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A molecular basis for developmental plasticity in early mammalian embryos

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Summary

Early mammalian embryos exhibit remarkable plasticity, as highlighted by the ability of separated early blastomeres to produce a whole organism. Recent work in the mouse implicates a network of transcription factors in governing the establishment of the primary embryonic lineages. A combination of genetics and embryology has uncovered the organisation and function of the components of this network, revealing a gradual resolution from ubiquitous to lineage-specific expression through a combination of defined regulatory relationships, spatially organised signalling, and biases from mechanical inputs. Here, we summarise this information, link it to classical embryology and propose a molecular framework for the establishment and regulation of developmental plasticity.

Key words: Chimaera, Developmental plasticity, Regulative development, Stochastic gene expression, Twin

Introduction

One of the most intriguing observations in developmental biology was reported by Hans Driesch in 1892 when testing the dogma of the time, which had been established by W. Roux (Roux, 1888; see Sander, 1991), that potencies, or 'prospective cell fates' (see Glossary, Box 1) as we call them today, are progressively and irreversibly restricted from the first cleavage of an embryo (Driesch, 1892). Driesch established a clean experimental protocol to split the early blastomeres of sea urchin embryos and analyse their fates during development. Not without surprise he observed that, upon separation, individual blastomeres from the 2- and 4-cell stages could give rise to a complete sea urchin larva (Fig. 1A). This indicated that the fates of the first blastomeres were not fixed, as had been suggested by Roux, but exhibited a large degree of plasticity (see Glossary, Box 1), i.e. the blastomeres were totipotent (Sander, 1991; Sander, 1992). As a result of these experiments he could experimentally induce twins and quadruplets. Similar results were obtained through related experiments in other embryos, including frogs (Morgan, 1895) – which had been the subject of Roux's work – revealing that the full developmental potential of the zygote (totipotency, see Glossary, Box 1) is maintained through at least the first divisions of the embryo.

These experiments highlight a transient maintenance of developmental potential (see Glossary, Box 1), which is not restricted to the early stages of development, and indicate that, within an embryo, the potential of a cell or group of cells is greater than its actual fate (Fig. 1B) (Wolpert and Tickle, 2011). Furthermore, this potential can be captured and replicated, as in the

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Box 1. Glossary

Cell fate. The developmental destination of a cell if left undisturbed in its environment. It is revealed through lineage-tracing experiments in which a cell is labelled and its progeny followed. The fate of a cell is more restricted than its potential.

Cell state. A transient condition with a variable degree of stability; a stepping stone in the chain that configures a path to a fate. Development is characterised by branching sequences of cell states that culminate in specific fates. At the molecular level, a cell state is associated with a 'state-specific' gene regulatory network.

Determination. The process whereby a cell adopts a particular state or fate; it is 'irreversible'.

Differentiation. The process of expression of a developmental fate. It is often associated with cell cycle exit and with the stable expression of proteins that will lead a cell to execute specific functions. Differentiation is essentially irreversible, except under forced reprogramming conditions.

Multipotency. The ability of a cell to give rise to multiple cell types or lineages within a tissue or an organ, e.g. the skin, gut or haematopoietic system.

Plasticity. The ability of a cell, tissue or organ to react to an external input or injury by altering its state or even its fate. It reveals the potential of the cell and its regulative capacity. Regeneration is often associated with plasticity.

Pluripotency. The ability of a cell to give rise to all cell types of an embryo (but not the extraembryonic tissues) and to propagate this ability in culture. Pluripotent cells are typically derived from epiblast cells in the mammalian blastocyst, which is also pluripotent, but only transiently so.

Potential. The range of fates into which a cell can develop. It is reduced during development and can only be found experimentally. It is obscured by the mere observation of events in, for example, lineage-tracing experiments, which only reveal fates.

Priming. The seeding of a particular fate on the way to a commitment. At the molecular level, it is revealed in the low-level, reversible activation of particular gene regulatory networks; more recently, it is becoming clear that specific epigenetic marks can be read as signs of commitment in the absence of sustained transcription. Priming is a reversible process. The sequence is usually priming → commitment → determination → state ... determination → fate.

Specification. The process that restricts the potential of a cell, committing it to a particular state or fate. It is reversible.

Stem cell. A single cell that can give rise to a variety of states or fates while propagating this capacity through replication in culture.

Totipotency. The ability of a cell to give rise to all the tissues of an organism, both embryonic and extraembryonic, e.g. a zygote or an early mammalian morula are said to be totipotent.

Transition state. An intermediate during cell fate decision in which a cell exhibits a mixed identity between two or more states, which often represents the state of origin (i.e. the initial state the cell is in) and that of destination (i.e. the identity that the cell is adopting). It is highly unstable and reversible.

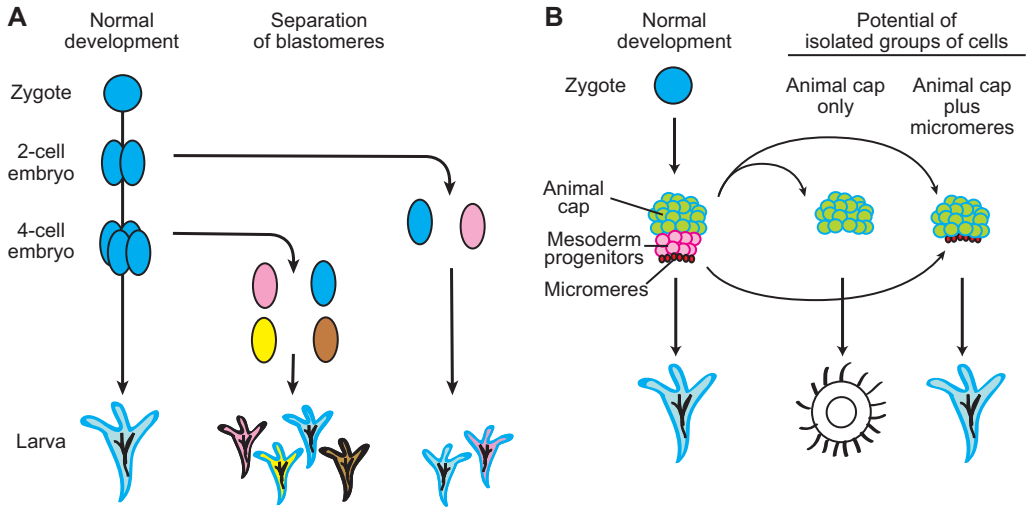

Fig. 1. Regulative capacities of early embryos. (**A**) Summary of the experiments by H. Driesch, which tested the potential of individual blastomeres of the sea urchin embryo. Driesch demonstrated that, upon separation, individual blastomeres from 2- and 4-cell stage embryos could give rise to complete sea urchin larvae. (**B**) Experiments from S. Hörstadius showing the role that cell interactions play in regulating the plasticity of the sea urchin embryo. At the morula stage, the embryo is organised into three tiers of cells (left): the top tier (green) is the animal cap and gives rise to the ectoderm; the middle tier (pink) gives rise to the mesoderm; and the micromeres (red, bottom) give rise to some skeletogenic structures. Together, they give rise to the larva. However, on their own, the animal cells can only give rise to an epidermal ball (middle), but when placed together with the micromeres (right) they reconstitute the larva, giving rise to multiple lineages including mesodermal derivatives. These experiments highlight that the potential of the animal cells is greater than their fate and that cells can regulate each other's fate.

case of embryonic stem cells (ESCs) (see Glossary, Box 1) derived from mouse embryos (Evans and Kaufman, 1981; Martin, 1981), in which the ability to give rise to all cell types of an organism (pluripotency, see Glossary, Box 1) can be propagated over a large number of generations (reviewed by Silva and Smith, 2008; Nichols and Smith, 2011). Cells that can replicate in an undifferentiated cell state (see Glossary, Box 1) can also be found in adults (adult stem cells). In this case, however, potential is restricted to giving rise to particular lineages of a specific cell type (e.g. skin, blood or gut), is maintained throughout life, and allows an organ to withstand ageing and injury. This property is called multipotency (see Glossary, Box 1) (Morrison and Spradling, 2008; Simons and Clevers, 2011).

Driesch was quick to realise that his observations on the developmental potential of early blastomeres posed fundamental questions in biology: what are the mechanisms that generate, maintain and restrict these potentials? How do cells encode the information to make an organism (Driesch, 1908)? In recent years, advances, particularly in the genetics and molecular biology of early mouse embryos, have begun to shed light on these matters. Here, we review what is known about the developmental potential of cells in the early mouse embryo and summarise recent advances in understanding the underlying molecular events at this stage. We propose that unregulated, heterogeneous gene expression in the blastomeres of early embryos provides a substratum for the flexible assembly of lineage-specific gene regulatory networks under the control of mechanical and chemical signals. The term 'mechanical signal' refers to the information created by the organisation of cells into ensembles that results from the integration of adhesion and the activity of the cytoskeleton and creates stresses and strains. Naturally, mechanical signals are transduced by molecular devices but, as A. Turing pointed out, they can be formally separated from the better understood chemical events (Turing, 1952) that arise from the interactions between, for example, growth factors and their receptors. The integration of these types of signals allows cells to react flexibly to their environment and endows the embryo with the capacity of regulative development. We discuss the implications of this idea for a mechanistic understanding of the maintenance and propagation of developmental potential in cells of the embryo and in culture.

Defining developmental potential: mosaic versus regulative development

Biology, particularly developmental biology, is plagued with ill-defined terms that try to capture the elusive identity of cells in developmental or culture systems. Arguments about whether a cell is 'determined', 'committed' or 'primed' often take interesting discussions into gridlock. For this reason, definitions are important. Since much of our discussion is associated with the notion of 'developmental potential', which lies at the heart of these terms, it is important that we explain what we mean by this term.

Embryonic development has conceptually been divided into two different modes: mosaic and regulative. Embryos that are capable of compensating for the loss of some cells are called 'regulative' because they are able to repair or regulate their losses (Fig. 2A), and Driesch's type of experiments revealed an extreme form of this behaviour. In contrast to this mode, deterministic development driven by intrinsic cellular cues is known as 'mosaic' (Fig. 2A). Regulative and mosaic development are therefore manifestations of the developmental potential of cells within an embryo (Lawrence and Levine, 2006); cells in regulative embryos are endowed with a broad fate potential coupled to the ability to receive and respond to extracellular cues, whereas cell fates in mosaic embryos are largely determined intrinsically by the content that they receive from their mother cell, i.e. the cell's history and relative position in an embryo.

The passive observation of development, as performed throughout much of the nineteenth century, supports the concept of

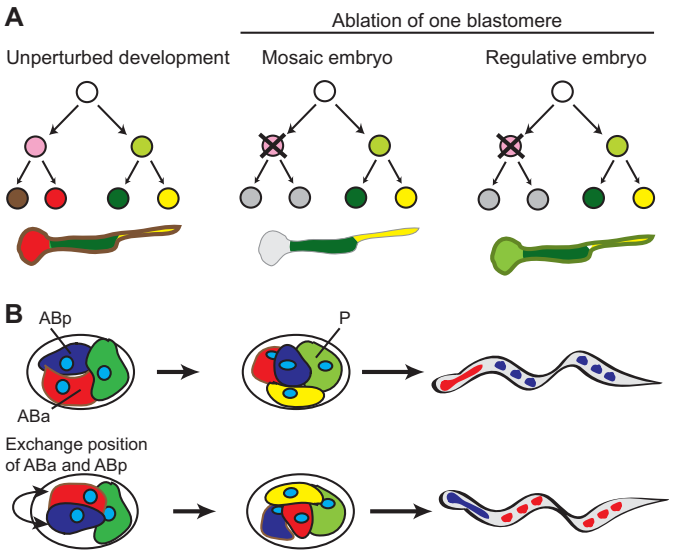

Fig. 2. Mosaic and regulative development. (**A**) The difference between mosaic and regulative development can be illustrated in an organism in which the length of the body plan is derived from four different progenitor cells (left): the skin (brown), the head (red), the trunk (green) and the tail (yellow). If ablation of one of the cells that results from the first cleavage leads to the loss of the structures that derive from it (middle), the development is said to be 'mosaic'. If, on the other hand, the descendants of the remaining cell can give rise to the structures that would have developed from the lost cell (right), the embryo is said to be 'regulative'; in this case, the green cell is able to regenerate the head structures as well as giving rise to the trunk. (**B**) Experiment in *C. elegans* to test the regulative ability of the embryo (Priess and Thomson, 1987). During normal development (top) the ABa blastomere (red) gives rise to the pharyngeal muscles (red in the worm on the right), while its sister, the ABp cell (blue), gives rise to the somatic muscles (blue in the worm). The relative spatial arrangement of the blastomeres at the 4-cell stage is important for this pattern of development; deletion of the P blastomere (green) abolishes the development of the muscles from ABp, which normally comes to be adjacent to P. To test the importance of this relative positioning, it is possible to exchange the relative positions of ABa and ABp with respect to P, such that ABa (rather than ABp) is now adjacent to P. If the fate of each blastomere were determined solely by lineage, this exchange would result in abnormal development of the muscles. However, this experiment does not alter the pattern of muscle fate assignations: after the positional exchange, ABa gives rise to the somatic muscles while ABp gives rise to the pharyngeal muscles. This indicates that the overriding influence on the fate of ABa and ABp is whether they interact with P or not, rather than their lineage.

mosaic development because it reveals a highly organised and reproducible process. Inevitably, this leads to the deterministic views that inspired Roux's conclusions: the cleavage of a zygote is associated with restrictions of cell fates and therefore it must be associated with the allocation of fate determinants to particular cells. However, the behaviour of cells in an undisturbed embryo does not allow us to infer their developmental potential; this needs to be determined through experiments. Whether lineage determinants are allocated exclusively to specific cells can only be tested by removing early blastomeres from the embryo and investigating whether the remaining cells can compensate for the loss and generate a whole organism (Fig. 2A). Furthermore, to assess directly the developmental potential of a single cell it is necessary to take it out of its normal context and investigate which lineages it can form. When these experiments were first performed on ascidian embryos, they supported Roux's contention: the loss of a blastomere resulted in the loss of specific lineages of the organism, and the embryos followed a stereotyped and reproducible cleavage pattern associated with fate restriction (Conklin, 1905; Nishida, 1987). A particularly clear example of a corresponding molecular lineage determinant can be found in the 'myoplasm' of ascidian embryos – a yellow cytoplasm associated with particular blastomeres that segregates to the tail muscles (Conklin, 1905) and which molecular analysis has shown to correlate with mRNA for a zinc-finger protein necessary for muscle development (Nishida and Sawada, 2001).

A similar line of deterministic lineage-based thinking coloured the initial understanding of the development of *C. elegans* embryos, which exhibit a precise final cell number and a stereotyped lineage allocation process (Sulston et al., 1983), and provided strong support for the mosaic view of development. These studies suggested that the fate of a cell is progressively and strictly determined through the provision of instructions linked to the cell divisions associated with its differentiation (see Glossary, Box 1). However, closer inspection and refinement of these experiments suggests that a strict mosaic mode of development might be more the exception than the norm. In a classic experiment in *C. elegans*, positional exchange of the early sister blastomeres ABa and ABp shows that what determines their fate is their position relative to adjacent blastomeres rather than their absolute lineage (Priess and Thomson, 1987) (Fig. 2B). This demonstrates that even the rigid cell lineage of the *C. elegans* embryo is not strictly driven by cell-intrinsic factors, but might arise from a reproducible geometry of cell interactions that determine cell fates**.** Furthermore, there is a certain degree of variation between individuals in the pattern and timing of the cleavages (Schnabel et al., 1997), which supports the contention that corrective mechanisms exist. Even in ascidians, the fate of some early blastomeres and the development of some tissues require cell interactions (Lemaire, 2009).

Therefore, even in classical examples of mosaically developing embryos, the developmental potential of a cell in an embryo is greater than its fate, and the fate of a cell is, for the most part, conditional and relies on that of its neighbours. In the sea urchin, these principles were dramatically demonstrated by the experiments of Driesch and were explored further by Hörstadius using older embryos in which the development of particular regions of the embryo was shown to depend on the fate of their neighbouring cells (Hörstadius, 1973) (see Fig. 1B). The observation that there are cell populations whose main function is to instruct a fate of naïve cell populations during the development of an organism underlies the behaviour of the gastrula organiser in vertebrate embryos (Robb and Tam, 2004; De Robertis, 2009) and suggests that cell-cell inductions are a general principle of developmental systems. Therefore, at the level of single cells, development is predominantly regulative, and in embryos cells have "invariant cell lineages but conditional fates" (Davidson, 1989), i.e. even though the patterns of cell division are stereotyped and often conserved from individual to individual, in early embryos the fate of each cell is reliant and conditional on interactions with its neighbours and its relative position in the embryo. These concepts are particularly relevant to the early development of mammalian embryos, as we will outline in the next section.

It is important to bear in mind, in particular when attempting to determine experimentally the key influences driving lineage choices and differentiation in embryos, that what is being observed is always a response of the system to a specific experiment. Few experimental perturbations will reflect situations encountered by

the embryo during normal development and therefore will not reveal behaviours that have been selected during evolution. Yet, because the response of the embryo to experimental perturbation is dependent on the properties of the underlying cellular machinery, a regulative response will be indicative of the presence of cellular machinery that is reactive and plastic. Understanding the molecular organisation of this reactive device – either in the context of intact embryos or in the case of ESCs in culture – is an important element of modern developmental biology.

Assessing developmental potential: merging and splitting embryos

Having established a conceptual framework to discuss the behaviour of early embryos in experimental settings, we can now turn to the main question of this article: what is the molecular basis of cell fate decisions in early mammalian development? Owing to their intrauterine development, the first lineage decisions in mammalian embryos set aside tissues that interact with the maternal environment and function to pattern the embryo. The first cell fate decision involves specification (see Glossary, Box 1) of the cells of the trophectoderm (TE) and inner cell mass (ICM); the TE forms the first physical interaction with the uterus, secretes inducing factors required for gastrulation and germ cell specification, and gives rise to the embryonic parts of the placenta. The ICM then further segregates into primitive endoderm (PrEnd), which controls the anterior/posterior orientation of the embryo and goes on to form parts of the yolk sac (Beddington and Robertson, 1999), and the epiblast (Epi), which forms the embryo proper. The process of cell lineage specification in the preimplantation embryo, and how and when developmental potential becomes restricted, have been the subject of intense scrutiny over the last ten years.

The existence of chimaeras generated by the combination of cells from different embryos provides strong evidence for the regulative ability of the early mammalian embryo (Gardner, 1968; Gardner and Munro, 1974; Mayer and Fritz, 1974; Tucker et al., 1974; Fehilly et al., 1984b; Fehilly et al., 1984a; Tachibana et al., 2012). Chimaeras occur naturally during the development of marmosets (Gengozian et al., 1964; Ross et al., 2007) and their existence in humans has been dramatically revealed in various court cases and paternity suits (Ainsworth, 2003). In particular, the use of *in vitro* fertilisation for assisted conception, in which more than one embryo is transferred, has resulted in an increase in the number of chimaeras arising from the aggregation of embryos (Quintero et al., 2003; Souter et al., 2003). In many cases, chimaerism results from the transportation of cells between individuals via the shared circulating blood and involves organs with a good blood supply (van Dijk et al., 1996); in some instances, it extends to the germ cells (van Dijk et al., 1996; Yunis et al., 2007).

Murine chimaeras have been generated by the aggregation of embryos (Tarkowski, 1961; Mintz, 1962; Gardner, 1968; Gardner and Munro, 1974). This technique, when performed with embryos of different genotypes, has become a useful experimental device for generating mosaics and allows studies of gene function by creating organisms with cells of different genotypes (Fig. 3). In some instances, more than two embryos have been aggregated and, perhaps surprisingly, the resulting animal exhibits a size appropriate for a single mouse, with the size regulation occurring at around the start of gastrulation (Rands, 1986; Power and Tam, 1993). An extreme example of the plasticity of early development can be observed in the ability of ESCs to integrate within the embryo before implantation and resume normal development, even after genetic modification, contributing to all tissues of the adult animal. Although these studies reveal an extraordinary capacity to regulate development throughout the early stages, it is clear that there are species differences. Interestingly, interspecies chimaeras generated by implanting mouse induced pluripotent stem cells into rat embryos can result in adult animals of varying sizes that are proportional to the level of contribution from the donor cells (Kobayashi et al., 2010). Recent work (Tachibana et al., 2012) has revealed that, in contrast to mice, Rhesus monkey ICM cells do not integrate into host embryos to form chimaeras and instead sort from the embryo and form twins. However, cells from the morula stage can integrate and form chimaeras. Whether the behaviour of ICM cells is a characteristic of the species or depends on the exact experimental techniques employed awaits further investigation.

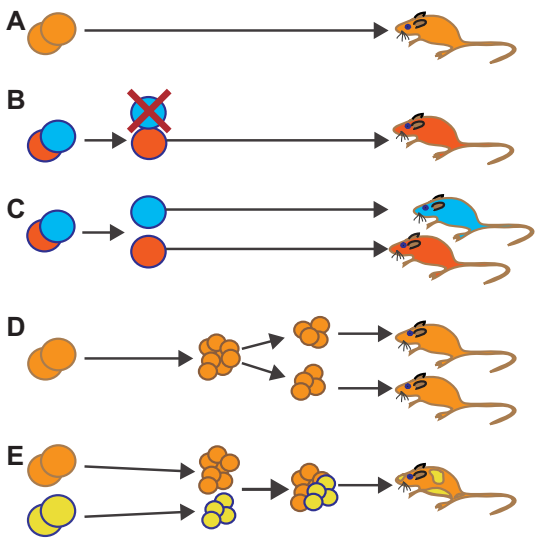

Fig. 3. Outcomes of experimentally interfering with the early stages of mouse development. (**A**) If left undisturbed, the first two blastomeres will continue to develop and will give rise to a mouse. (**B**) If one of the blastomeres is ablated then the remaining one will compensate and give rise to a complete mouse. (**C**) If separated, individual blastomeres at the 2-cell stage give rise to two twin mice. The frequency of this event is low but has been reported more than once (see text and Table 1 for details). (**D**) This regulative ability extends into the morula: if bisected, a morula can give rise to two twin mice. (**E**) If two different embryos are aggregated, they merge into a single embryo and give rise to a balanced single mouse.

The ability of an embryo to allow for chimaerism proves the ability of an ensemble of cells to react to the addition of other cells. However, it does not test directly for the existence of cell-intrinsic lineage cues that might normally guide embryonic development in unperturbed conditions. In the early 2000s it was suggested that events during the first two cleavages determine the fate of the first two blastomeres: the one that divides first, which is thought to derive from the sperm entry position in the zygote, having a much higher chance of contributing to embryonic rather than to extraembryonic tissue (Gardner, 2001; Piotrowska et al., 2001; Piotrowska and Zernicka-Goetz, 2001). This suggestion has implications for our understanding of the development of mammalian embryos, including human embryos, and it is for this reason that it has received much attention. A strict embryonic-extraembryonic decision at the 2-cell stage would prevent the formation of twins from the daughters of the first division. The ‘Driesch test’, which interrogates the behaviour of separated

blastomeres, as well as an examination of the phenomenon of twinning, is a good way to investigate this prediction and the degree of determination (see Glossary, Box 1) of the blastomeres.

Natural monozygotic twinning occurs occasionally in a few mammalian species, including humans and cattle. Furthermore, double twinning is observed in the nine-banded armadillo, which regularly produces four individuals out of every zygote (Loughry et al., 1998). The few available studies on this organism indicate that this double twinning occurs after implantation of the blastocyst (Enders, 1962; Enders, 2002), when the single-layered Epi expands and then thickens in specific regions. Each of these new foci develops into a cup-shaped epithelium and growth of the interfocal regions results in the four clones becoming evenly spaced around the uterus. The large fraction of naturally occurring twins in humans and cattle that are monochorionic are likewise thought to arise late in development after blastocyst formation (Hall, 2003). Although these cases make a point for the regulative ability of the early mammalian embryo, they do not rule out a possible early separation of extraembryonic lineages by cell-intrinsic cues. Dichorionic twins, by contrast, are thought to arise from the spontaneous splitting of embryos prior to the segregation of extraembryonic lineages (Hall, 2003) and would therefore hint at the existence of totipotency in early blastomeres. Given the uncertainties of the exact timing and mechanisms of natural twinning, the experimental generation of twins provides a clearer test of this feature. In several mammalian species, this has been achieved through the separation of blastomeres at different early stages of development, e.g. in cows and sheep (Willadsen, 1981; Fehilly et al., 1984a; Willadsen, 1989), horses (Allen and Pashen, 1984), goats (Ozil, 1983; Tsunoda et al., 1985), rats (Matsumoto et al., 1989) and rabbits (Moore et al., 1968).

In mouse embryos the experimental generation of twins is not easy to perform as the formation of the blastocyst in this organism is determined by a cell division clock, i.e. cavity formation and lineage segregation take place after a defined number of cell divisions (McLaren and Smith, 1977; Morris et al., 2012). Thus, separated blastomeres form blastocysts ‘on time’ from the moment of fertilisation and therefore with fewer cells than normal embryos; these embryos appear to have less chance of successful development (Morris et al., 2012). Notwithstanding this difficulty, there is experimental literature on this subject. The splitting of morulae, for example, produces twin blastocysts with ease and many of these develop into twin mice at a reasonable frequency (Nagashima et al., 1984; Kim et al., 1986; Lawitts and Graves, 1988). Although these experiments provide a hint of the totipotency of early blastomeres, they do not rule out the possibility that only some of the cells in each half-morula contribute to the embryo, i.e. they do not test the potency of individual blastomeres. Killing or removing one blastomere at the 2-cell stage produces blastocysts and mice, suggesting that the embryo can compensate for the loss of one blastomere (Tarkowski, 1959; Papaioannou et al., 1989; Papaioannou and Ebert, 1995). However, although yields can be very high, like the morulae bisections these experiments do not test the potential of both blastomeres, which is the crucial element of the Driesch test and the only way to rule out an early determination event.

The all important experimental test of the developmental potential of sister blastomeres from 2-cell embryos in the mouse has been performed in at least five independent experiments, with different strains and by different experimenters (Mullen et al., 1970; Tsunoda and McLaren, 1983; Togashi et al., 1987; Wang et al., 1997; Morris et al., 2012). The reported yield of viable embryos and mice is variable but the results are clear: twin blastocysts are obtained at high frequency and twin live births are not infrequent (Table 1). In these experiments, the number of live embryos appears to depend on reproductive and genetic variables and is altered by experimental conditions. A recent study (Morris et al., 2012) showed that developmental failure often correlates with reduced numbers of cells in the Epi lineage at the blastocyst stage. The authors could significantly improve the success rate of obtaining monozygotic twins from separated 2-cell blastomeres by pharmacological treatment to expand the number of precursors of the foetal lineage in embryos before transfer (Nichols et al., 2009; Morris et al., 2012). Further experiments testing the potential of single blastomeres have been performed with tetraploid chimaeras, in which diploid cells are mixed with tetraploid cells and only the diploid cells contribute to the embryo. In these experiments, twins and multiplets from 2-, 4-, 8- and even 16-cell embryos have been reported (Tarkowski et al., 2001; Tarkowski et al., 2005; Tarkowski et al., 2010).

Table 1. Experimental production of twins from the bisection of mouse embryos at the 2-cell stage

Data source	Total embryos (twins)	Total live (twins)	Success of twins (%)
Experiment (a)*	322 (161)	173 (78)	48
Experiment (b)*	25 (10)	23 (6)	60
Experiment (a)‡	124 (62)	23 (3)	4.8
Experiment (b)‡	126 (63)	47 (13)	20.6
Untreated§	16 (4)	ND	25
2i treated§	16 (8)	ND	50

*Tsunoda and McLaren (Tsunoda and McLaren, 1983). The values presented combine all the embryos from the experiment in table 5 (a) and table 6 (b).
‡Wang et al. (Wang et al., 1997). The numbers presented combine all the embryos from the experiment in table 2 for the CD1 strain (a) and the F1 resulting from the F1 cross from C57BC × CBA (b).
§Morris et al. (Morris et al., 2012). Data are taken from the table in figure 3D, columns ‘GFP twin recovery’.
ND, not done.

The outcomes of these experiments (Fig. 3) rule out a strict early determination event during the first cleavages but they still allow for the existence of a ‘bias’ in the fate of the blastomeres (Bruce and Zernicka-Goetz, 2010), which is supported by quantitative analysis of the fate of different blastomeres at different stages (Piotrowska-Nitsche et al., 2005; Tabansky et al., 2013). At present, it is unclear whether these biases are driven by the asymmetric allocation of lineage-determining factors or whether they arise as a result of the physical constraints of the early cleavages of an embryo, which develops without growth in a confined space (Motosugi et al., 2005; Kurotaki et al., 2007; Alarcón and Marikawa, 2008; Honda et al., 2008). Irrespective of these open questions, the sum of experimental evidence from both twining- and chimaera-based studies shows that mouse blastomeres, at least until the compaction of the morula, and probably later (see below), can be deemed totipotent.

Early lineage specification and biases

The restriction of totipotency is as important as its establishment and maintenance. In the mammalian embryo this occurs between compaction and the formation of the blastocyst. Shortly after compaction, mouse blastomeres separate into outer and inner cells in order to fill the volume of the embryo, and it is at this stage of ~16 cells that the seeds for the three lineages emerge (Fig. 4A). The outer cells become polarised, with an asymmetric distribution of microvilli and organelles, whereas the inner cells remain apolar

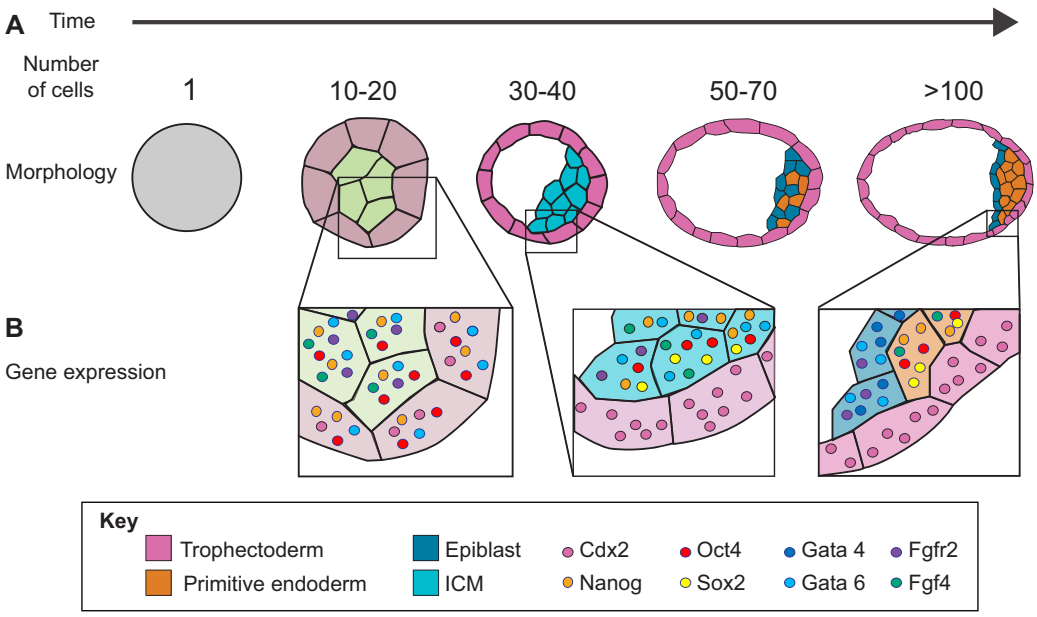

Fig. 4. Stages and molecular events associated with early development of the mouse embryo. (**A**) Sequence of the stages spanning fertilisation and implantation. The approximate number of cells at each stage is indicated. Mouse embryos divide asynchronously and, although it is possible to associate specific morphologies with the time elapsed since mating (e.g. E3.0 or E4.0 for early or late blastocysts, respectively), it is often best to define developmental stage by the number of cells in a particular embryo (Plusa et al., 2008). The first differences between blastomeres are visible at the 8- to 16-cell stage when, due to constraints in space and the geometry of the embryo, some blastomeres lie on the outside of the ball of cells and some inside; those located outside form an epithelium (pink) that envelops the cells located inside (green). This distinction has implications for fate assignment as the cells located outside become committed to the trophectoderm (TE, purple) fate whereas those inside form the inner cell mass (ICM, light blue). Between the 20-cell and the 40-cell stage, a fluid-filled cavity emerges within the embryo, which leads to asymmetric localisation of the ICM. The cavity arises from a fusion of intracellular vacuoles of the cells in the morula and is expanded by the polarised function of Na-K ATPases and aquaporins in the TE. During these stages a sequence of molecular events leads to the segregation of the ICM into two lineages: the epiblast (orange), which will give rise to the soma and the germ line of the mouse, and the primitive endoderm (dark blue), which will give rise to the yolk sack and make some contribution to the endoderm. (**B**) Gene expression events associated with lineage segregation during the early stages of development. The genes are colour coded to indicate their expression in the different cell types at each stage. Note that the allocation of cell type-specific patterns of gene expression is always preceded by multilineage expression at the level of single cells.

(Johnson and Ziomek, 1981). At this stage, cells are not committed to particular fates as shown in the pioneering experiments of Tarkowski and colleagues (Tarkowski and Wróblewska, 1967; Tarkowski et al., 2010): when presented with the alternative environment, i.e. from outside to inside or vice versa, cells can assume the characteristics of their new neighbours (Hillman et al., 1972). At around the 32-cell stage, when the cavity is clearly visible, cells that lie outside have a defined epithelial appearance and are apparently irreversibly determined to TE fate, whereas the cells that lie inside form the ICM. The segregation of TE and ICM fates requires cell-cell interactions, as recently shown in elegant experiments in which blastomeres were immediately separated after division for the first five division cycles of the embryo (Lorthongpanich et al., 2012). All resultant long-term separated blastomeres assumed a similar molecular identity that was distinct from both TE and ICM. These findings suggest that the opposing lineage-specific gene expression programmes emerge as cells signal to each other, and argue against a strict asymmetric distribution of lineage specification factors in cells within the early blastocyst. Such asymmetric distributions of determinants would reveal itself in the maintenance of individual fates when the cells are separated. The interactive and regulative nature of these early embryos is revealed by the ICM, or a subpopulation of its cells, which retains the potential to differentiate into TE if the original TE is eliminated (Handyside, 1978; Spindle, 1978; Nichols and Gardner, 1984; Grabarek et al., 2012). Whether this capacity is utilised during undisturbed development (to ensure sufficient cells in the TE during expansion) is not easy to interpret because experimental intervention might trigger a repair response that is not an element of the normal developmental programme (Cruz and Pedersen, 1985; Gardner and Nichols, 1991).

As the blastocyst matures, the ICM becomes subdivided into Epi and PrEnd. Several hypotheses have been put forward to account for this segregation of fates: (1) that it depends on the position of a cell in the ICM (Rossant, 1975); (2) that cell lineage determines or biases fate, with cells arising from later differentiative divisions of the blastocyst outer layer being predisposed to become PrEnd (Chisholm and Houliston, 1987; Rossant et al., 2003); and (3) that a random mixture of cells with either Epi or PrEnd identity emerges and then sorts out as the blastocyst matures with positional cues playing a leading role in the sorting process (Chazaud et al., 2006). A further extension to this last hypothesis suggests that the ICM initially consists of a population of uncommitted cells that express markers of both lineages, and that the two fates are assigned by a combination of positional cues and intercellular signals prior to the sorting event (Plusa et al., 2008; Meilhac et al., 2009).

To distinguish between these possibilities, it is first necessary to trace the lineage of blastomeres and test how it correlates with Epi or PrEnd fate. The origin of any possible lineage bias then needs to be investigated by directly testing the developmental potential of individual blastomeres through experimental manipulation. Two studies have used elegant blastomere labelling and lineage-tracing

experiments *in vivo* to achieve the first goal. Whereas one study failed to detect any correlation between cell lineage and fate in the ICM (Yamanaka et al., 2010), a second study (Morris et al., 2010) revealed a certain degree of spatiotemporal order in the assignation of ICM cells to either fate: ICM cells that divide early and come to be located in the centre of the ICM tend to adopt the Epi fate, whereas cells that emerge from later divisions and lie closer to the TE and the emerging cavity preferentially adopt the PrEnd fate (Morris et al., 2010). The authors conclude that although there is an important positional element to the assignation of fates, there is also a significant role for lineage, specifically the time of cell birth and asymmetric cell division, in the assignation of fate. Whether this observed lineage bias depends on cell-intrinsic factors or is driven by extrinsic cues was more recently addressed by Grabarek et al. (Grabarek et al., 2012), who tested the developmental potential of blastomeres at different stages between the 32-cell stage and the mature blastocyst in morula aggregation experiments. Donor cells were isolated at various stages from embryos carrying a Pdgfra-H2B-GFP (Hamilton et al., 2003) transgene, which is heterogeneously expressed in the ICM at the mid-blastocyst stage and marks the PrEnd at the late blastocyst stage. Cells were classified according to Pdgfr-H2B-GFP expression levels, transplanted into appropriate hosts and their lineage and fate followed until the end of preimplantation development using an ubiquitously expressed marker in the donor cells (Grabarek et al., 2012). When isolated from early blastocysts, cells can contribute to all three lineages of the blastocyst irrespective of the Pdgfra-H2B-GFP expression level. As development proceeds, GFP-negative cells lose the ability to contribute to the PrEnd fate earlier than the GFP-high cells lose the ability to become Epi, but full commitment of cells to a particular fate is only achieved from the late blastocyst stage. These results argue that the lineage biases that were previously detected in undisturbed embryos might not be mediated by intracellular factors, but instead arise from the dynamic architecture of the signalling interactions between cells in the blastocyst.

Fibroblast growth factor (FGF) signalling is an important player in these early fate decisions. The establishment and maturation of PrEnd is dependent on FGF/MAPK signalling (Yamanaka et al., 2010; Frankenberg et al., 2011; Goldin and Papaioannou, 2003; Nichols et al., 2009; Kang et al., 2013), and Fgf4 is expressed in Nanog-positive cells of the ICM that are fated to become Epi (Messerschmidt and Kemler, 2010; Frankenberg et al., 2011). These molecular events provide a possible explanation for the observations of Morris et al., which suggest that a first ‘wave’ of cell division is associated with the Epi fate, whereas later waves generate PrEnd (Morris et al., 2010). The first cells to enter the inside of the embryo during differentiative divisions would do so when FGF signalling is low in the embryo as a whole, a condition that favours the Epi fate. These cells would then start to produce an FGF signal of their own that will accumulate over time. Cells that are internalised during the second round of differentiative divisions would thus be exposed to this signal from their birth, and this might favour differentiation into PrEnd. The change in cell fate bias as cleavage proceeds might therefore reflect the changing milieu of the embryo rather than a lineage influence.

Taken together, these observations suggest that regulative development prevails during the early stages of mouse development, and provide support for the notion that, in embryos, cells have “invariant cell lineages but conditional fates” (Davidson, 1989). Therefore, as is the case in early *C. elegans* embryos, a reproducible fixed or biased lineage does not imply autonomous determination, but cells become determined to specific fates only very late. Having established the behaviour of the cells, the question remains: what are the molecular mechanisms that mediate this behaviour?

A molecular framework for regulative development

Regulative behaviour is a property of embryos as a whole but, naturally, it needs to have a molecular basis at the level of single cells. To understand this, we need to identify the molecular elements that encode the fate of a cell. In mosaic embryos this is easy as it relies on the biased distribution of lineage-determining transcription factors (Lemaire, 2009; Nishida and Sawada, 2001; Maduro, 2010) to particular cells and their descendants to trigger specific developmental programmes. In regulative embryos, by contrast, independently of how it is initiated, a cell fate is determined in a non-cell-autonomous manner over a large multicellular domain to account for its sensitivity to the size of the embryo. A mechanism capable of underpinning this behaviour requires a molecular device present in each cell that: (1) senses, measures and integrates global properties of the embryo, such as the total number of cells, polarity, the identity of neighbours, strains and stresses, and transmits this information to the nuclei of individual cells; and (2) endows individual cells with the ability to respond to changes in their chemical and physical environment.

The early mouse embryo offers several advantages in understanding how mechanical and chemical signals interact during fate assignation and patterning in a regulative system: the embryos start from a naïve state of gene expression; the onset of gene expression can be followed at the level of single cells; the fate assignment events take place within a system comprising a small number of cells (from 1 to 100); and the system can be manipulated. Below, we outline the molecular makeup of the cells of the blastocyst upon which these signalling systems act and we propose how this makeup, particularly heterogeneous gene expression, can contribute to developmental plasticity.

Signal transduction and transcription factor networks in early embryos

Advances in analysing the molecular genetics of mouse preimplantation development have provided detailed information about a small group of transcription factors that are associated with particular lineage assignations at the early stages of development (Rossant and Tam, 2009). For example, Cdx2 and Tead4 are associated with the TE, whereas the ICM is characterised by varying degrees of expression of Gata6, Sox17, Oct4 (Pou5f1) and Nanog at different times of development. As mentioned above, the ICM resolves into the PrEnd, which expresses Gata4, Gata6, Sox17 and Sox7, and the Epi, which expresses Nanog, Oct4 and Sox2. Most of these genes are already expressed at the 8-cell stage (Guo et al., 2010) in heterogeneous patterns that vary from cell to cell and embryo to embryo.

During the first two cleavages there is low-level expression of many early genes, with no significant differences in expression profiles between individual blastomeres and an overall bias toward the TE fate (Tang et al., 2011). By the 8- to 16-cell stage, low-level but clear expression of many lineage markers can be detected, but there is no restriction of expression to particular cells. Thus, *Nanog*, *Oct4*, *Cdx2* and *Gata6* transcripts can be observed in all cells of the embryo (Fig. 4B) (Guo et al., 2010). By the 32-cell stage, most cells simultaneously express *Cdx2*, *Gata6*, *Nanog* and *Oct4* mRNA and protein to variable degrees (Dietrich and Hiiragi, 2007;

Dietrich and Hiiragi, 2008; Plusa et al., 2008; Guo et al., 2010). If we define priming (see Glossary, Box 1) as the reversible expression of a gene without functional consequences for the cell, at this stage individual blastomeres can be considered to be primed for all lineages before restrictions occur. This situation is reminiscent of that observed in haematopoietic precursors and reflects the notion of multilineage priming (Hu et al., 1997), which states that the low-level expression of markers of different lineages at the level of single cells can act as a template for lineage decisions (Martinez Arias and Brickman, 2011). There is also evidence that other genes involved in lineage specification (e.g. *Fgf4*, *Fgfr2*, *Pdgfra*, *Sox2*, *Sox17* and *Klf4*) are also expressed at this stage (Plusa et al., 2008; Guo et al., 2010; Niakan et al., 2010; Kurimoto et al., 2006; Artus et al., 2011).

Lineage restriction events, which are associated with the progressive increased expression of lineage 'determinant' genes concomitant with reduced expression of determinants of alternative fates, first occur around the 32-cell stage. At this time, the outer cells become committed to the TE lineage through upregulation of Cdx2 expression and concomitant downregulation of PrEnd- and Epi-associated genes. Additionally, an asymmetric distribution of *Cdx2* mRNA at the 8- to 16-cell transition might contribute to elevating Cdx2 protein levels in outside cells and reducing them in inside cells (Skamagki et al., 2013). In the ICM, gene expression differences that are associated with emerging lineage restrictions can be detected by the 64-cell stage, when Nanog and Gata6 expression become mutually exclusive in individual cells (Plusa et al., 2008; Guo et al., 2010; Frankenberg et al., 2011). This early separation of PrEnd and Epi fate is then further promoted by FGF expression in the Nanog-positive Epi precursor cells. This signal is required in a paracrine fashion to sustain Gata6 expression and to support maturation of the presumptive PrEnd, which eventually becomes marked by Sox17 and Gata4 expression (Kurimoto et al., 2006; Kang et al., 2013).

The regulative capacity of the mammalian embryo is associated with multilineage priming

The available experimental evidence that we have discussed above indicates that totipotency of the early embryo is maintained throughout the early cleavage stages into the morula, during which all cells express variable low levels of different lineage markers. On this basis, we propose that the regulative ability of the mammalian embryo is associated with this multilineage priming at the level of single cells. This priming from an initial 'uncontrolled' pattern of expression might be the result of the onset of zygotic gene expression and of the epigenetic events that are associated with the initiation of transcription in the early embryo. In other words, gene expression events between the 2- and 64-cell stages are probably not driven by specific signals, but instead can be thought of as resulting from uncontrolled transcriptional bursts. Cell fates thus emerge as these noisy gene expression events are slowly patterned through a combination of specific transcriptional regulatory interactions between factors that establish defined networks with the participation of extracellular signals. Given the low levels of activity from the first bursts of 'uncontrolled' transcription, there is likely to be significant cell-to-cell variation in the networks that become activated, i.e. different cells have different active networks, as has been suggested for ESCs (Trott et al., 2012). We believe that mechanical and chemical signals arising from cell interactions will then favour and amplify some of these interactions and thus promote the activity of certain gene regulatory networks (Fig. 5) that, in turn, will promote particular cell fates.

The segregation of TE and ICM cells provides an example of how mechanical signals impinge on a fate decision. As summarised above, early blastomeres respond to their relative position by forming a polarised epithelium if they find themselves at the outside of the embryo. It is not clear how a cell is able to detect that it is on the outside, but once it does, this triggers the assembly of adherens and tight junctions, which will create strains and stresses on these cells that are not present in inner cells. There is little information about the molecular devices that can sense this physical property, but once the outer cells form an epithelium there are hints as to how this can be transmitted to the nucleus to mediate differential gene expression. The Hippo pathway, which is under the control of cell polarity cues and is able to sense cell density and associated physical parameters (Schroeder and Halder, 2012), is active in inner cells of the morula, where it inhibits Tead4 activity through cytoplasmic sequestration of its co-factor Yap (Yap1). In outside cells Hippo signalling is low, allowing Yap to translocate to the nucleus and, together with Tead4, to promote Cdx2 expression (Nishioka et al., 2009). Thus, Hippo provides a candidate for the transduction of the mechanics of the cell, as represented in the cytoskeleton and adhesion system, to the transcriptional networks. Cell polarity and adhesion are also required for the asymmetric localisation and inheritance of *Cdx2* mRNA at the 8- to 16-cell transition (Skamagki et al., 2013). Together, these two mechanisms will, over time, restrict Cdx2 expression to outside cells, where this transcription factor in turn can act to downregulate ICM-specific factors such as Oct4 (Niwa et al., 2005).

The second lineage decision taken by the ICM cells in terms of PrEnd versus Epi relies, in comparison, much more on chemical signals. Although the initial expression of Gata6 in the embryo is independent of Fgf4 signalling (Kang et al., 2013), Fgf4 is required to maintain the expression of Gata6, to promote the expression of later PrEnd markers such as Sox17 and Gata4, and to allow for the maturation of this tissue (Goldin and Papaioannou, 2003). In fact, it has been proposed that several rounds of interactions between elements of FGF signalling and the transcription factor networks take place to establish the PrEnd fate and separate it from the Epi (Yamanaka et al., 2010; Frankenberg et al., 2011). In addition to Fgf4 signalling, Pdgfrα signalling has a quantitative effect on the number and proliferation of PrEnd cells (Artus et al., 2010), suggesting that FGF is not the only signal involved in the establishment of the PrEnd fate. The segregation of the Epi and PrEnd is also likely to have a mechanical input: PrEnd cells ultimately need to be positioned along the blastocoel cavity and there is evidence that the correct positioning of these cells plays a role in the maintenance of marker expression and the survival of cells (Plusa et al., 2008; Meilhac et al., 2009). In fact, cells specified as PrEnd migrate towards the cavity to become incorporated into the emerging tissue (Plusa et al., 2008). FGF signalling is likely to determine the migratory behaviour, as it does in other systems, but the directionality must be impacted by some global characteristics of the tissue, such as differential adhesion to the cavity.

Computational models that take the combined mechanical and chemical inputs into account provide novel insights into the actual cell fate allocation process (Krupinski et al., 2011) and show that the combination of the two signalling inputs acting on self-organised transcriptional networks provides a more robust way to implement regulative development than those controlled purely by chemical inputs.

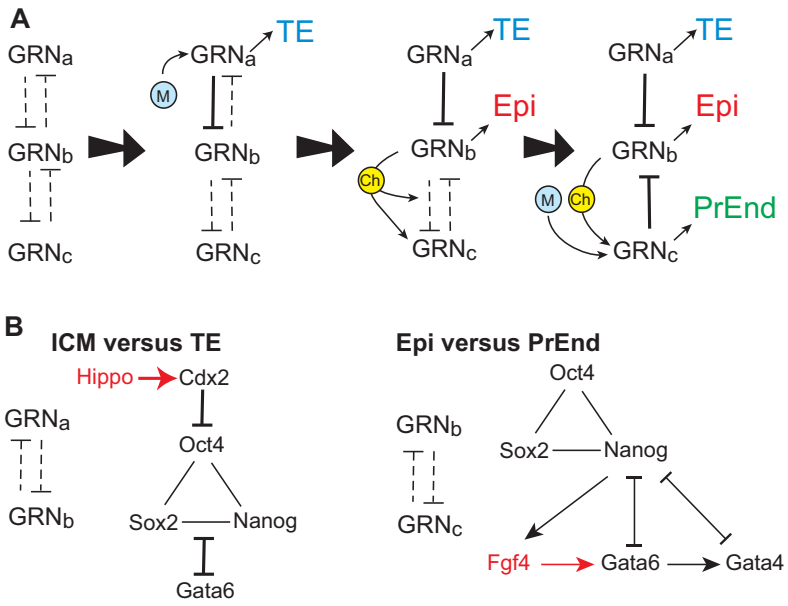

Fig. 5. The sequence of interactions between gene regulatory networks (GRNs) during lineage specification in the early mouse embryo. Three lineages are specified during the early stages: trophectoderm (TE), epiblast (Epi) and primitive endoderm (PrEnd), and each is associated with a specific GRN (a, b and c). (**A**) Genetic analysis suggests that these networks antagonise each other, that they do so sequentially, and that their regulation and interactions combine mechanical (M) and chemical (Ch) signals. Initially, all blastomeres express low levels of the basic elements of all networks, which have antagonistic cross-regulatory interactions. We propose that lineage segregation is driven by the resolution of the interactions between networks at the level of single cells under the influence of mechanical and chemical signals, i.e. the interactions between the individual elements of a network and between networks will be influenced by the mechanical environment in which a particular cell finds itself and the chemical signals that it receives. First, the TE emerges through GRNa suppressing the activity of GRNb and GRNc, and is reinforced by mechanical inputs associated with the epithelialisation of the cells on the outside; the Epi and PrEnd lineages are then specified from the ICM. Since acquisition of the PrEnd fate depends on chemical signals produced by Epi-fated cells (see main text for details), the primary fate of ICM cells can be considered to be Epi. The chemical signals produced by some ICM cells promote the activity of GRNc in a different subset of ICM cells, leading to the establishment of the PrEnd fate. The PrEnd fate is further supported by mechanical signals that arise from localising prospective PrEnd cells at the surface of the blastocyst cavity. (**B**) Molecular details of the interactions between the individual elements of each network and between networks. It is likely that the Hippo pathway acts as a conduit for the mechanical component during TE establishment (left). The establishment of the PrEnd fate (right) requires a collection of mechanical and chemical inputs, which act on elements of the PrEnd-specific GRNc, as well as on the interactions between GRNc and the Epi-specific GRNb. The Fgf4 signal is crucial for sustained Gata6 expression and for the initiation of Gata4 expression, and expression of Fgf4 is Nanog dependent. Activation of the PrEnd circuit GRNc can therefore be deemed to be dependent on prior establishment of the Epi-specific GRNb.

Heterogeneous gene expression and plasticity in the embryo and in culture

We have argued that the unregulated, heterogeneous expression of genes relating to multiple lineages at the level of individual cells of early embryos functions as a substratum for the spontaneous assembly of gene regulatory networks that determine specific fates. The notion that developmental potential is expressed in multilineage priming, as reflected in heterogeneities in gene expression, was first raised in the context of haematopoietic precursors (Hu et al., 1997). Over the last few years, this concept has become adopted, knowingly and unknowingly, for other systems (Enver et al., 2009), and fittingly in the case of ESCs, which exhibit large heterogeneities in gene expression that have been linked to their pluripotency (Chambers et al., 2007; Hayashi et al., 2008; Toyooka et al., 2008; Enver et al., 2009; Graf and Enver, 2009; Kalmar et al., 2009; Canham et al., 2010; MacArthur et al., 2012). In ESCs, these heterogeneities have been shown to be dynamic and it has been suggested that cells use them to explore developmental potential (reviewed by Huang, 2011; Martinez Arias and Brickman, 2011). It is possible that early gene expression heterogeneities in embryos will exhibit similar dynamic fluctuations, although this is still an open question. In embryos, the heterogeneities could reflect different rates and timings of gene expression bursts associated with differential kinetics of rising gene expression in individual cells rather than the periodic patterns observed in culture. An important difference between the two systems is that, in contrast to the situation in ESC cultures, heterogeneities in embryos are transient and are quickly resolved by interactions between gene regulatory networks and signalling inputs, thus resulting in the directionality of the developmental programmes. Blocking the resolution of these heterogeneous states and maintaining them over time could therefore be considered a hallmark of establishing successful culture conditions for pluripotent cells. In line with this idea, we have previously suggested that the heterogeneities in ESCs represent trapped transition states (see Glossary, Box 1), with profiles of gene expression associated with cell fate decisions (Muñoz-Descalzo et al., 2012). The fact that these states can be ‘captured’ experimentally in culture (Silva and Smith, 2008) suggests that the corresponding transition states in embryos at this stage have long enough life spans to be transformed into the self-propagated states associated with ESC cultures. Thus, the plasticity of early embryos might reflect the life span of these heterogeneities, and their disappearance might be associated with the resolution of the networks that underlie them and the susceptibility of these underlying transcriptional networks to be modulated by signalling pathways.

Summary and perspectives

In the face of the available evidence, we believe that the preimplantation mouse embryo is a highly regulative biological system and that transcriptional multilineage priming during the early cleavage stages underlies totipotency and, later, the pluripotency of cells in the embryo. Commitment and determination events at the level of individual cells are guided by the spatiotemporal shaping of this priming through an emergent wiring of specific gene regulatory networks under the molecular restrictions of the proteins involved and the influence of mechanical and chemical signals. The geometrical constraints of the small number of cells that make up the early embryo limit the repertoire of possible patterns of cell division and interactions and could easily lead to a deterministic view of cell fate allocations. However, this does not imply that there is any sort of lineage-dependent allocation of particular fates to specific blastomeres linked to asymmetric cell divisions, but rather that what is at work is a probabilistic and regulative process in which global forces interact with cell-autonomous biochemical processes. Simulations of the early cleavage events support this last possibility (Honda et al., 2008; Krupinski et al., 2011) and experimental manipulation reveals that, even when cells appear to be committed to a particular fate, actual determination and irreversibility is a late event (Grabarek et al., 2012). The convergence of mechanical and chemical signals on these events is likely to provide robustness to the process. In this regard, it is interesting to note that, although there is some evidence that ESC cultures contain a small fluctuating population of cells that will differentiate into PrEnd derivatives when introduced into embryos (Canham et al., 2010; Niakan et al., 2010), differentiation toward a PrEnd phenotype is rare in standard ESC cultures. However, when cells are allowed to form embryoid bodies (EBs) an outer layer of PrEnd-like cells is readily assembled (Coucouvanis and Martin, 1995; Coucouvanis and Martin, 1999). One difference between the two situations is the formation of a cell mass with tissue-like characteristics in the EBs, which is absent in the ESC culture. It might be that, in order to reveal a stable PrEnd-like population in an ESC culture, one needs to impose a number of mechanical constraints that would act in concert with the better characterised chemical signalling. Experiments varying physical parameters, such as cell density, compliance, surface tension and local adhesion, in ESC cultures could be used to test this hypothesis.

Our observations and suggestions raise two questions. The first concerns whether there is some advantage to this mode of development. It is possible that sustained multilineage priming is a strategy associated with the evolution of placental development rather than a general one. Selection might have favoured a mechanism that delays lineage commitment until the extraembryonic tissues that will support the development of the embryo have been established. In this case, it would be advantageous to commit cells to the embryonic fate only when there are enough cells in the zygote to minimise the risk of losing the embryo. A second question concerns whether our proposal of a molecular basis for pluripotency based on a generalisation of the notion of 'transition states' is relevant to other mammals. This is not easy to answer. However, although it is clear that the cellular and embryological basis for twinning and regulation will vary from one species to another, the notion of sustained multilineage priming in a transition state might be a general principle of early mammalian embryogenesis. In this case, it will be important to analyse the mechanisms that generate and control the heterogeneities. It might be that, as has been suggested, there are mechanisms and, in particular, signalling systems, that are dedicated to the control of the distributions generated by the heterogeneities (Muñoz-Descalzo et al., 2012).

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Competing interests statement

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