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In vitro organogenesis in three dimensions: self-organising stem cells

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Summary

Organ formation during embryogenesis is a complex process that involves various local cell-cell interactions at the molecular and mechanical levels. Despite this complexity, organogenesis can be modelled in vitro. In this article, we focus on two recent examples in which embryonic stem cells can self-organise into three-dimensional structures – the optic cup and the pituitary epithelium; and one case of self-organising adult stem cells – the gut epithelium. We summarise how these approaches have revealed intrinsic programs that drive locally autonomous modes of organogenesis and homeostasis. We also attempt to interpret the results of previous in vivo studies of retinal development in light of the self-organising nature of the retina.

Key words: Crypt, Pituitary development, Retinal development, Self-organisation, Stem cells, Three-dimensional culture

Introduction: 3D stem cell biology for studying organogenesis

Over the past two decades, much progress has been made in understanding the mechanisms of genetic regulation of cellular differentiation, as well as stem cell maintenance and reprogramming. However, different approaches are necessary for understanding the dynamics of organogenetic processes. New tools have been applied to in vivo organogenesis studies, including conditional knockouts, cellular ablation, multi-colour lineage tracing and in vivo deep imaging. However, these in vivo tools are not always applicable to the complex organogenetic field in the embryo, which involves various multicellular interactions in a four-dimensional context (time and space).

If mammalian organogenesis could be studied in vitro under defined conditions, it would enable us to analyze spatiotemporally dynamic intercellular interactions more precisely. But the issue is whether this is possible. Can an in vitro system recapitulate the robust regulatory systems of organogenesis, in particular with regard to spatial patterning and morphogenesis? In this Review, we introduce recent examples of in vitro stem cell studies of organogenetic dynamics and discuss their contribution to our understanding of organ development.

First, we will consider self-organising phenomena (see Glossary, Box 1) of ectodermal organ buds, the optic cup (see Glossary, Box 1) and Rathke's pouch (see Glossary, Box 1), from aggregates of embryonic stem (ES) cells, addressing the biological meaning of local autonomy in complex organogenesis. Then, we discuss in vitro formation and maintenance of crypt-like structures using clonal culture of adult intestinal stem cells. Collectively, these studies demonstrate how in vitro and in vivo studies can be combined to elucidate the dynamics of tissue formation in stem cell systems.

The optic cup

Retinal development in vivo

Morphogenesis and patterning of the eye is highly conserved throughout vertebrates. Here, we briefly summarise eye development in mammals (for a more in depth review, see Graw, 2010; Fuhrmann, 2010). During the early stages of eye development (Fig. 1), the retinal neuroepithelium expands outwards (evaginates) from the diencephalic wall to form an optic vesicle (see Glossary, Box 1). The distal part of the vesicle, which is in contact with the surface ectoderm, becomes fated to the neural retina (NR; see Glossary, Box 1), whereas the proximal part differentiates into the retinal pigment epithelium (RPE; see Glossary, Box 1). The optic vesicle then folds inwards (invaginates) at its distal region to form a two-layered cup-like structure, the optic cup, with the NR and RPE being the inner and outer layers, respectively. In the postnatal eye, the neural retina shows a stratified structure with six different types of components: photoreceptors, horizontal cells, bipolar cells, amacrine cells, ganglion cells and Müller glia (for their diversification mechanisms, see Livesey and Cepko, 2001; Cayouette et al., 2006).

Over recent years, several studies have provided evidence that neighbouring tissues – including the lens, periocular mesenchyme, cornea and surface ectoderm, and diencephalon – influence optic cup formation (reviewed by Martinez-Morales and Wittbrodt, 2009; Fuhrmann, 2010; Adler and Canto-Soler, 2007). However, conflicting results have been reported in such analyses. Some studies in chick and mouse embryos have indicated that the surface ectoderm and its derivatives play roles in optic cup formation (Lopashov, 1963; West-Mays et al., 1999; Smith et al., 2009), whereas other mouse studies have indicated that the surface-ectoderm derivatives, at least the lens, are not essential for NR invagination (Hyer et al., 2003). In addition to the surface ectodermal derivatives, roles for periocular mesenchyme and neural crest derivatives (which may include mesenchymal cells) have been suggested (Fuhrmann et al., 2000; Grocott et al., 2011). These tissues surrounding the forming optic cup could influence physical environments of retinal morphogenesis, as well as biochemical signalling environments; for example, they produces various signalling molecules that affect retinogenesis, such as Wnts, fibroblast growth factors (FGFs) and TGFβ family factors (Adler and Canto-Soler, 2007; Esteve and Bovolenta, 2006; Fuhrmann, 2008; Müller et al., 2007; Yang, 2004; Nguyen and Arnheiter, 2000; Diep et al., 2004; Zhao et al., 2001; Martinez-Morales et al., 2005; Grocott et al., 2011).

Recapitulating optic cup formation in vitro

Recently, it has been demonstrated that substantial aspects of retinogenesis can be recapitulated by 3D culture of mouse ES cells (Eiraku et al., 2011). In particular, the 3D structure of the optic cup

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Box 1. Glossary

+4 cells. Slow-cycling cells located at position +4 directly above the Paneth cells in the small intestine crypt with long-term DNA-label retention.

Adenohypophysis. Anterior region of pituitary gland containing hormone-producing cells for adrenocorticotropic hormone, growth hormone, melanocyte-stimulating hormone, thyroid-stimulating hormone, luteinizing hormone/follicle stimulating hormone and prolactin.

Crypt of Liberkühn. Epithelial tubular structure that invaginates into intestinal walls and functions as a stem cell reservoir and gland.

Crypt-base columnar cells. Small undifferentiated cycling epithelial cells located at the intestinal crypt base. In the small intestine, they are typically sandwiched between the Paneth cells.

Hinge. Boundary domain between neural retina and retinal pigment epithelium in the optic cup, where retinal epithelium curves in an acute angle. Later, this region gives rise to the ciliary margin, where retinal stem cells are located.

Infundibulum. Hypothalamic neuroepithelium adjacent to Rathke's pouch. It protrudes downwards from the ventral floor of the hypothalamus and gives rise to part of the neurohypophysis.

Matrigel. Laminin-containing extracellular matrix proteins solublised and extracted from Engelbreth-Holm-Swarm mouse sarcoma cells.

Neural retina. Functional part of the retina that develops from the inner region of the optic cup. When it matures, it has a multi-layered structure containing photoreceptors, three kinds of interneurons (bipolar, horizontal and amacrine cells), ganglion cells and Müller glia.

Neurohypophysis. Posterior region of pituitary gland containing axons of vasopressin- and oxytocin-producing cells, which are located in the hypothalamus.

Optic cup. Early retinal anlage that forms by deformation of the optic vesicle, with a double-walled brandy glass-like structure.

Optic vesicle. Early retinal anlage that appears as an epithelial vesicle protruding laterally from the embryonic diencephalon.

Organoid. Three-dimensional cell mass resembling an organ in some aspects. In particular, this term is often used by Hans Clevers and colleagues to describe an intestinal stem cell-derived epithelial cyst with crypt-like structures.

Paneth cells. Large eosinophilic cells located at the base of the small-intestinal crypt, secreting bacteriocidal factors such as α-defensin and lysozyme. Recently, they have been shown to have a niche-creating function for intestinal stem cells.

Pituitary placode. Presumptive ectodermal placode for Rathke's pouch located in the midline rostral to the anterior neural ridge.

Rathke's pouch. Pituitary anlage derived from oral ectoderm under the influence of inductive signals from neighbouring hypothalamic neuroepithelium. It forms by invagination of pituitary placode.

Retinal pigment epithelium. The supporting part of the retina that develops from the outer region of the optic cup.

Self-organisation. Spontaneous formation of a highly ordered structure or pattern from elements (in this case, cells) with no or minimal pre-pattern.

SFEBq culture. Serum-free floating culture of embryoid body-like aggregates with quick reaggregation. This culture is suitable for inducing various ectodermal derivatives from mouse and human embryonic stem cells.

Villus. Small finger-like protrusions of intestinal epithelium directed towards the lumen that contribute to surface expansion for efficient absorption.

was generated, presumably following self-organising principles, as well as the fully stratified structure of the postnatal neural retina: including all six cell types and synaptic zones corresponding to the external and internal plexiform layers (Fig. 2A).

To give a brief overview of the method (for details, see Eiraku and Sasai, 2012), this culture was started by reaggregating dissociated mouse ES cells (3000 cells), which were then grown in medium containing Matrigel (see Glossary, Box 1) and minimal growth factors. By day 6, retinal epithelia (expressing the specific marker Rx) were efficiently generated and had evaginated to form hemispherical vesicles. The distal region subsequently underwent invagination, forming a two-layered cup-like morphology characteristic of the optic cup (Fig. 1, Fig. 2A). The invaginated distal epithelium became neural retina, whereas the proximal epithelium, now forming the outer shell, expressed pigment epithelium markers and later accumulated pigmentation. No lens, surface ectoderm or periocular mesenchyme was present around the forming cup, demonstrating that dynamic morphogenesis is self-driven and does not require external forces imposed by surrounding tissues.

Successful formation of optic cups in this 3D culture reproducibly involves three changes in local tissue mechanics leading to invagination (Fig. 2B). First, a local reduction in phospho-myosin light chain 2 (pMlc2) levels probably leads to increased flexibility in the distal epithelium (presumptive NR) of the optic vesicle (a similar reduction in the embryonic NR in vivo has been observed) (Eiraku et al., 2011; Eiraku et al., 2012). Second, there is a strong apical constriction of the hinge epithelium (see Glossary, Box 1). This causes the hinge cells to have an apically narrow wedge shape, contributing to a slight apical (inward) bending of the NR epithelium. Third, the NR expands tangentially during the late phases of optic cup morphogenesis, which generates compression within the tissues. The expansion of the flexible NR in a limited space surrounded by the lower yielding shell of RPE would lead to buckling of the NR tissue or to invagination. These three local changes in tissue mechanics, when applied to vertex model-based computer simulation, induce optic cup morphogenesis in silico (Eiraku et al., 2011), supporting the idea that a relatively simple internal program involving local mechanical changes is sufficient to create characteristic epithelial deformation without external forces. The mechanistic aspects of this process are discussed more in depth in a recent review (Eiraku et al., 2012), which explains self-driven morphogenesis with a relaxation-expansion model involving three local mechanical rules.

More recently, this work has been extended to show self-formation of the optic cup and stratified NR from human ES cell cultures (Nakano et al., 2012). This study reports several intriguing species-specific features seen in the human ES cell-derived optic cup. The human ES cell-derived optic cup had a much larger and thicker epithelium than the one generated in mouse ES cell culture, in accordance with the actual difference in size between human and mouse embryonic optic cups. The larger epithelial size of the human ES cell-derived retina implies that higher bending forces would be required for the invagination of such a thick retinal epithelium. Interestingly, our data suggest that optic cup morphogenesis in human ES cell culture, but not in mouse ES cells, involves a fourth local rule of tissue mechanics. Human NR tissue has an intrinsic tendency to curve in an apically convex manner during invagination (Fig. 2B). This spontaneous bending property requires integrin-mediated signals from the basement membrane (Nakano et al., 2012), which promote strong apical deviation of progenitor cell positions and help the apically predominant expansion of human NR. How the integrin signals translate into the cell position change is an important topic for future study. Interestingly, a crucial role for integrin-dependent basal constriction has also been demonstrated in the developing NR

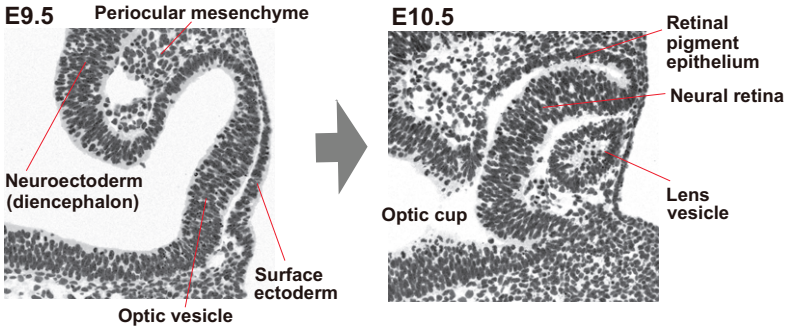

Fig. 1. Optic cup development in vivo. Cross-sections of optic vesicle (left; E9.5) and optic cup (right, E10.5) in the mouse embryo. Nuclei are marked by DAPI staining. In vivo, the environment in which the retina forms is complex, surrounded by surface ectoderm, periocular mesenchyme and diencephalic neuroectoderm in close proximity.

of fish embryos (Martinez-Morales et al., 2009), raising the possibility that this mechanism may be more evidently conserved between human and fish, but clearly not in rodents. However, it is also possible that the mechanism could be more complex because biased accumulation of actomyosin or active myosin on the basal side, which contributes to basal bending in the fish NR, was not seen in human ES cell-derived NR (Nakano et al., 2012).

In vitro generation of stratified human retina by self-organisation enhances the possibility of its future medical application in regenerative medicine or pathogenesis studies of retinal degeneration. Importantly, for such applications, an efficient cryopreservation method has been also invented to store these human ES cell-derived 3D NR tissue (Nakano et al., 2012). The layer-forming nature of NR derived from human induced pluripotent stem (iPS) cells was also reported in a recent independent study (Phillips et al., 2012), supporting the reliability and robustness of this strategy.

Reconciling in vitro and in vivo data

The formation of the optic cup and laminated neural retina in vitro demonstrates the presence of a 'latent intrinsic order' in retinal progenitor cells, which emerges when cells work together via cell-cell interactions under permissive conditions. Does it then follow that optic cup morphogenesis in vivo is totally self-organising? This intriguing question has been a matter of debate for more than a century, since the time of Hans Spemann. On the basis of his and

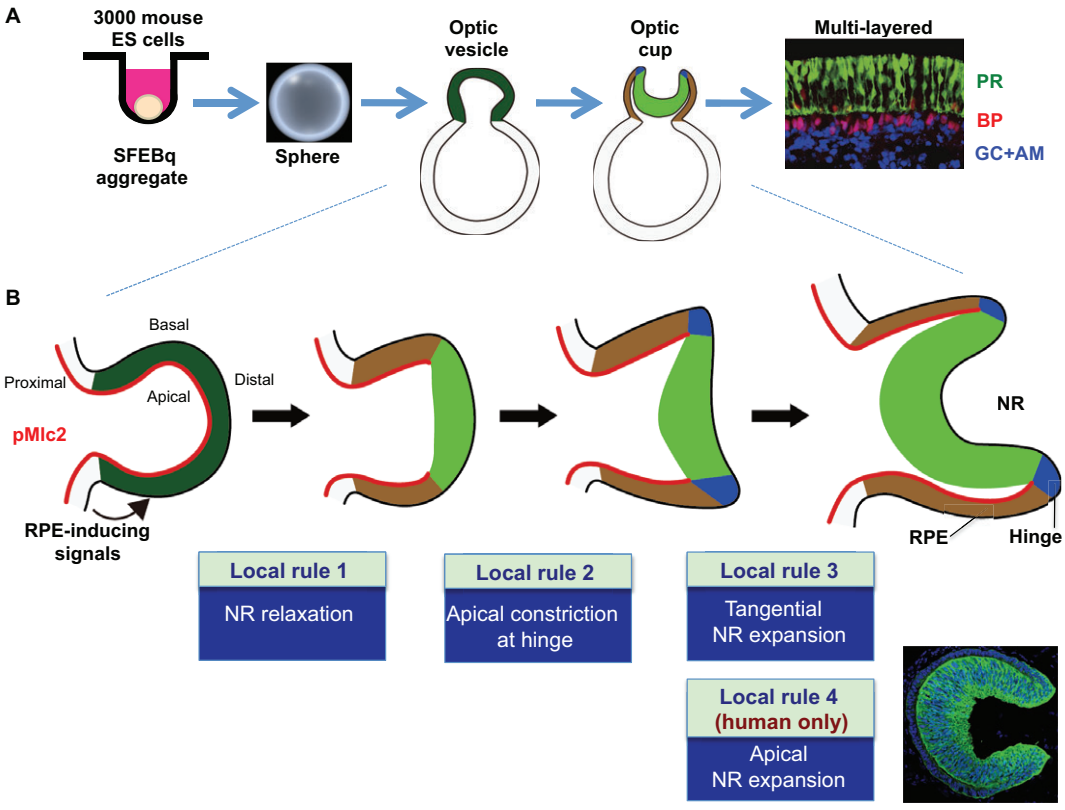

Fig. 2. Self-organisation of optic cup in vitro. (**A**) Self-formation of optic cup stratified from an embryonic stem (ES) cell aggregate. Schematics summarise the protocol and stages of optic cup formation. Photomicrograph shows a cross-section of mouse ES cell-derived stratified neural retina. Modified, with permission, from Eiraku et al. (Eiraku et al., 2011). PR, recoverin^{+} photoreceptors; BP, Chx10^{+} bipolar cells; GC, ganglion cells (Pax6^{+}); AM, amacrine cells (Pax6^{+}). (**B**) Relaxation-expansion model for self-driven optic cup morphogenesis. Three local rules are common for mouse ES cell and human ES cell cultures, whereas the fourth local rule is specific to large and thick human retinal epithelium. Right panel shows a cross-section of human ES cell-derived optic cup. NR, neural retina; pMlc2, phospho-myosin light chain 2 (pMlc2); RPE, retinal pigment epithelium.

Warren Lewis' famous lens-induction experiments in amphibian embryos (Spemann, 1938), Spemann concluded that lens invagination has little to do with optic cup formation. Unlike head ectoderm, which has the competence to receive a lens-inducing signal from the retina, trunk ectoderm placed in contact with the optic vesicle cannot differentiate into lens tissue. However, even when the lens vesicle is absent, an optic vesicle transplanted into the trunk still forms the optic cup, showing that invagination of the NR is not necessarily coupled with that of the lens. Moreover, more recent experiments in lower vertebrates have demonstrated that misexpression of crucial transcription factors such as Pax6 and Six3 can induce ectopic 'mini eyes' in various tissues (Onuma et al., 2002; Zuber et al., 2003; Loosli et al., 1999), again suggesting that accurate information from the local environment is not necessarily essential for optic cup formation. However, and as discussed above, there is a body of evidence that implicates important roles for neighbouring tissues in directing retinal development.

How can these arguments be reconciled with the in vitro observation of the self-organising nature of the optic cup? Given the complex interdependencies in a developing system, secondary effects are inevitable in embryonic studies using tissue ablation and transplantation. In 3D free-floating culture, an optic cup structure forms from an optic vesicle without external forces. However, the mechanical environment in the developing embryonic head is more restrictive. For example, if an optic vesicle were placed in the wrong 3D topology, its evagination would be hindered by the tension in the surface ectoderm, as well as by the high viscosity created by periocular mesenchyme. In addition, embryonic head tissues produce numerous extracellular signals, e.g. Wnt, bone morphogenetic protein (BMP), FGF, Shh and their antagonists, with complex expression patterns (Adler and Canto-Soler, 2007; Esteve and Bovolenta, 2006; Fuhrmann, 2008; Müller et al., 2007; Yang, 2004; Nguyen and Arnheiter, 2000; Diep et al., 2004). Some of these signals may be favourable for optic cup formation, whereas others could be suppressive of retinogenesis. Moreover, even signals that are used in retinal morphogenesis could perturb the system if they are present at the wrong time and place or at the wrong level.

We propose that the internally programmed local signalling communications within the forming optic cup are rather delicate and mutually dependent, given that no 'organiser'-equivalent signalling centre has been known in early retinal formation. Such a delicate signalling network can self-form easily in culture with minimal external signals. However, in the signal-rich embryonic environment, it would easily be perturbed, thus disrupting autonomous eye formation. Therefore, in addition to the self-organising program in the retinal epithelium, it would be necessary during in vivo development to have substantial cooperation between the forming optic cup and its neighbouring tissues (Fig. 3). For example, in the mouse embryo, the lens-specific depletion of Pax6 not only causes severe defects in the lens, but also impairs optic cup invagination and secondarily affects the adjacent NR tissue in a non-cell-autonomous fashion, making it thinner and/or split (Ashery-Padan et al., 2000; Smith et al., 2009). This phenomenon makes sense if we postulate the presence of local extracellular signals that are inhibitory to NR development, which are counteracted by lens-derived secreted factors (e.g. FGFs) that promote NR fate (Pittack et al., 1997; Hyer et al., 1998; Zhao et al., 2001; Martinez-Morales et al., 2005). Periocular mesenchyme is known to promote RPE development at the cost of NR development in a non-cell-autonomous fashion (Fuhrmann et al., 2000), whereas Wnt signals, provided by both retinal and non-retinal tissues (including periocular mesenchyme), also instruct retinal differentiation towards the RPE fate and contribute to its maintenance (Fuhrmann, 2008; Fuhrmann, 2010; Westenskow et al., 2009; Liu et al., 2010; Fujimura et al., 2009). Notably, periocular mesenchyme and surface ectoderm derivatives (lens, cornea) are located exclusively adjacent to RPE and NR, respectively (Fig. 3). Such interactions between retinal epithelium and its neighboring tissues may provide double assurance for robust and precise development of the eye as an organ. Accordingly, surgical ablation and transplantation experiments in classic studies might have led to conflicting results, partly because they perturbed mutual positioning of the tissues to various extents. For example, surface ectoderm removal, in some cases, could have substantially mislocated periocular mesenchyme into the position contacting NR, and this could interfere NR differentiation and optic cup formation.

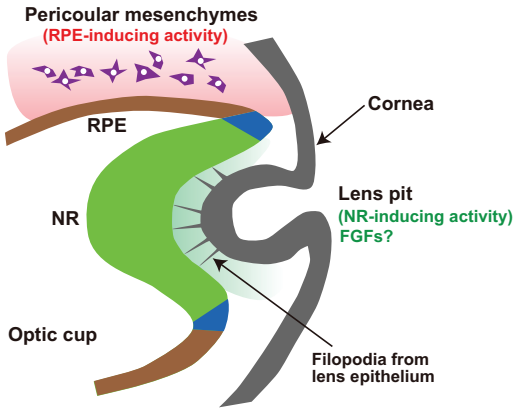

Fig. 3. Complex tissue interactions for robust development of optic cup in vivo. Possible interactions between the forming optic cup and the surrounding mesenchyme and surface-ectoderm derivatives. Periocular mesenchyme emanates pigment epithelium (RPE)-inducing activity that suppresses neural retina (NR) differentiation, while the surface ectoderm derivatives secrete NR-inducing factors such as fibroblast growth factors (FGFs). Direct contact between lens epithelium and NR may also transmit chemical or mechanical cues. These tissue interactions presumably contribute to robust patterning and positional adjustment of NR and RPE tissues by working together with their own self-organising functions.

To have accurate vision, it is absolutely essential that the axis of the optic cup is in exactly the same line as that of the lens; otherwise, incoming light does not form a focus. A recent study has suggested a coordinating role of neural crest derivatives in the positioning of lens and retina via Wnt and TGFβ signals (Grocott et al., 2011). In addition to these biochemical interactions, mechanical links may play a role in the coordinated eye development. Another recent report has shown an intriguing connection between lens and neural retinal epithelium, which invaginate at the same time (Chauhan et al., 2009). Close observations of this process reveal that there is a gap between the basement membranes of the two tissues, refuting the idea of mechanical pushing between them. There are, however, many thin cellular protrusions of lens epithelium (from its basal side) that reach the basement membrane of neural retina across the gap. These thin protrusions extend straight out, raising the possibility of the presence of tension. Although it remains unknown whether these extensions are used mechanically to transmit pulling forces

from the neural retina to the invaginating lens epithelium, their function is certainly an attractive topic for future investigation.

Another intriguing issue is how far optic cup formation in the embryo is recapitulated in the in vitro culture. In fact, the optic cup in vivo is not radially symmetrical and exhibits molecular and architectural differences along the nasal-temporal and dorsal-ventral axes. For example, in addition to differential expression of region-specific markers (Graw, 2010; Fuhrmann, 2010), the ventral-most region of the optic cup in the embryo has a characteristic gapped structure called the choroid fissure, which is formed by complex morphogenetic processes, including NR fanning and RPE back-folding (Adler and Canto-Soler, 2007; Martinez-Morales et al., 2009; Martinez-Morales and Wittbrodt, 2009) (this fissure later closes by fusion and defects in this process cause coloboma; Taylor, 2007). The proximal region of the embryonic optic cup becomes the optic stalk (Fuhrmann, 2010), which could provide an intrinsic constraint for morphogenesis, whereas the hinge region gives rise to the ciliary margin, which harbours retinal stem cells later (Reh and Fischer, 2006). In future studies, it will be important to examine the similarities and differences between the in vivo and in vitro situations in such detailed aspects of optic cup morphogenesis and development.

The adenohypophysis

The optic cup self-forms from initially homogenous cells without external interactions. However, many cases of organ development are known to involve some tissue-tissue interactions for their initiation. Can 3D ES cell culture recapitulate such complexity in a self-organising manner? A recent study addressed this question by attempting the generation of a pituitary anlage in 3D ES cell culture (Suga et al., 2011).

Pituitary development in vivo

The main part of the pituitary gland is the adenohypophysis (see Glossary, Box 1), which secretes several important systemic hormones, such as adrenocorticotropic hormone (ACTH) and growth hormone, into the bloodstream in response to a releasing stimulus from the hypothalamus. The rest of the pituitary gland is the neurohypophysis (or posterior pituitary; see Glossary, Box 1), which contains axons of hypothalamic vasopressin- and oxytocin-containing neurons that release these hormones there. The development of the adenohypophysis is a complex process. It arises from the dorsal region of the oral ectoderm during early mouse embryogenesis (roughly embryonic day 9 in mice), first as a thickened placode, then invaginating to form an epithelial pouch called Rathke's pouch (Fig. 4A). Specification of the pituitary placode (see Glossary, Box 1) that will form the adenohypophysis occurs in response to inductive signals from the overlying ventral hypothalamus (Gleiberman et al., 1999; Brinkmeier et al., 2007; Bharti et al., 2011). The molecular nature of this local inductive interaction at the initial phase of pituitary development still remains elusive, although FGF and BMP signals appear to be involved (Takuma et al., 1998; Brinkmeier et al., 2007).

In addition to inductive cues, there appear to be important inhibitory signals that spatially define the pituitary field. The Vax1 transcription factor is expressed in the ventral hypothalamus. In *Vax1*-knockout mice, a second Rathke's pouch develops in addition to the orthotopic anlage (Bharti et al., 2011), suggesting that the juxtaposition of oral and hypothalamic epithelia has the potential forms more than one pituitary morphogenetic field (Fig. 4). Thus, Vax1 probably limits the area of hypothalamic neuroepithelium that is competent to generate pituitary-inducing signals; indeed, *Vax1* expression in vivo is eliminated near the infundibulum (see Glossary, Box 1), which has inducing activity for pituitary development. Consistent with this idea, ectopic expression of Fgf10, which is expressed in the infundibulum and implicated in pituitary induction, is also detected in the hypothalamic neuroepithelium overlying the second pouch in the *Vax1* mutant mouse. This pituitary-inducing competence is more intricately regulated in vivo; for example, partial formation of a secondary adenohypophysis has also been reported in *Nog*$^{-/-}$ mutant mice (Davis and Camper, 2007).

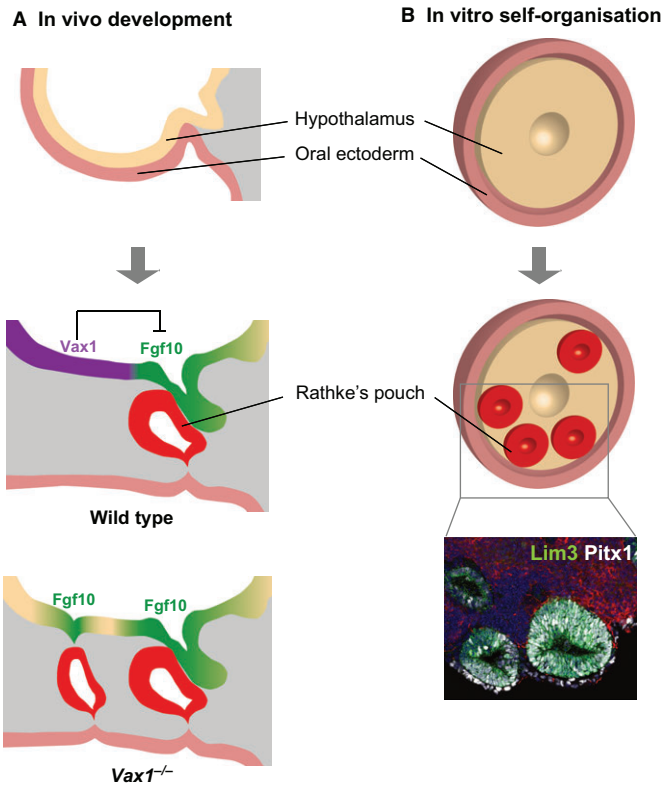

Fig. 4. Induction of Rathke's pouch in vitro and in vivo. (**A**) Rathke's pouch development from oral ectoderm in response to hypothalamus-derived inductive signals such as Fgf10. In the absence of Vax1 (*Vax1*$^{-/-}$), which limits the inductive competence in the hypothalamus, a secondary pituitary (green) is induced rostral to the primary adenohypophysis. (**B**) In vitro self-formation of multiple Rathke's pouches (Lim3^{+}, Pitx1^{+}) in an embryonic stem (ES) cell aggregate, in which oral ectoderm and hypothalamic neuroepithelium are juxtaposed. Photomicrograph shows a cross section of ES cell-derived Rathke's pouch. Modified, with permission, from Suga et al. (Suga et al., 2011).

Adenohypophysis generation in culture

Recently, we have been able to induce pituitary development in a single 3D aggregate of ES cells in which the interactions between the oral ectoderm and hypothalamic neuroectoderm were recapitulated (Suga et al., 2011) (Fig. 4B). Previous work has shown that the default fate of mouse ES cells in our floating aggregate culture of ES cells (SFEBq culture; see Glossary, Box 1) is rostral hypothalamus; differentiation along this lineage occurs efficiently when ES cell aggregates are cultured in growth factor-free, chemically defined medium (Wataya et al., 2008). Therefore, a technical modification required here was to co-induce

differentiation of oral ectoderm in addition to hypothalamic differentiation. In brief, this was achieved by increasing the initial plating cell number from 3000 to 10,000 per aggregate. This increase in cell number was sufficient to cause an elevation of endogenous BMP signalling levels in the aggregate, which only slightly inhibited hypothalamic differentiation and induced non-neural (oral ectodermal) tissue in the same aggregate. The co-induced oral ectoderm ($Pitx1/2^+$) and hypothalamic neuroepithelium ($Rx^+/Sox1^+$) spontaneously sorted out to form the outer and inner layers of the aggregate, respectively. A similar self-sorting phenomenon (neural in/non-neural out) was also observed in a repetition of the famous reaggregation study by Townes and Holtfreter (Townes and Holtfreter, 1955) using dissociated embryonic amphibian tissues (Steinberg and Gilbert, 2004).

As in the embryo, oral ectodermal tissue was juxtaposed to hypothalamic tissue in this modified culture. In addition, as the pituitary develops on the midline, a high level of Shh signalling (a feature of the midline) (Treier et al., 2001) was given to the aggregate. Under these simple culture conditions, parts of the oral ectoderm epithelium on the surface of the aggregate became thickened and started to express the pituitary placode marker *Lim3* (Ellsworth et al., 2008; Sheng et al., 1996). These thickened parts subsequently underwent invagination and formed pouches by detaching from the surface epithelium, as seen in adenohypophysis development in vivo. These $Lim3^+$ pouches expressed a set of markers that were specific to the embryonic Rathke's pouch.

With respect to pouch morphogenesis in this culture, $Lim3^+$ pouches reproducibly formed at a roughly fixed size, ~150 μm in diameter, which is similar to the size of the early Rathke's pouch in the embryo. Interestingly, a single aggregate often contained several pouches (Fig. 4). These findings suggest that several morphogenetic fields for pituitary placodes can be independently generated within oral ectoderm epithelium on the surface of the ES cell aggregate – reminiscent of the situation in the *Vax1* knockout and, again, suggesting that inhibitory signals must exist to limit pituitary placode formation.

Previous studies have shown that $Lim3^+$ epithelium of Rathke's pouch gives rise to all endocrine cell lineages in the adenohypophysis (Davis et al., 2011). Generation of cells with different hormone types is mostly instructed by a combination of localised extrinsic signals (BMP, Wnt, Shh, etc.) from neighbouring tissues, including ventral hypothalamus (Dasen and Rosenfeld, 2001; Ericson et al., 1998; Norlin et al., 2000; Rizzoti and Lovell-Badge, 2005; Potok et al., 2008). Among these endocrine cell types, differentiation of corticotropes ($ACTH^+$) is controlled in a relatively simple manner, via inhibition of the Notch-Hairy/Enhancer of Split pathway (Zhu et al., 2006; Kita et al., 2007). Consistent with this idea, treatment of ES cell-derived Rathke's pouch tissue with the Notch inhibitor DAPT was sufficient to induce an efficient differentiation of $ACTH^+$ cells. These corticotropes were functional, and, when transplanted under the kidney capsule, could rescue the hormone levels (ACTH and glucocorticoid), survival and spontaneous motor activity in hypophysectomised mice (Suga et al., 2011).

In general, mature endocrine cells have two basic functions. First, they need to secrete hormones by selectively responding to a proper stimulus. Second, they need to function as a part of homeostatic regulation at the systemic level. For example, when hormone activity is too high, they must stop secretion. The ES cell-derived endocrine cells display these properties: responding appropriately to hormonal stimuli in both inductive and negative-feedback contexts (Suga et al., 2011). This finding was somewhat surprising, as pancreatic β-cells derived from ES cells often show inefficient hormone secretion and/or an incomplete responsiveness to glucose levels (Raikwar and Zavazava, 2009; Noguchi, 2010). We suggest that the endocrine organoid (see Glossary, Box 1) formed in these 3D culture conditions may better reflect the in vivo micro-environment than isolated neuroendocrine cells derived from 2D ES cell cultures, and that such approaches may be beneficial in producing other functionally mature endocrine tissues.

Re-interpreting in vivo development in the light of the in vitro findings

In the in vitro 3D ES cell culture, multiple placodes self-form at random sites where oral ectoderm contacts hypothalamic neuroectoderm in the ES cell aggregate. An interpretation of these in vitro findings from a general viewpoint is that the pouch formation process per se (including morphogenesis and rough size determination) is driven locally by an intrinsic self-organising mechanism, whereas the specific place and number of pituitary placodes are under control of the complex positional information given by region-specific regulators. The latter process in the embryo is also tightly controlled by a combination of various other transcription factors (e.g. Tcf4) (Brinkmeier et al., 2007) and inductive signals (e.g. Shh, Wnt, BMP, noggin) (Ericson et al., 1998; Takuma et al., 1998; Rizzoti and Lovell-Badge, 2005; Davis and Camper, 2007) downstream of them. In addition, another spatial constraint to be considered for in vivo pituitary development may be physical separation between oral ectoderm and hypothalamic neuroectoderm; the lack of prechordal mesoderm and neural crest derivatives between them in the pituitary-forming field could contribute to positional preference for pituitary placode differentiation in the embryo.

Intestinal crypt architecture self-forms from a single stem cell

The above examples both make use of ES cells to generate organoids in vitro, recapitulating the in vivo developmental process. Besides the initial formation of organ buds, the growth and maintenance of such compound tissues are important for the development of functional organs. Our third example, the gut endoderm, concerns adult stem cells and their local structure-dependent maintenance. As described below, a recent series of studies on adult intestinal stem cells, including both in vivo and in vitro approaches, have provided novel insights into the local relationship between stem cells and their niche (Sato et al., 2009; Sato et al., 2011; Takeda et al., 2011; Tian et al., 2011; Cao et al., 2011; Spence et al., 2011). These studies revealed the dynamic self-sustaining nature of intestinal crypt structures, which are crucial for the development and homeostasis of the gut stem cell system.

Homeostasis of the adult intestine

The absorptive epithelium of the small intestine is known to undergo rapid cell turnover (typically, with a lifespan of 5 days or less), and this massive cell renewal depends on the stem cell system present at the bottom of the crypt of Liberkühn (crypt, hereafter; see Glossary, Box 1) (Fig. 5A). This system supports stem cell renewal and also generates 'transit-amplifying' (TA) cells, which are located just above the stem cell domain (Barker et al., 2010; Simons and Clevers, 2011). TA cells rapidly cycle and differentiate into all types of intestinal epithelial cells. Enterocytes (the main absorptive epithelial cells), entero-endocrine cells and goblet (mucus-producing) cells migrate upwards towards the villus (see Glossary, Box 1) in a conveyer belt-like fashion, whereas

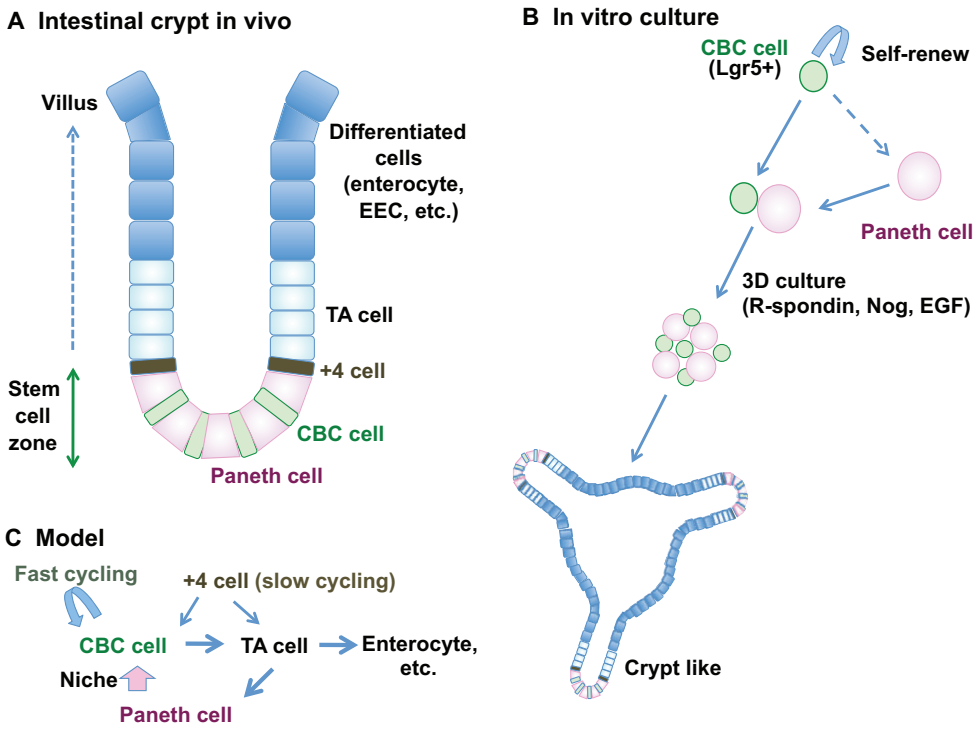

Fig. 5. Self-sustaining system of intestinal crypt architecture and its stem cells. (**A**) Dynamic maintenance of intestinal crypt tissue by two stem cell populations (fast-cycling CBC cells and slow-cycling +4 cells) in vivo. CBC, crypt-base columnar; TA, transit-amplifying; EEC, entero-endocrine cell. (**B**) Self-organised formation of crypt-like structures from single Lgr5$^+$ cells. Although CBC cells can self-renew in the presence of strong Wnt signalling, its efficacy is greatly improved when CBC cells receive niche-signals from Paneth cells, which can be also generated from CBC cells (presumably via TA cells). In 3D culture, these cells grow to form crypto-like organoids in a self-organising fashion. (**C**) The inter-relationship between the two stem cell populations and Paneth cells.

Paneth cells (see Glossary, Box 1) descend to the crypt bottom (Cheng and Leblond, 1974) and persist there for several weeks, unlike the short-lived enterocytes.

In the small intestine, the crypt bottom domain contains three major cell types: Paneth cells, crypt-base columnar (CBC) cells (see Glossary, Box 1) and special cells just above the upper-most Paneth cells, at position +4 from the crypt-base centre (hereafter, +4 cells; see Glossary, Box 1) (Barker et al., 2010; Carlone and Breault, 2012). Earlier studies raised two distinct theories for the nature of the crypt stem cells. The so-called classic model proposes that +4 cells are the true stem cells (Cairnie et al., 1965; Potten, 1977; Potten et al., 2012). These cells are sensitive to radiation and, more importantly, exhibit substantial retention of nucleotides labelled with tritium or bromine, a signature that is characteristic of slow-cycling stem cells. In addition, some +4 cells (but not all) show strong expression of Bmi1 and mTERT (B lymphoma Mo-MLV insertion region 1 homologue and mouse telomerase reverse transcriptase) (Sangiorgi and Capecchi, 2009; Montgomery et al., 2011; Yan et al., 2012), which are often associated with stem cells. The second theory, suggested by genetic marking studies, is that CBC cells (Cheng and Leblond, 1974; Bjerknes and Cheng, 1999), which are relatively slender and immature-looking epithelial cells wedged between large Paneth cells, are the crypt stem cells.

Over the past few years, strong evidence in favour of the second model has accumulated. A crucial breakthrough was the identification of the cell-surface molecule Lgr5 (leucine-rich G-coupled receptor 5) as a downstream target of canonical Wnt signalling and bona fide marker of CBC cells by Hans Clevers and colleagues (Barker et al., 2007). *Lgr5*-expressing CBC cells display both fundamental properties of a stem cell: self-renewal and multipotency. Lgr5 and its relative Lgr4 have been recently shown to potentiate canonical Wnt signalling by associating Wnt receptors and mediating the activity of R-spondin (Wnt agonist) (de Lau et al., 2011; Carmon et al., 2011; Glinka et al., 2011), consistent with the idea that Wnt signalling plays a crucial role in the maintenance of intestinal stem cells (Ootani et al., 2009).

More recent reports have provided experimental evidence that +4 cells also directly participate in the crypt stem cell system. Conditional ablation of *Lgr5*-expressing cells demonstrated that elimination of the rapidly cycling CBC cells did not substantially perturb homeostasis of the intestinal epithelium (Tian et al., 2011), indicating that CBC cells are dispensable, at least in this particular situation. In this work, the authors posited the presence of a reserve stem cell population in the crypt, and attributed it to a Bmi-positive population of +4 cells that expanded after ablation and compensated for the loss of *Lgr5*-expressing CBC cells.

Gut organoid formation in culture

As described above, the stem cell system in the crypt is intricate and involves multiple interactions between different types of cells via multiple local signals. Notably, however, understanding of the in vivo roles and relationship of these cell types has been greatly advanced by recent in vitro studies using 3D culture of isolated intestinal stem cells (Fig. 5B). In particular, the stem cell nature of CBC cells was strongly suggested by the finding that a single Lgr5$^+$ cell can grow to generate a crypt-like ‘organoid’ when cultured in the presence of R-spondin, Noggin and EGF (Sato et al., 2009). Importantly, this single-cell suspension culture also requires the addition of ROCK inhibitor, which is known to suppress dissociation-induced apoptosis and promote proliferation in single epithelial cells, including human ES cells (Watanabe et al., 2007; Ohgushi et al., 2011). Another point worth emphasising is that this organoid formation does not require the presence of mesenchymal cells, which play important roles in various aspects of endoderm-derived organs in vivo. The tubular evagination from the epithelial cyst in this culture is superficially similar to that seen in 3D culture of MDCK (Madin-Darby Canine Kidney) cells with HGF (hepatocyte growth factor), a model for mesenchymal signal-driven tubulogenesis (Montesano et al., 1991). However, a fundamental difference is that the Lgr5$^+$ cell culture recapitulates not only tubular morphogenesis but also the formation of the intestinal stem-cell zone, the TA cells and differentiated cell

compartments. In other words, this intestinal stem cell culture co-generates both the stem cell niche and tissue architecture in a self-organising fashion.

A recent study using this organoid culture also revealed the role of the Paneth cell as a niche-creating cell (Sato et al., 2011) (Fig. 5B). At the crypt base, CBC cells and Paneth cells ($CD24^+$) are located side by side, forming a checkerboard pattern (Snippert et al., 2010). Relatively gentle dissociation of crypt epithelium tended to produce doublets consisting of a CBC cell and a Paneth cell, suggesting a tight physical association between the two types of cells. Importantly, crypt-like organoids were generated much more efficiently in suspension culture of these doublets (or co-culture of two cell types) than of a CBC cell alone. Paneth cells express stem cell-supporting signalling molecules (Wnt3, Dll4, EGF and TGFα), and genetic removal of Paneth cells in vivo decreases the number of CBC cells (Sato et al., 2011) [although the latter point may still require careful argument in light of a new report by Kim et al. (Kim et al., 2012)]. The stem cell-supporting activity of Paneth cells can be substituted, at least in part, by strong Wnt signalling. These in vivo and in vitro findings together demonstrate a direct role of Paneth cells in creating a stem cell niche for CBC cells.

Importantly, the organoid culture also demonstrated that +4 cells (which express Hopx) and CBC cells can generate one another in vitro, showing the inter-convertible nature of these two populations (Takeda et al., 2011; Tian et al., 2011) (Fig. 5). These in vitro studies support the idea that two intestinal stem cell populations with different cell-cycling dynamics co-exist in distinct niches, reconciling the two models for crypt stem cells.

Although the studies described above mainly dealt with adult intestinal stem cells, crypt-like organoids have been also successfully generated from ES cell and iPS cells (Cao et al., 2011; Spence et al., 2011). These studies using pluripotent stem cell culture suggest the possibility that the 3D gut organoid culture could be used to analyze the developmental aspects of gut epithelia besides the maintenance mechanism.

A unified model for the dynamic self-sustaining system of crypt stem cells

Based on these in vitro and in vivo observations, the following mechanism seems to emerge for the robust and dynamic maintenance of the crypt stem cell system. In a steady-state situation, $Lgr5^+$ CBC cells (~14 cells/crypt) mainly function as active stem cells that rapidly proliferate symmetrically. They are supported by the micro-environment created by adjacent Paneth cells (typically 20 cells/crypt or fewer). CBC cells that fail in neutral competition for the niche (Snippert et al., 2010; Lopez-Garcia et al., 2010) exit from the stem cell zone to the compartment above +4 and differentiate into TA cells. By contrast, after injury of CBC cells, slow-cycling +4 cells proliferate more efficiently and support TA cell generation to compensate. Eventually, the +4 reserve cells also restore $Lgr5^+$ CBC cells, whereas CBC cells can contribute to de novo production of +4 stem cells. The strong mutual coupling among stem cell populations (mediated by as yet unknown mechanisms), together with the self-formation of their niche, probably contributes to robust generation and maintenance of crypt-like organoids from single $Lgr5^+$ cells in 3D culture under Wnt-enhanced conditions.

An important question remains: how is the constant size of the stem cell zone at the crypt base determined? An assumption in the model above is that the number of Paneth cells, which determines the niche size, must somehow be kept constant. However, as Paneth cells can be also generated from CBC cells, this could create a positive-feedback loop in which an increase of Paneth cell number increases the number of CBC cells, generating more Paneth cells; this cycle would lead to divergence of the system and not make the cell number constant. In future investigations, the 3D stem cell culture system should be useful for analyzing the cellular dynamics and potential regulatory factors, including feedback inhibitors and mechanical signals. Another intriguing issue is the molecular control of the checkerboard-like spatial arrangement of CBC and Paneth cells. This type of pattern formation is also seen in the spatial arrangement of hair cells and supporting cells in the inner ear epithelium, for which differential heterophilic affinities of nectin subtypes play an essential role (Togashi et al., 2011). Perhaps a similar principle is at work for the spatial arrangement in the intestinal stem cell zone. Although a preliminary attempt at computer simulation of the biomechanics of gut organoid formation has been recently reported (Buske et al., 2012), it will be intriguing to unravel this process using a combination of real-time 3D imaging and computer modelling.

In addition, 3D stem cell culture of intestinal stem cells is expected to contribute to new tissue-replacement therapy. A recent report showed successful application of intestinal ‘organoids’ expanded from an $Lgr5^+$ colon stem cell to functional transplantation into the mouse colon epithelium (Yui et al., 2012). This strategy may be applied to regenerative medicine for intractable ulcers (e.g. inflammatory bowel diseases) by promoting epithelial wound healing. As intestinal organoids can be also generated from human iPS cells in 3D culture (Spence et al., 2011); they will be also useful for establishing disease models of genetic disorders (e.g. malabsorption syndrome), as well as studying oncogenesis.

Common principles of these in vitro systems

In this Review, we discuss a novel in vitro approach to the study of organogenesis using 3D stem cell culture, in which unexpected levels of self-organisation are seen. The ES cell cultures recapitulate the development of retina and adenohypophysis, while adult intestinal stem cell culture mimics the dynamic maintenance of somatic stem cells and their niche in the crypt.

These experimental systems have three common features. One is that they begin with cells having no pre-pattern. ES cell culture is started by reaggregation of undifferentiated ES cells, whereas intestinal stem cell culture can be initiated from a single cell. In both cases, cells are cultured in homogenous medium with no signal gradient. Another common feature is that the cells are grown as floating aggregates. The floating 3D culture is crucial for analyzing both shape formation and spontaneous patterning in organogenesis. For example, recent studies have demonstrated that culturing cells on stiff plastic or glass surfaces strongly influences not only the cytomechanics but also growth and differentiation (e.g. via Hippo-related pathways) (see Dupont et al., 2011; Wada et al., 2011; Sansores-Garcia et al., 2011). Floating culture allows a high level of freedom of tissue deformation with no external restriction. In other words, the boundary conditions of the aggregate can vary along the time line according to the internal conditions and rules. This is characteristic of these 3D cultures, and contrasts with strict constraint in conventional tissue-engineering microfabrication. The third shared feature is that these three organoids (optic cup, Rathke’s pouch and gut crypt) are epithelial structures with a clear apical-basal polarity. The epithelial nature may have an advantage in facilitating growth, collective behaviours, controlled deformation and intra-tissue communications, whereas the apical-basal polarity could help the ordered alignment of cells and the directionality within the tissue.

Table 1. Self-organisation of various tissues in culture

Self-organised tissue	Cell type used	Biological observations	References
Optic cup	Mouse and human ES cell aggregates	Generation of appropriate 3D morphology and stratified NR tissue	Eiraku et al., 2011; Nakano et al., 2012
Adenohypophysis	Mouse ES cell aggregates	Rescue of hormonal levels, daily activity and survival after transplantation into hypopituitary mice	Suga et al., 2011
Gut	CBC cells (±Paneth cells)	Facilitation of ulcer healing in the colon when grafted	Sato et al., 2009
Cerebral cortex (foetal type)	Mouse and human ESC aggregates	Projection of axons to subcortical targets after grafting; synchronized spontaneous firing	Eiraku et al., 2008
Tooth germ	Embryonic oral ectodermal cells and mesenchymal cells	Development of fully grown teeth after oral transplantation	Nakao et al., 2007; Ikeda et al., 2009
Skin	Karatinocytes, dermal mesenchyme and endothelial cells	Three-dimensional self-formation of dermis and epidermis	Sun et al., 2005; Lee et al., 2011
Hair follicle	Bulge stem cells (or embryonic epidermal cells) and dermal (or deep papilla) mesenchyme	Hair-type specific growth (pelage and vibrissa) after transplantation	Toyoshima et al., 2012

CBC, crypt-base columnar; ES, embryonic stem; NR, neural retina.

A substantial difference between the two ES cell cultures and the intestinal stem cell culture is that the latter is strongly dependent on various growth factor signals. By contrast, self-organisation in ES cell culture occurs best when growth factor signals are kept minimal. One reason for the difference may be that tissue stem cells are less self-sustaining and more tightly regulated in an environment-dependent fashion; in fact, self-sustaining growth of tissue stem cells must be restricted in vivo, because they would otherwise form a tumour. Addition of growth factor cocktails affects differentiation from pluripotent ES cells, although it may not disturb the cellular fate of somatic stem cells as much because they are oligopotent.

Conclusions and perspective

Recent findings discussed in this Review suggest that self-organising mechanisms play substantial roles in establishing local tissue architecture, while interactions with neighbouring tissues contribute to robust determination of the location and number of organ buds. Local autonomy should play more profound roles than we have expected before; the three examples shown here probably represent only a tip of the iceberg. Table 1 summarises other examples of organogenesis studies in vitro, which we have not considered in detail here, and describes the cell types used and the biological observations seen there. For example, human ES cell aggregates in SFEBq culture have been shown to generate a laminated structure of foetal cortical tissue in a self-organising fashion (Eiraku et al., 2008). Other remarkable examples for in vitro self-formation of 3D structures include: tooth germs from reaggregates of oral ectoderm placed next to tooth mesenchyme in collagen gel (Nakao et al., 2007; Ikeda et al., 2009); skin-like tissue from a simple mixture of skin mesenchymal cells and keratinocytes, with (or without) endothelial cells on a scaffold (Sun et al., 2005; Lee et al, 2011); and hair follicles from a conjugation of budge stem cells and dermal mesenchymal cells (Toyoshima et al., 2012; Zheng et al., 2005).

The strength of this in vitro approach is its relative simplicity as an experimental system. At least with the examples discussed here, an unexpected level of robustness in the local organogenetic program could be seen even when the system was ‘cut out’ from its in vivo context. Studying organogenesis in vitro facilitates detailed observations of defined elements with much reduced complexity, and is compatible with many perturbation approaches for testing dynamic models. Three-dimensional stem cell culture may also become a versatile tool for mechanical analysis of tissue deformation during early organ development, e.g. with respect to internal forces and mechano-chemical coupling. Last, this system is also suitable for synthetic biology at the cell and tissue levels, as seen by the effects of tissue juxtaposition on pituitary self-formation and of Paneth cells on gut organoid formation from CBC cells. These ‘additive’ approaches should give crucial information in a complementary manner to the subtractive approaches in reverse genetic studies.

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Competing interests statement

The authors declare no competing financial interests.

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