreover the expression of genes involved in cell cycling, those that were reported to be repressed in terminal fibroblasts, were also used as markers (Grassilli et al. 1996); the authors could not find any evidence for the presence of terminal cells in the skin from centenarians. The gene profile determined by cDNA microarrays also showed that distinct sets of genes are observed in cells close to the end of their proliferative life in vitro and in cells obtained from old human donors (Park et al. 2001).

Recently it was reported that a marker for telomere-associated DNA damage increased exponentially in baboon skin fibroblasts cultivated from animals of different ages; it reached a value of 15–20% in cells grown from very old animals (Herbig et al. 2006). The number of divisions between explantation and the time the cells were checked for DNA damage was not reported. The proliferation status of the cells carrying the DNA damage marker, was not ascertained either. The tests performed to check the health status of the baboons were not mentioned. The cellular marker used is associated with so called senescent cells in cultured human fibroblasts aged in vitro (Herbig et al. 2004). In any case these data show that the large majority of skin fibroblasts of old baboons belong to the proliferation compartment.

5 Conclusions

The concept that the evolution through serial divisions of the fibroblast cell compartment is a succession of differentiating steps is appealing and worthy of further investigation. As it now stands the relevance for organism aging is not the final arrested state but rather the functional evolution of the fibroblast compartment created by asymmetric divisions, leading the cell through functional changes. This drift creates new regulations and interactions with other cell compartments and is an important component of the permanent evolution of a mammalian organism. It fits into what is known of the physiology of the organism.

Holtzer (1970) hypothesized that differentiated cells become committed in the course of a last mitosis the “quantal mitosis”. The symmetry breaking during the last mitoses of the human fibroblast proliferation life span could be the final commitment step of the differentiation program.

References

Allsop RC, Vaziri H, Patterson C, Goldstein S, Younglali EV, Futcher AB, Greider CW, Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci USA 89:10114–10118

Bemiller PM, Miller JE (1979) Cytological changes in senescing WI-38 cells. A statistical analysis. Mech Ageing Dev 10:1–15
Berumen L, Macieira-Coelho A (1977) Changes in albumin uptake during the life span of human fibroblasts in vitro. Mech Ageing Dev 6:165–172
Bittles AH, Harper N (1984) Increased glycolysis in aging cultured human diploid fibroblasts. Biosci Rep 4:751–756
Bond JA, Haughton M, Blaydes J, Gire V, Wynford-Thomas D, Wyllie F (1996) Evidence that transcriptional activation by p53 plays a direct role in the induction of cellular senescence. Oncogene 13:2097–2104
Bosmann HB, Gutheil RL Jr, Case KR (1976) Loss of a critical neutral protease in ageing WI-38 cells. Nature 261:499–501
Brissenden JE, Cox DW (1982) Alpha-2 macroglobulin production by cultured human fibroblasts. Som Cell Genet 8:289–305
Celis JE, Bravo R (1984) Synthesis of the nuclear protein cyclin in growing, senescent and morphologically transformed human skin fibroblasts. FEBS Lett 165:21–25
Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO (2002) Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc Natl Acad Sci USA 99:12877–12882
Dell'Orco RT, Whittle WL, Macieira-Coelho A (1986) Changes in the high order organization of DNA during aging of human fibroblast-like cells. Mech Ageing Dev 35:199–208
Dick JE, Wright JA (1985) On the importance of deoxyribonucleotide pools in the senescence of cultured human diploid fibroblasts. FEBS Lett 179:21–24
Dimri G, Lee G, Basile M, Acosta M, Scott G, Roskelly C, Medranos E, Linskens M, Rubellj I, Pereira-Smith OM, Peacocke M, Campisi J (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92:9363–9367
Ding W, Gao S, Scott RE (2001) Senescence represses the nuclear localization of the serum response factor and differentiation regulates its nuclear localization with lineage specificity. J Cell Sci 114:1012–1016
Grassilli E, Bellesia D, Salomoni P, Croce MA, Sikora E, Radiszewska E, Tesco G, Vergelli M, Latorraca S, Barbieri D, Fagiolo U, Santacaterina S, Amaducci L, Sorbi S, Franceschi C (1996) C-fos/c-jun expression and AP-1 activation in skin fibroblasts from centenarians. Biochem Biopys Res Commun 226:517–523
Gupta RS (1980) Senescence of cultured human diploid fibroblasts. Are mutations responsible? J Cell Phys 103:209–216
Hards RG, Patterson D (1986) Variation of glycinamide ribonucleotide synthetase levels during in vitro aging of human fibroblasts. Implications for gene dosage studies. Mech Ageing Dev 36:65–70
Harley CB (1991) Telomere loss: mitotic clock or genetic time bomb? Mutat Res 256:271–283
Hayflick L, Moorhead PS (1961) The serial cultivation of human diploid cell strains. Exp Cell Res 25:585–621
Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM (2004) Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, p21 (CIPI) but not p16 (INK4a). Mol Cell 14:501–513
Herbig U, Ferreira M, Condel L, Carey D, Sedivy M (2006) Cellular senescence in aging primates. Science 311:1257

Holtzer M (1970) Myogenesis. In: Schejde A, DeVellis J (eds) Cell differentiation. VanNostrand, Reynhold and Co, New York, pp 476–503
Icard-Liepkalns C, Dolly J, Macieira-Coelho A (1986) Gene reorganization during serial proliferation of normal human fibroblasts. Bioch Bioph Res Comm 141:112–123
Kahn A, Meienhofer MC, Guillouzo A, Cottreau D, Baffet G, Henry J, Dreyfus JC (1982) Modifications of phosphoproteins and protein kinases occurring with in vitro aging of cultured human cells. Gerontology 28:360–370
Kaji K, Matsuo M (1983) Responsiveness of human lung diploid fibroblasts ageing in vitro to epidermal growth factor: saturation densities and life span. Mech Ageing Dev 22:129–133
Krishna DR, Sperker B, Fritz P, Klotz U (1999) Does pH 6 β-galactosidase activity indicate cell senescence? Mech Ageing Dev 109:113–123
Kuo CH, Wells WW (1978) Galactosidases from rat mammary gland. J Biol Chem 253:3550–3556
Lee HC, Paz MA, Gallop PM (1982) Low density lipoprotein receptor binding in aging human diploid fibroblasts in culture. J Biol Chem 257:8912–8927
Liu AYC, Chang ZF, Chen KY (1986) Increased level of cAMP-dependent protein kinase in aging human lung fibroblasts. J Cell Phys 128:149–154
Macieira-Coelho A (1983) Changes in membrane properties associated with cellular aging. Intl Rev Cytol 83:183–220
Macieira-Coelho A (1988) Biology of normal proliferating cells in vitro. Relevance for in vivo aging. Karger SA, Basel
Macieira-Coelho A (1991) Chromatin reorganization during senescence of proliferating cells. Mutat Res 256:81–104
Macieira-Coelho A (1995) The last mitoses of the human fibroblast proliferative life span, physiopathologic implications. Mech Ageing Dev 82:91–104
Macieira-Coelho A, Berumen L (1973) The cell cycle during growth inhibition of human embryonic fibroblasts in vitro. Proc Soc Exp Biol Med 144:43–48
Macieira-Coelho A, Puvion-Dutilleul F (1989) Evaluation of the reorganization in the high-order structure of DNA occurring during cell senescence. Mutat Res 219:165–170
Macieira-Coelho A, Taboury F (1982) A reevaluation of the changes in proliferation in human fibroblasts during ageing in vitro. Cell Tiss Kinet 15:213–224
Macieira-Coelho A, Garcia-Giralt E, Adrian M (1971) Changes in lysosomal enzymes associated structures in human fibroblasts kept in resting stage. Proc Soc Exp Biol Med 138:712–718
Macieira-Coelho A, Bengtson A, Van der Ploeg M (1982) Distribution of DNA between sister cells during serial subcultivation of human fibroblasts. Histochemistry 75:11–24
Martin GM, Sprague CA, Norwood TH, Pendergrass WR (1974) Clonal selection, attenuation and differentiation in an in vitro model of hyperplasia. Am J Path 74:137–154
Matsumura T, Miyashita S, Ohno T (1979) Conversion of proliferation and production of the colony stimulating factor during serial passage of mouse fibroblasts in culture. Cell Struct Funct 4:267–274
Mitsui Y, Schneider EL (1976) Characterization of fractionated human diploid fibroblast populations. Exp Cell Res 103:23–30

Park WY, Hwang CI, Kang MJ, Seo JH, Chung JH, Kim YS, Lee JH, KimH, Yoo HJ, Seo JS (2001) Gene profile of replicative senescence is different from progeria or elderly donor. Exp Cell Res 282:934–939

Paz MA, Torrelio M, Gallop PM (1981) X-linked processes in serially passaged aging human diploid cells. J Geront 36:142–151

Pignolo RJ, Cristofalo VJ, Rotenberg MO (1993) Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 stage. J Biol Chem 268:8949–8957

Puvion-Dutilleul F, Macieira-Coelho A (1982) Ultrastructural organization of nucleoproteins during aging of cultured human embryonic fibroblasts. Exp Cell Res 138:423–429

Raes M, Genens G, Brabander M, Remacle J (1983) Microtubules and microfilaments in ageing hamster embryo fibroblasts in vitro. Exp Gerontol 18:241–254.

Schneider EL, Fowlkes BJ (1976) Measurement of DNA content and cell volume in senescent human fibroblasts utilizing flow multiparameter single cell analysis. Exp Cell Res 98:298–302

Severino J, Allen RG, Balin S, Balin A, Cristofalo VJ (2000) Is β-galactosidase staining a marker of senescence in vitro and in vivo? Exp Cell Res 257:162–171

Staiano-Coico L, Darzynkiewicz Z, Melamed MR, Weksler M (1982) Changes in DNA content of human blood mononuclear cells with senescence. Cytometry 3:79–83

Steinhardt M (1985) Effect of donor age on clonal differentiation of human skin fibroblasts in vitro. Gerontology 31:27–38

Takahashi S, Zeydel M (1982) Alpha-glutamyl transpeptidase and glutathione in aging IMR-90 fibroblasts and in differentiating 3T3 L1 preadipocytes. Archs Biochem Biohys 214:260–268

Van't Hof J, Bjerknes CA (1982) Cells of pea (pisum sativum) that differentiate from G2 phase have extrachromosomal DNA. Mol Cell Biol 2:339–345

Yamagishi H, Kunisada T, Iwakura Y, Nishimuno Y, Ogiso Y, Matsuhiro A (1983) Emergence of extrachromosomal circular DNA complexes as one of the earliest signals of cellular differentiation in the early development of mouse embryo. Devel Growth Different 25:563–569

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