From disease to development to cell biology and back

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Summary

Nowadays, the focus of developmental studies is shifting away from formal models of developmental pathways that are characterised by flow charts of controlling factors connected by arrows, to mechanistic models that explain developmental processes at the cellular level. Surprisingly, this shift towards a cellular view of developmental biology is occurring simultaneously across a range of model organisms. One consequence of taking such a cell biological view of development is that many model organisms are now becoming good models for studies of human disease and therapy.

These are times of change in three fields: cell biology, development and pathology. Indeed, this is a time in which they are merging. In recent years, developmental studies on the cell biology of morphogenetic events have made it clear that it is no longer possible to distinguish between the factors that endow developing cells with information (such as transcription factors and signalling molecules) from those that ensure the basic survival of cells (such as metabolic factors, cytoskeletal and membrane trafficking effectors, and extracellular matrix proteins). This trend is reflected in cell biological and developmental conferences at present: people who have never met before are starting to realise that they speak the same language. What is emerging from these meetings is the recognition that disease is beginning to be explained in terms of development, and developmental problems are beginning to be explained in terms of cell biological processes. This was the focus of a recent Jacques Monod conference, 'Cells in embryonic development and disease', which was held in June 2003 near Toulon, France.

Disease, developmental genetics and cell biology

How do cells manage to integrate complex information during development? How do they deploy all the weaponry that they need to accomplish a distinct developmental program? It was in *Drosophila* that the Selector genes were first discovered: master genes that encode transcription factors that directly, or, through the action of intermediate transcription factors, coordinate the expression of target genes that execute different developmental fates. Such transcription factors have since been found to play similar roles during development from fly to fish and mouse, revealing their evolutionary conservation. Now such essential genes seem to underlie a variety of diseases. One such gene, *Runx2*, was discussed by Gerard Karsenty (Baylor College, Houston, TX, USA). Runx2 is a transcription factor that controls osteoblast differentiation (reviewed by Karsenty, 2001). In its absence, bone fails to form, and its ectopic expression is sufficient to cause ectopic bone differentiation. Mutations in *RUNX2* have since being discovered to be responsible for cleidocranial dysplasia, a hereditary disease in humans: individuals heterozygous for a *RUNX2* mutation have a hole in their skull. Another example of a key transcriptional regulator involved in disease is the transcription factor IPF1 (insulin promoter factor 1), which is implicated in diabetes. Helena Edlund (University of Umea, Umea, Sweden) discussed how IPF1 (also known as Pdx1 in mouse and other model organisms) controls the differentiation of stem cells into pancreatic β cells and, later, directly activates the transcription of the insulin gene in these cells. In the $Pdx1^{-/-}$ knockout mouse, the pancreas is specified normally, but then fails to develop (Jonsson et al., 1994). At least one homozygous $IPF1^{-/-}$ human shows the same pathology (Stoffers et al., 1997b). In a Cre/*lox* conditional $Pdx1^{-/-}$ knockout, in which the gene is inactivated late in the development of the β cells, insulin production and glucose sensing are impaired (Ahlgren et al., 1998). Again, some diabetics that have a similar phenotype have been found to be mutant for *IPF1* (Stoffers et al., 1997a). IPF1 activates fibroblast growth factor receptors (FGFRs) in β cells, and FGF signalling in turn is required for the expression of a glucose transporter, Glut2 and the prohormone convertase, PC1/3 (Hart et al., 2000). Glut2 triggers a cascade of events that lead to insulin secretion, whereas PC1/3 controls the processing of pro-insulin into active insulin. The implications of these findings are obvious: FGFRs and MAPK cascades downstream of FGF signalling might be potential targets for new drugs for diabetes. Thus, new strategies to treat diabetes might result as a consequence of improving our understanding of the developmental biology of β cells.

Hedgehog and Wnt signalling may also be implicated in the development of skin cancer because of their involvement in the specification of sebaceous and follicular stem cells in the epidermis. Fiona Watt (Cancer Research UK, London, UK) has developed an interesting whole-mount procedure for studying epidermal stem cells in the epidermis of mouse tails. Using this approach, she has mapped populations of stem cells in this tissue and has studied their proliferative and differentiation capabilities (Fig. 1). She has found that stem cells do not necessarily coincide with label-retaining cells (those that incorporate BrdU) in neonatal epidermis and do not subsequently divide (Braun et al, 2003). Using this procedure, Watt has found that both Indian hedgehog and Wnt proteins are involved in the development of the sebaceous and the hair follicular progenitors. These events might also be affected during the progression of a cell into a state of uncontrolled growth in tumours such as squamous cell carcinoma, sebaceous carcinoma and trichofolliculoma.

Morphogen gradients and myogenesis

During the past decade, the concept of the morphogen – a secreted signalling molecule that spreads from its source and forms a concentration gradient – has boosted our understanding of morphogenesis during development. Depending on their distance from the source of a morphogen, target cells encounter different concentrations of the molecule and respond with the expression of different target genes. Philip Ingham (University of Sheffield, Sheffield, UK) showed how this principle also

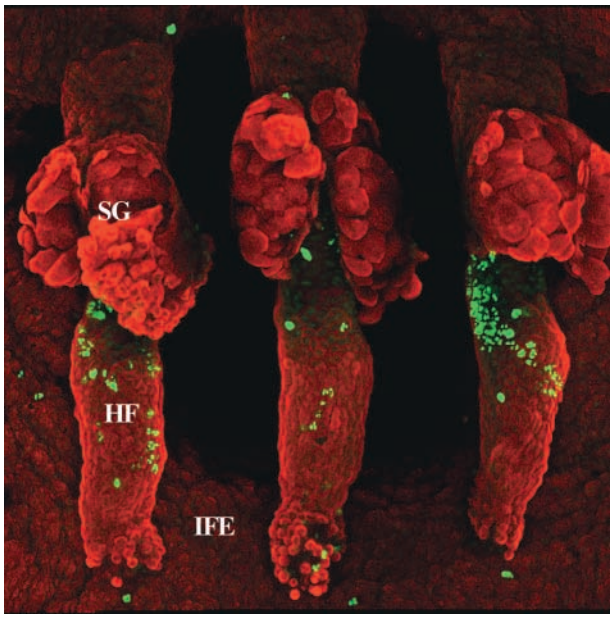

Fig. 1. Localisation of DNA label-retaining cells in three whole-mount preparations of a mouse tail epidermis. In this procedure, 10-day old mice receive four injections of BrdU over 48 hours to label mitotic cells. After a 70-day chase period, a whole-mount preparation of tail epidermis is immunolabelled for keratin 14 (red) and BrdU (green; to detect label-retaining cells, LRCs). HF, hair follicle; IFE, interfollicular epidermis; SG, sebaceous gland.

applies during early vertebrate myogenesis. In zebrafish, two muscle types with different contractility behaviour are spatially segregated: mononucleated slow twitch muscles expressing slow myosin heavy chain (MHC) and multinucleated fast twitch muscles expressing fast MHC. It turns out that the slow twitch precursors are induced by the sonic hedgehog (Shh) signal but differentiate into distinct muscles depending upon the levels of Shh to which they are exposed (Wolff et al., 2003). This implies that the myoblasts read a Shh morphogenetic gradient, and Ingham described how, as in *Drosophila*, the interpretation of signal strength requires the activity of the serine/threonine kinase Fused. In addition, it seems the competence of the muscle precursors to respond to Shh changes with time, such that at later developmental stages, high levels of Shh induce the differentiation of a specific class of fast-twitch muscles. These findings indicate that time might also influence how morphogenetic gradients are interpreted by cells.

The syncytial character of muscles is conserved from insects to vertebrates. What factors are involved in the fusion of myoblasts to become multinucleated muscles? Work from Mar Ruíz-Gómez (CSIC, Madrid, Spain) has shed light on the cell biology of myoblast fusion in *Drosophila*. Each somatic muscle derives from the fusion of a single muscle founder cell with a set number of fusion-competent cells. In collaboration with Michael Bate (Cambridge University, Cambridge, UK), Mar has isolated Dumbfounded (Duf), a transmembrane protein that contains an immunoglobulin (Ig) domain that is specifically expressed in muscle founders and is sufficient to target the specific fusion of the fusion-competent cells with their founder (Ruíz-Gómez et al., 2000). How general is the function of Duf? For example, is it also required in the visceral mesoderm from which the gut muscles derive? Gut muscles in the fly were thought to develop somewhat differently to somatic muscles: the visceral mesoderm forms an epithelium in contact with the gut, and then gives rise to the circumferential muscles of the gut, which were presumed to be mononucleated. The work of Mar and others has recently shown that the epithelium of the visceral mesoderm is the source of the gut muscle's founder cells (Klapper et al., 2002). They express *Duf* and are surrounded by a cloud of mesenchymal mesodermal cells that correspond to the fusion-competent myoblasts. Thus, myoblast fusion does indeed occur in the gut muscle, and uses the same molecular machinery as do somatic muscles. The partner of Duf in both systems is another Ig-containing protein, called Sticks-and-stones (Sns), originally identified by Abmayr's laboratory (Bour et al., 2000). Consistently, Sns expression is confined to the fusion-competent myoblasts.

The many roles of membrane trafficking and lipid metabolism in development

Endocytic pathway in flies

The interface between membrane trafficking and developmental signalling has become a major area of research in recent years (González-Gaitán, 2003). In former times, it was believed that trafficking through the biosynthetic pathway was merely involved in the secretion of ligands, while the endocytic pathway degraded cell-surface receptors to downregulate a signalling event. In recent years, the endocytic pathway has emerged as a site of signal transduction: the initiation of the intracellular kinase cascades during epidermal growth factor and transforming growth factor β (TGFβ) signalling seem to take place, at least in part, from the endosome (reviewed by González-Gaitán, 2003). Other aspects of signalling, such as the spreading of morphogens to form concentration gradients, are also believed to make use of the endocytic pathway, as in the case of TGFβ and Wnt signalling (Dubois et al., 2001; Entchev et al., 2000).

Although the concept of the morphogen has pervaded studies of morphogenesis, little is known about how morphogen gradients actually form. Previous theories have suggested that morphogens spread across tissues by freely diffusing through the extracellular matrix; a morphogen thus forms a gradient as it moves away from its source. How could this scenario generate a stable gradient that robustly encodes positional information? Marcos González-Gaitán (Max-Planck Institute, Dresden, Germany) has explored the role of the endocytic trafficking during morphogen gradient formation. He showed that the formation of a stable, long-range Decapentaplegic (Dpp) gradient in the fly imaginal disc requires endocytosis in the cells where the gradient is formed (Entchev et al., 2000). Dpp is internalised into endosomal structures in the receiving cells. Such internalised Dpp can follow different fates: it can be recycled back to the plasma membrane/extracellular space or degraded in the lysosome. These different destinations are controlled by small GTPases of the Rab family. By studying flies mutant for these GTPases, González-Gaitán has shown that the range of the gradient depends on the rates of Dpp recycling and degradation in the

receiving cells (Entchev et al., 2000). The model that arises from these findings is that Dpp travels through the cells via the endocytic pathway and not around the cells in the extracellular matrix.

Another example of how cells control signalling by controlling the trafficking of the signalling molecules was provided by Barry Dickson (IMP, Vienna, Austria), who studies axon guidance at the midline of the *Drosophila* central nervous system (CNS). At the midline of the developing CNS, a secreted ligand, Slit, repels growth cones that express high levels of the Slit receptor, Robo at the plasma membrane (Rajagopalan et al., 2000). It is unclear whether Slit secreted from the midline forms a gradient of concentration, so acting as a morphogen. However, Robo levels at the growth cone are known to be controlled by its degradation in lysosomes (Keleman et al., 2002). This process involves Commisureless (Comm), which is required in neurones to downregulate Robo. Comm targets Robo to the late endosomes directly from the Golgi, before it reaches the plasma membrane (Keleman et al., 2002). An E3 ubiquitin ligase, Nedd4, is also involved in this process (Myat et al., 2002). E3 ubiquitin ligases have recently being shown to be involved in downregulating receptors through endocytosis and lysosomal targeting (Hicke, 1999). However, in this case, ubiquitination of Robo itself is not required for its degradation, as a Robo mutation that precludes ubiquitination does not cause axon pathfinding defects (B. Dickson, unpublished). Some other molecule must, in this case, be the target of Nedd4-dependent ubiquitination.

The secretory pathway in fish

The zebrafish provides an ideal model system with which to study trafficking through the secretory pathway during development, as exemplified by the studies of Derek Stemple (NIMR, London, UK) on the role of Golgi trafficking during notochord development in zebrafish. The notochord is the major skeletal element of the zebrafish embryo and forms from a single row of cells that also act as a source of midline signals during early development. By studying notochord mutants in zebrafish, Stemple's group has shed light on the role of vesicular trafficking and the basement membrane in notochord formation. As the mutations cause the fish to be very short, they have been named after Snow White's seven dwarfs.

Normally, as it differentiates, a thick basement membrane forms around the notochord, whereas a large vacuole inflates within each cell. Three of the dwarf mutations directly affect sheath formation (see below), while three others indirectly affect both formation of the vacuole and the sheath, as they encode proteins essential for vesicular trafficking. These loci, called *sneezy*, *happy* and *dopey*, encode the α, β and β′ subunits of the coatomer complex, which forms part of the coat of a major class of Golgi vesicles (D. Stemple, unpublished). Surprisingly, the α subunit mRNA is upregulated in notochord progenitors, while it is downregulated in other places in the embryo. Stemple showed that this upregulation involves a negative-feedback loop, which is the basis of a molecular mechanism that elevates, upon demand, the transcription levels of the coat components: their transcription levels increase if expression of one of the coatomer subunits is disrupted, either by mutation or by morpholino injection, or if coatomer assembly is impaired with a drug (e.g. Brefeldin A). These observations suggest that a homeostatic mechanism, which regulates transcription levels, is exploited during development to generate tissue-specific expression patterns of a key membrane trafficking factor.

Sphingolipid and cholesterol metabolism in development

Membrane rafts – lipid microdomains that are enriched in sphingolipids and cholesterol, and that possibly act as signalling platforms – have garnered particular attention in studies that connect development with vesicular trafficking (Simons and Ikonen, 1997). In both fish and worms, studies on the developmental role of these domains is being addressed using genetic and cell biological approaches. Steven Farber (Thomas Jefferson University, Philadelphia, PA, USA) has developed a number of fluorescent reagents to study, in vivo, the dynamics of phospholipid and cholesterol metabolism during zebrafish development. For example, BODIPY FR-PC is a phosphatydilcholine analogue that uses FRET (fluorescence resonance energy transfer) to report when it is enzymatically modified. This probe contains a fluorophore on each acyl chain: the release of one chain by phospholipase A2 activity terminates the FRET between the two fluorophores and changes the spectral properties of the probe. To study cholesterol metabolism, Farber uses a fluorescent cholesterol analogue, called NBD-cholesterol, which he feeds to 5-day-old zebrafish to visualise liver function and lipid metabolism. Using a forward genetic screen with these reagents, Farber has identified mutants with altered lipid synthesis, degradation and trafficking.

In his talk, Adam Antebi (Max-Planck Institute, Berlin, Germany) discussed the connection between cholesterol and worm development (Gerisch et al., 2001). When deprived of cholesterol, *C. elegans* larvae enter the dauer state. A key regulator of this process is Daf12 (Antebi et al., 2000), a steroid hormone receptor. Could cholesterol be involved in this steroid receptor signalling event? Although the mechanism of action remains unclear, Adam has found that Daf36, which is an FeS ketosteroid hydroxylase involved in steroid biosynthesis, is a key regulator of dauer formation that acts upstream of Daf12.

Cell junctions in development

Studies of diverse morphogenetic events across a range of organisms have highlighted that cellular junctions have several key functions during the development of epithelial primordia; these functions are required during morphogenetic signalling, during embryonic morphogenetic movements and for asymmetric cell division. Marcos González-Gaitán proposed a model for the formation of morphogen gradients where Dpp is deployed in a space in the extracellular matrix, between the apical adherens and the more basal septate junction, the homolog of the vertebrate tight junction. In this model, morphogens are confined to a narrow plane of the apicobasal axis of the developing epithelium where the gradient formation can be more effectively built up and controlled. Thomas Lecuit (LGPD, Marseille, France) talked about a different developmental role for junctions: morphogenetic movements. Lecuit has found that cell junction remodelling is essential for the convergence/extension movements that occur during germband elongation in the *Drosophila* embryo. What is novel about his findings is that they reduce the problem of convergence/extension of a whole tissue to the behaviour of tetrads of cells. The embryonic epidermal cells are arranged in

tetrads in the plane of the epithelium with a dorsal, ventral, anterior and posterior cell in each tetrad. Before tissue extension occurs, the anterior and posterior cell in each tetrad are connected by a cell junction, while the dorsal and ventral cells are not in contact. During extension, the contact junction between the anterior and posterior cells shrinks, while a new junction appears between the dorsal and ventral cell. This planar remodelling of junctions is associated with the recruitment of a contractile actin-myosin cytoskeleton at the junctions. Myosin is specifically enriched in disassembling junctions and its activity is required for planar cell intercalation (T. Lecuit, unpublished).

Another role for cell junctions in development has emerged from the study of spermatogenesis in *Drosophila*. During this process, cell-cell contacts via the adherens junctions seem to orchestrate asymmetric cells divisions. In the adult testis, a group of cells, the somatic hub, instructs nine cells to behave as asymmetrically dividing stem cells that produce gonioblasts, which become sperm upon further proliferation and differentiation. Margaret Fuller (Stanford University, Stanford, CA, USA) showed how the hub itself induces the stem cells to divide asymmetrically. The nine stem cells divide radially with respect to the hub: the stem cell spindles align along radii where the hub is in the center. This is achieved through a mechanism in which the stem cell centrosome is located adjacent to the hub; upon centrosomal duplication, the second centrosome migrates to the opposite pole. In *centrosomin* mutants, the spindle is misoriented leading to circumferential divisions that cause the two daughter cells (instead of only one) to become gonioblasts. Fuller showed that the stem cells are polarised, express E-cadherin and establish adherens junctions with the hub. APC2 (adenomatous polyposis coli 2), a factor that is associated with the adherens junctions of embryonic and larval epithelia, is also in the hub/stem cell interphase and colocalises with the centrosomes. APC2 connects the junctions with the centrosomes. This way, by being connected to the stem cells through adherens junctions, the hub organises the spindle orientation and the asymmetric division of the stem cell.

Structural integrity during development: collagen and laminin in fish and worms

Three of the notochord mutants studied by Stemple, *bashful*, *grumpy* and *sleepy*, carry mutations in genes that encode the α1, β1 and γ1 laminin chains, which form a tripartite complex that contributes to the basement membrane that surrounds the notochord. Loss of any of these laminin chains leads to loss of this basement membrane. Without a basement membrane, the notochord fails to differentiate, early marker genes are persistently expressed and vacuoles that normally appear in the notochordal cells during the process of elongation fail to form (Parsons et al., 2002). In fact, the vacuoles and the basal lamina are essential during notochord elongation: the thick peri-notochordal basement membrane provides a mechanical barrier against which the vacuolated cells can exert turgor pressure during the process of elongation. This explains the short-axis phenotype of the laminin mutants.

The final elongation of the *C. elegans* embryo also involves a similar mechanism. Michel Labouesse (IGBMC, Strasbourg, France) has found that two main forces act during the final elongation of the worm: circumferential forces, which are mediated by a circumferential actin ring; and anterior-to-posterior longitudinal forces, which are mediated by the muscles. Labouesse reported that two Spektraplakin protein isoforms, which are encoded by a single *vab-10* gene, protect the animal from these tearing forces, thereby maintaining its structural integrity (Bosher et al., 2003; Poinat et al., 2002). VAB-10A and VAB-10B show an alternative banding pattern in muscles, which correlates with myosin and actin banding, respectively. VAB-10A and VAB-10B have different mechanisms of action. VAB-10A is associated with intermediate filaments in the muscle. In *vab-10a* mutants, the intermediate filaments are disorganised and the muscles collapse. The tearing phenotype of VAB-10A is thus due to ripping caused by muscle activity. VAB-10B binds microtubules in the epidermis; microtubular organisation is disturbed in *vab-10b* mutants. As a consequence, the connection of muscles to the epidermis is lost, presumably because the VAB-10A and VAB-10B networks are interconnected in the epidermis, uncoupling the actin ring in the epidermis from the muscles. Thus, while VAB-10A protects the embryo from radial muscle forces against the epidermis, VAB-10B protects from longitudinal tearing forces.

The zebrafish lateral line

Alain Ghysen (INSERM, Montpellier, France) studies the development of the zebrafish lateral line. The lateral line in the freshly hatched zebrafish is formed by five neuromasts, which are composed of hair cells enclosed in a cupula. Ghysen illustrated how during early development in the lateral line, a group of neuromast precursor cells migrate posteriorwards, following a pathway that is defined by the expression of Sdf1, a chemokine (David et al., 2002). The neuromast precursor cells in turn express Cxcr4b, the Sdf1 receptor.

Although the freshly hatched fish has a single lateral line, older fish carry four parallel lateral lines. Ghysen showed how these multiple lateral lines are formed (Sapede et al., 2002). Mosaic experiments show that once the first round of differentiated neuromasts is deployed, the neuromasts migrate ventrally. Then, a second round of neuromast precursors migrate posteriorwards from the anterior region of the embryo to form a second lateral line that will also eventually drift ventrally. This process is then repeated several times. In the case of the Mexican blind cavefish *Astyanax fasciatus*, they get much of their information about the outside world from dozens of neuromasts that decorate their entire epidermis. They might develop their elaborated lateral pattern of neuromasts from many iterations of the same developmental theme: anterior to posterior migration plus ventral drift.

Concluding remarks

When the transcriptions factors that encode patterning information were elucidated in *Drosophila*, fly geneticists began to study the signalling mechanisms that mediate positional information, such as lateral inhibition, induction and the action of morphogens. In recent years, this field has moved to the cell biological bases of these signalling mechanisms, an approach that was already well under way in worms. The analysis of developmental problems in fish is now also reaching the cellular level: at this symposium, for example, we learnt that notochord elongation can be explained in terms of trafficking and extracellular matrix deposition/function, and that the development of the lateral line can be explained in

terms of planar polarity. The understanding of the cell biological basis of a developmental process in fly, worm and fish provide hints for the developmental cell biology of the same process in mouse and human. Ultimately, although the developmental scenarios might look very different between worms and mammals, the cellular structures are often equivalent. Who is therefore profiting most from this trend? Most probably those studying the molecular and cellular bases of disease.

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