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REVIEW

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Early human development: new data raise important embryological and ethical questions relevant for stem cell research

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Abstract Recent research has considerably changed our views about the developmental biology of early mammalian embryos compared with the ideas that were predominant throughout the previous 30 years or so. New data obtained recently suggest that the mammalian embryo uses traits of axes determination mechanisms that are not too different from the modes used by other vertebrates. In particular, it appears that asymmetry cues derived from the oocyte cytoplasm and modified/specified during sperm penetration appear to be crucial in normal embryogenesis, rather than the environmental influences exerted, e.g. at embryo implantation in the uterus. On the other hand, recent advances in research on the equivalents of a Spemann-Mangold organizer in species other than amphibia (including mammals) provide a background for new discussions of early embryonic patterning (axis formation) processes in the embryonic disc. In combination, these new views appear to be of considerable interest in the debate on the developmental properties and the ethical status of embryonic stem cells. The present review focuses specifically on the new aspects of axis determination and pattern formation processes in early mammalian embryos and relates this to questions about the developmental potential of embryonic stem cells (totipotency vs pluripotency/omnipotency), i.e. facts that appear to be worth considering in the recent debate about the ontological status of the early human embryo as well as of human embryonic stem cells.

Introduction

The biological and ethical status of early human embryos has become a topic of much attention and debate not only in the scientific community but also among the general public. After an upsurge of interest at the advent of human in vitro fertilization and embryo transfer (IVF-ET) in 1978, much more intense discussions were stirred up when human embryonic stem cells (ESC) became available (Thomson et al. 1998), followed by debates about the acceptability or non-acceptability of pre-implantation diagnosis (PID) in recent years. While these discussions have predominantly centred on general ethical, sociobiological and technical aspects, they have largely omitted aspects of the developmental biology of early embryos. This is in contrast to the fact that developmental biology is recently making impressive and very rapid progress, including not only work on invertebrate and non-mammalian vertebrate species but also on mammalian embryos. The present review intends to bridge this gap with a focus on topics that are of specific actual interest, i.e. aspects of totipotency and pluripotency, raised in the context of research on human embryonic stem cells.

One central topic of developmental biology is pattern formation. Recently, considerable progress has been made with respect to the molecular basis of early embryonic pattern formation in vertebrate embryos, e.g. the Spemann-Mangold organizer (De Robertis and Aréchaga 2001). It would appear to be timely to make use of this progress and to apply this knowledge to a discussion of the developmental biology of ESCs and blastomeres potentially used in PID. Surprisingly, however, this has rarely been done, in spite of the fact that it had been proposed to focus on these aspects (Denker 1997, 1999) even before the first paper on human ESCs (Thomson et al. 1998) was published. This may be due to the fact that the main interest of stem cell workers is on application-related aspects of cell differentiation, not pattern formation, and that the discussion that has come up specifically in Germany after 1998 has been led by reproductive biologists, not by developmental biologists. This might also be the reason why aspects of, for instance, the formation of the Spemann-Mangold organizer and principles of axis formation were largely omitted from this discussion. The Spemann-Mangold organizer and its equivalents will be much in the focus of the present review, since the formation of an organizer is instrumen-

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tal in individuation, a topic of utmost importance in developmental biology as well as in ethical considerations. This can be illustrated by taking another look at Spemann and Mangold's original report (1924; cf. Spemann 1936). In discussing their classical experiments on transplantation of an organizer to the presumptive ventral region, they were talking about a "second embryo" or "second embryonic anlage" (even though this was a, chimeric, Siamese twin).

The early embryonic pattern formation potential of embryonic stem cells is insufficiently known

The ability of ESCs to differentiate in cell culture (or in the teratoma model) into derivatives of all three germ layers is well known (for human and non-human primates see Thomson et al. 1998; Thomson and Marshall 1998; Reubinoff et al. 2000; Amit et al. 2000; Itskovitz-Eldor et al. 2000; Schuldiner et al. 2000; for the voluminous literature on the differentiation of mouse ESCs see Wobus and Boheler 1999). Surprisingly, however, it is rarely studied and rarely discussed that, in order to be able to enter these various pathways of differentiation, the cells seem to require the transitional formation of so-called embryoid bodies (EBs), a specific form of colonies that resemble in certain respects early embryos, and which we will discuss in detail later. It is generally assumed that in these EBs complex cell–cell interactions are initiated, which somehow correspond to cascades of events of induction and sequential gene activation as take place during germ layer formation and organogenesis during embryonic development. This is a historically old assumption which had already been expressed at the time of the pioneering experiments on mouse EBs using teratocarcinoma cells rather then ESCs (work by Stevens and Pierce, for references see Sherman and Solter 1975). Unknown, however, is what exact role any processes of pattern formation may have in these EBs and what degree of similarity to normal embryogenesis might be required here with respect to cell migration, cell–cell interactions and the formation of microenvironments and niches in order to allow the sequential differentiation events to occur. Of specific interest is the question whether there is any requirement for a morphological equivalent of an embryonic disc, in particular of an epiblast with the equivalent of a primitive streak, i.e. the structure where a process of localized and regulated epithelial-mesenchymal transition takes place. Or, to take another example, how important may it be that transitorily neural tube-like structures are formed in these ESC cultures, in order to promote the formation of neuronal (progenitor) cells (see Zhang et al. 2001)?

Embryonic stem cells: in vitro equivalents of the embryoblast/epiblast

ES cell lines are usually produced by isolating the embryoblast of a blastocyst from the trophoblast, followed by propagating the former in vitro under appropriate conditions. One important element among these in vitro culture conditions is to provide direct contact to a layer of feeder cells, usually mitotically blocked fibroblasts obtained from mouse embryos (not necessarily a homogeneous population of cells, since purification and cloning of these feeder cells is usually not attempted). It been reported recently that mouse feeder cells could perhaps be replaced by fibroblasts from human embryos (Richards et al. 2002) or foreskin (Amit et al. 2003) or by extracellular matrices (Xu et al. 2001). In the case of mouse ESCs, but not in human or monkey ESCs, feeder cells can be replaced by the cytokine LIF (leukaemia inhibitory factor) (Nichols et al. 2001; Reubinoff et al. 2000; Thomson et al. 1998). Under all these conditions ESCs are, more or less efficiently, prevented from differentiating and continue proliferating, at least in a subpopulation. To remain in this undifferentiated stem cell state is in marked contrast to what the cells would have done in the embryo from which they were derived. Here they would have initiated a cascade of differentiation and pattern formation events culminating in the formation of the embryo proper. Thus, apparently the specific in vitro culture conditions chosen keep these embryoblast-derived cells in some type of artificial stem cell "niche". As with other postulated stem cell "niches", e.g. in case of somatic stem cells, it is still not completely clear what types of cell–cell interactions and signalling processes are involved here (Fuchs and Segre 2002; Watt and Hogan 2000). The fact that LIF is effective in mouse, but not in primate and human, ESCs probably reflects a speciality of the mouse, the ability to enter the peculiar state of diapause (delayed implantation) in which development is stopped (Nichols et al. 2001). This may be the reason why in the mouse the production of ESC lines is particularly efficient if diapause blastocysts are used rather then blastocysts from normal pregnancy (Robertson 1987). Success rates reported for generating ESC lines in humans and monkeys are surprisingly high, however (Thomson et al. 1998; Reubinoff et al. 2000; Pera 2001), in spite of the fact that here blastocysts are not able to enter a state of diapause and that LIF is not effective here. In these species the regulation of the undifferentiated ES cell state may be dominated by other regulatory pathways, some of which may be involved also in the mouse (Chambers et al. 2003; Mitsui et al. 2003).

It is not clear which cell type of the early mammalian embryo may be represented by ESCs. Since they are mostly produced from the embryoblast of blastocysts, it is of course tempting to assume that they simply continue to express the properties of the embryoblast in vitro. This assumption, however, finds only partial support from the observed patterns of marker molecule expressions and likewise by their developmental potential. In the mouse,

many authors have assumed that ESCs correspond to epiblast cells (Gardner and Brook 1997; Smith 2001). On the other hand, there are many observations that cannot easily be reconciled with this view. For example, ESC-like cells can on principle already be produced experimentally when one starts with pre-blastocyst (cleavage) stages (for a review of the literature, see Prelle et al. 1999). It is not clear, however, to what extent the choice of embryonic stages determines whether permanently growing cell lines can be derived or only non-permanent cultures. Interestingly, long before the term ESCs had been coined, successful cell cultures derived from cleavage stages had already been reported, although these cultures were followed only for limited periods of time (Cole et al. 1966). Other reports from the older literature describe that teratomas can be produced already from cleavage stages (and not only later stages as usually done) if these are transplanted ectopically, although the reported success rates appear to be somewhat contradictory (for a review of the literature, see Damjanov and Solter 1974).

The trophoblast differentiation potential has often been used as a criterion when discussing the question of what type of early embryonic cells ESCs may represent. This argument usually extrapolates from the mouse, in which most experiments have been done (for a review of the literature, see Denker 2002). In this species the embryoblast of early blastocysts still has trophoblast differentiation potential, whereas in later stages the epiblast can only give rise to extra-embryonic endoderm (hypoblast). The epiblast/primitive ectoderm of the post-implantation stages, in contrast, has lost the potential to form these extra-embryonic cell types, in the mouse, and instead forms the three definitive germ layers. This would fit in with the observed properties of mouse ESCs which are reported by most authors to produce little (if any) trophoblast in vitro. All observations about the developmental potential of ESCs, however, seem to depend very much on the experimental conditions used in the individual investigations. Indeed the embryoblast (inner cell mass) of mouse blastocysts retains, for a while, quite a remarkable potential to regenerate experimentally removed trophoblast and to reconstitute viable blastocysts (for a review of the literature, see Denker 2002). Correspondingly it was observed by Beddington and Robertson (1989) that mouse ESCs are able to differentiate into trophoblast cells in chimeras, although only at a low rate (cf. also Hemberger et al. 2003). It was shown recently that they can do so even autonomously by themselves in vitro (Hübner et al. 2003). Thus there seems to be good reason to assume that the properties of ESCs are close to cells of early, rather than late, blastocysts, or even of the post-implantation stage epiblast. Recently attempts have been made to pinpoint this more exactly using markers for intermediate stages in the formation of the epiblast (Chambers et al. 2003; Lake et al. 2000; Mitsui et al. 2003; Pelton et al. 2002; Rathjen et al. 1999).

In contrast to the mouse, all the primate ESCs so far described (human and non-human) provide evidence for a pronounced ability to differentiate trophoblast in vitro (for a literature review, see Denker 2002). Under appropriate culturing conditions, trophoblast differentiation can even prevail (Xu et al. 2002). It can be argued, therefore, that human and non-human primate ESCs either represent earlier developmental stages than those of the mouse (and thus may be more close to truly totipotent cells) or that in these species the ability to form extra-embryonic cells, including trophoblast, may be maintained by the embryoblast physiologically for a longer period of time, e.g. until the post-implantation stages. A combination of both reasons could also be possible.

The recent finding that mouse ESCs can give rise to oocytes in vitro has been taken as an argument that they should be considered totipotent (Hübner et al. 2003) (it should be noted that what is meant here is a totipotency in the wider sense, or what I suggest calling “omnipotency”, describing the fact that all cell types, but not necessarily a basic body plan, can be formed autonomously by the cells; see Denker 2002). One might argue that this puts mouse cells close to very early developmental stages, i.e. totipotent blastomeres. However, germ line determination seems to require a cooperation (signal exchange) between extra-embryonic and epiblast cells (Lawson et al. 1999). Therefore, the observed oocyte formation could reflect the fact that extra-embryonic, as well as all types of embryonic, cells are indeed formed by ESCs, in vitro, as already mentioned above. Depending on the stage when the extra-embryonic and embryonic cell lineage, respectively, are closed in vivo in the mouse vs the primate, this can be used as an argument for an early or late stage of embryogenesis being represented by ESCs. Since human and non-human primate ESCs have a pronounced ability to form trophoblast and other extra-embryonic cell types in vitro (see above), they might be expected to have no less, but perhaps even more, of a germ line differentiation potential in vitro, as compared with mouse ESCs, but this has yet to be demonstrated experimentally.

Embryoid bodies: a model not only for cell differentiation but also for early embryonic pattern formation?

In order to allow ESCs to express their differentiation potential in vitro, it seems to be important to give them an opportunity for complex cell–cell interactions in embryoid bodies (EBs). The mechanism behind this is not really understood. Theoretically one would expect that it should be possible to stimulate differentiation of ESCs also in monolayer cultures. However, such a simple strategy has so far been found to be only moderately successful and, interestingly, it is helpful to include in the differentiation protocols an intermediate step of EB formation. This is therefore done even when attempting to stimulate differentiation of cells pharmacologically [e.g. by using retinoic acid (Rohwedel et al. 1999) or with cytokines or growth factors (Schuldiner et al. 2000)]. An EB step seems to be important for the induction of mesodermal and endodermal cell types, whereas this is not obviously

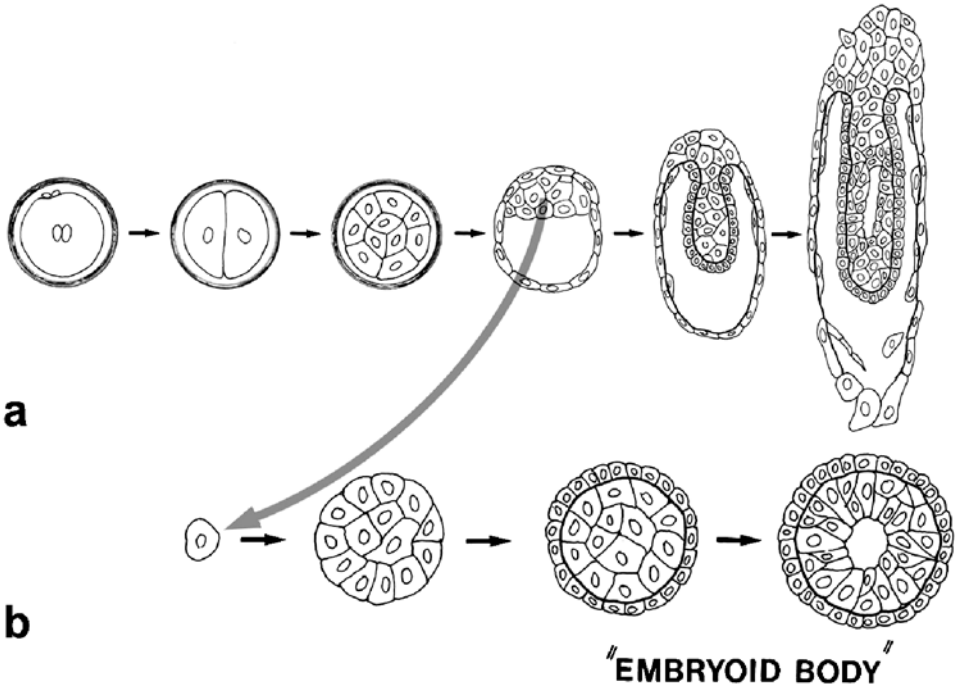


Fig. 1 Morphology of early embryonic development in the mouse (**a**) as compared to the formation of an embryoid body (*EB*) from mouse ESCs in vitro (**b**). Since ESCs are derived from the embryoblast of the blastocyst, the stages shown in **b** correspond to the last three of the stages shown in **a**. Note that the cell from which the EB is derived is much smaller than the zygote (first stage in **a**): it is lacking the abundant cytoplasm of an oocyte (with its cytoplasmic fields of factors that are to be segregated into the various blastomeres, cf. Figs. 3, 5, 6 and 7). Mouse ESCs form little trophoblast in vitro (for a discussion see text) so that a blastocyst stage is lacking here, and the outer (parietal) layer of extra-embryonic endoderm that would be underlying it at later stages is also missing. The EB, therefore, corresponds to just the egg cylinder (core structure in the last two stages in **a** and **b**). Both the egg cylinder in vivo and the EB in vitro develop a proamniotic cavity (last stage in **a** and **b**). The inner cell layer represents the epiblast, the outer epithelial layer the visceral (extra-embryonic) endoderm (from Denker 1983, by permission of S. Karger, Basel)

required for the formation of neuronal and glial cells. This is consistent with the fact that in normal embryogenesis the formation of the definitive endoderm and mesoderm requires the formation of a primitive streak, whereas this is not the case for neuronal and glial cells, which appear to represent a default pathway. This leads to the question of how far the similarity between EBs and early embryos must go, in morphological and functional terms, in order to allow the manifold differentiation processes to be initiated.

The term embryoid body was originally coined for the mouse system and refers to the fact that when mouse teratocarcinoma/embryonal carcinoma (EC) cells or ESCs are kept in mouse ascites or in vitro, they can form three-dimensional structures which more or less resemble in vivo developing embryos at the egg cylinder stage (see Fig. 1). Well-developed EBs consist of an epithelium that appears to correspond to the epiblast/primitive ectoderm, a central cavity corresponding to the proamniotic cavity, and an outer epithelial layer corresponding to the visceral endoderm (part of the extra-embryonic endoderm) (for references to the classical literature on EBs see Denker 1983; for more recent work see below). Trophoblast and parietal extra-embryonic endoderm are typically missing in such EBs of the mouse (although experimental evidence suggests that the potential to differentiate these types of cells is not completely lost in mouse ESCs; see above). This means that mouse EBs correspond primarily to those parts of the egg cylinder that form the embryo proper in vitro (epiblast), with few extra-embryonic cells (primitive/visceral endoderm), and that they are lacking other extra-embryonic cells, which play important roles in embryo implantation and yolk sac formation.

Data concerning the question whether the ESCs continue their in vitro differentiation/development by going through a primitive streak stage when forming the three germ layers are very limited. It is generally assumed that there must be processes somehow equivalent to primitive streak formation and gastrulation; at least this is suggested by the observed gene expression patterns (Rohwedel et al. 1999; Lake et al. 2000). However, there is little information available about morphological integrity or abnormality of any such primitive streak equivalents. A primitive streak has to show the phenomenon of an epithelial-mesenchymal transition (Burdsal et al. 1993; Hay 1995; Viebahn 1995). Thus the primitive streak provides the mechanism by which the definitive endoderm and mesoderm are formed. In addition a normal structure and positioning of the primitive streak is instrumental in making sure that a normal body plan with its anterior-posterior axis is laid down, that a singleton is formed and that the result is not a chaotic

mixture of tissues (teratoma). We return to this point below in connection with a discussion of the role of the Spemann-Mangold organizer. In EBs of the mouse, however, an ordered formation of a single and well-organized primitive streak does not normally seem to occur, and what one observes during further development in vitro usually resembles a teratoma much more than it resembles a normal embryo (for a discussion of teratomas see Sherman and Solter 1975; Denker 1983, 2000, 2002; Andrews 2002). Nevertheless many authors have pointed out that, in spite of the rather bizarre morphology of mouse EBs, the gene activation patterns seen in EB cultures imitate in many respects what happens during normal embryogenesis (Grabel et al. 1998; Rohwedel et al. 1999, see specifically their Fig. 1; Leahy et al. 1999; Lake et al. 2000; Maye et al. 2000; Murray and Edgar 2001).

In later stages of differentiation, ESCs demonstrate a considerable pattern formation potential with respect to organ anlagen. This is particularly impressive when ESCs are transplanted to various ectopic sites in host animals in vivo (teratoma model). In this case surprisingly regular organ anlagen can be formed (e.g. tooth anlagen or gut-like structures with a regular arrangement of mucosa, muscularis mucosae, submucosa, muscularis); this holds true for mouse as well as primate ESCs (Thomson and Marshall 1998; for references concerning the mouse see Sherman and Solter 1975; Andrews 2002). This organogenetic potential is basically also found in human ESCs (Thomson et al. 1998; Amit et al. 2000; Reubinoff et al. 2000). For our discussion it is of particular interest that, in addition, an early embryonic pattern formation potential may exist in primates that can possibly exceed that reported from the mouse: Thomson et al. (1996) observed that in dense cultures of marmoset monkey (*Callithrix jacchus*) ESCs, structures formed spontaneously that morphologically closely resembled normal embryos in early post-implantation stages of primates (Fig. 2c, d). The structures that were seen there were interpreted by Thomson et al. as an embryonic disc with epiblast and hypoblast as well as amnion with amniotic cavity and a yolk sac. Remarkably, those authors also identified on one end of the epiblast an area which showed all morphological signs of an early primitive streak with its epithelial-mesenchymal transition. One would have to assume, therefore, that this embryonic disc also seemed to have developed an anterior-posterior (cranio-caudal) axis. The morphological similarity to corresponding developmental stages of non-human primates and human embryos is in fact astonishing (Fig. 2a, b). Also of interest is that structures resembling these can from time to time be found even in spontaneously developing teratocarcinomas. For example Damjanow and Andrews have described a quite similar structure (referred to as an "embryoid body") that formed spontaneously in a human testicular teratocarcinoma (Andrews 2002; see Fig. 2e). Note the close similarity to the early human embryos in Fig. 2a, b.

The findings regarding a marmoset EB that so closely resembled a normal post-implantation stage primate embryo was interpreted as an indication that primate ESC colonies may serve as a very useful and promising in vitro model for experimental studies on normal early primate development (Thomson et al. 1996; Thomson and Marshall 1998; Thomson 1998). However, other studies published on the differentiation of non-human primate and human ESCs in vitro have not reported on the formation of such structures that would so closely resemble early post-implantation stage embryos (Thomson et al. 1995; Itskovitz-Eldor et al. 2000; Pera 2001). Itskovitz-Eldor et al. (2000) described vesicular structures containing epithelium that formed in in vitro cultures of human ESCs, but no appropriate markers have been used to clarify whether these epithelia could be identified as epiblast or hypoblast, amnion or even, for example, neural tube. At least derivatives of all three germ layers have been found. It must be seen, however, that in the investigations on human ESCs the culturing conditions were not the same as those used by Thomson et al. (1996) but rather were closer to those that have been developed and are in use for the mouse system. The three-dimensional suspension culture conditions developed for the formation of mouse EBs seem to be very appropriate for the mouse, with its germ layer inversion and the formation of an egg cylinder (cf. Fig. 1). However, since in the human and in non-human primates a flat embryonic disc is found instead (Fig. 2) (and many other differences exist), flat cultures as used by Thomson et al. (1996) could be more appropriate for embryonic pattern formation in primates. In this context the pronounced ability of human and non-human primate ESCs to differentiate extra-embryonic cell types (including trophoblast) also has to be considered (see above; for further discussions see Denker 2002). Pera (2001) emphasizes that, to the best of his knowledge, the spontaneous formation of embryonic disc-like structures similar to what Thomson et al. (1996) described for the marmoset monkey have never been observed in in vitro cultures of human ESCs; however, he does not give details about the range of different culture systems tried nor about the number of colonies studied morphologically in detail. On the other hand, he does consider such observations important and points out with respect to Thomson's findings of 1996: "There are questions regarding the reproducibility of this finding, and the precise identification of the structure observed, but if such structures were observed in cultures of human ES cells, there would be justified reason for concern, since such an entity might bear a very close resemblance to the embryo near the 14-day limit for observation in vitro" (Pera 2001).

Systematic investigations on the differentiation of human and non-human primate ESCs in vitro have indeed so far not been performed with a focus on aspects of early embryonic pattern formation potentials. The published work focuses on molecular markers, in particular, for the differentiation of germ layer derivatives, or on the morphology of teratomas formed in immunodeficient

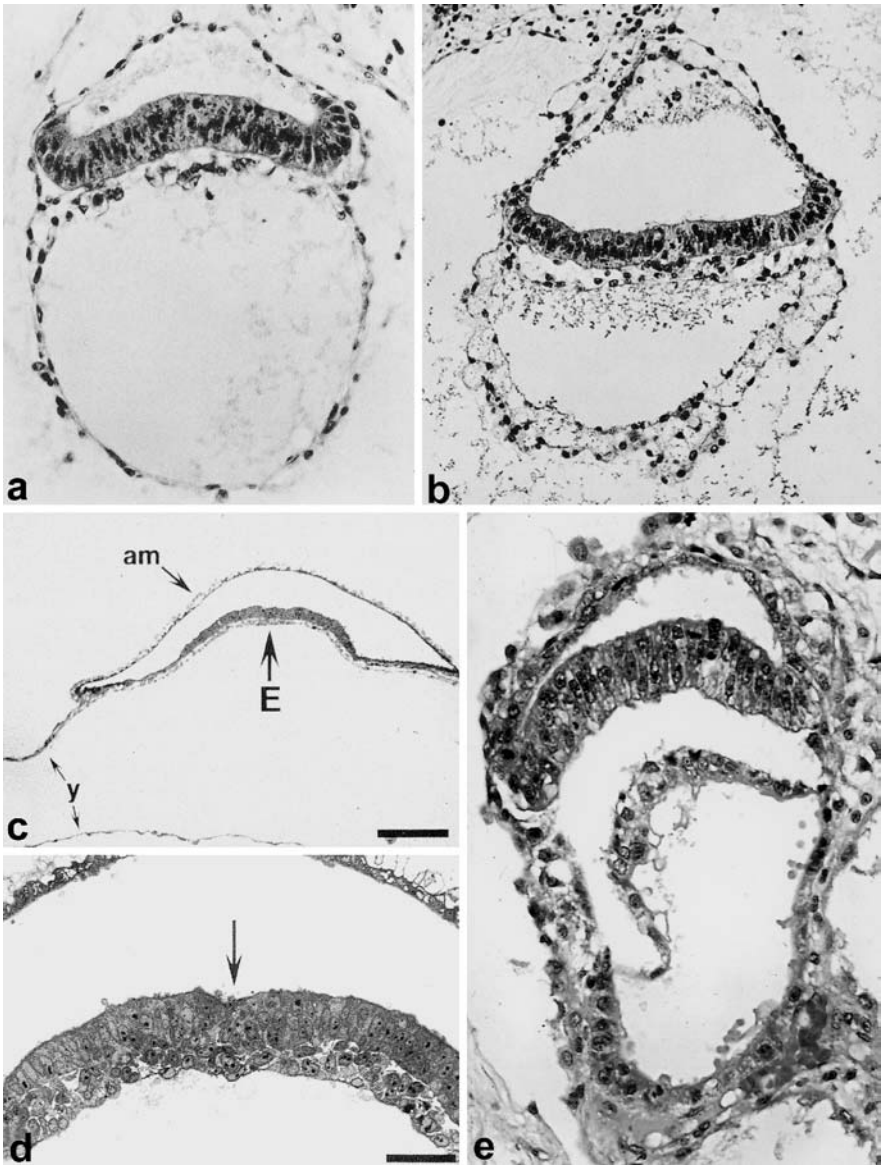


Fig. 2a–e Morphology of early post-implantation stage embryos as compared with embryoid bodies in the human and the marmoset monkey. Note that the human and the non-human primates have a flat germinal disc, in contrast with the egg cylinder of the mouse (cf. Fig. 1). **a**, **b** Normal human embryos (Carnegie stages 6a, 7b). Relevant structures, from above: *amnion*, *amniotic cavity*, *epiblast*, *hypoblast* = primitive extra-embryonic endoderm, *yolk sac*. The *primitive streak/node* can be seen in the middle of the epiblast (**b**) and *mesoderm cells* can be seen to emigrate between epiblast and hypoblast (from O'Rahilly and Müller 1987, by permission of the authors and of the Carnegie Institution of Washington). **c**, **d** "Embryoid body" that formed spontaneously in a culture of marmoset ESCs (**c** overview, *bar* 200 μm; **d** details, *bar* 50 μm); *am* amnion, *E* embryonic/germinal disc, *y* yolk sac (surrounded, as described in the legend, by extra-embryonic mesenchyme). The structure to which the arrow points in **d** was interpreted as a primitive streak, positioned at one end of the germinal disc (from Thomson et al. 1996, by permission of the authors and the Society for the Study of Reproduction). **e** "Embryoid body" formed spontaneously in a human teratocarcinoma. Note the close similarity to structures seen in **a** and **b**, i.e. amnion, amniotic cavity, epiblast, (cleft = artefact), hypoblast, yolk sac (from Andrews 2002, illustrating a specimen provided by I. Damjanow; with permission of both authors and the Royal Society of London)

mice, whereas the morphology of colonies and embryoid bodies forming in vitro has usually not been studied in much detail (Itskovitz-Eldor et al. 2000; Amit et al. 2000; Thomson et al. 1995; Reubinoff et al. 2000). Clearly, however, there is a problem with such studies should they be performed with human ESCs: if the findings reported for the marmoset monkey by Thomson et al. (1996) are indeed indicative of processes also going on in cultures of ESCs of other non-human primates or human ESCs, the use of such culture conditions under which these processes may occur spontaneously would have to be considered unacceptable for ethical reasons in the case of human ESCs (National Bioethics Advisory Commission 1999, p 71f; see examples of experiments listed as ethically unacceptable). Such an experiment would be, in essence, the cloning of a potentially viable human

embryonic anlage. Therefore, it cannot be envisaged that such investigations will ever be performed on human ESCs by any serious researcher. On the other hand, all the available data suggest that totipotency is a property that is inherent to the cells (or groups of cells) and not imposed on them by their environment, although the latter may be permissive or not for the expression of the developmental potential. The possibility always remains that even culture conditions not specifically chosen for the expression of totipotency might accidentally allow early embryonic patterning processes to be initiated. Should a finding like the structure depicted by Thomson et al. (1996) ever be made in an in vitro culture of human ESCs, this cannot be expected to be published, at least not in Germany, since the investigator would expect to face prosecution under the German Embryo Protection Law (ESchG). In order to tackle the questions raised here experimentally, the only acceptable way forward seems to be to do these investigations with non-human primate ESCs. However, our group appears to be the only one which is currently concentrating on this type of experimental investigation (Behr et al. 2003).

Natural cloning: The formation of monozygotic twins

The following sections give an overview of the theoretical basis on which the question about any early embryonic pattern formation potential of ESCs can be discussed. It briefly reviews the principles of monozygotic twinning, the basic processes involved in the formation of the basic body plan, and the specific role of the Spemann-Mangold organizer in this context.

Monozygotic twins are clones. Their spontaneous formation is not a particularly rare event in the human (3.5‰ of all live births; O'Rahilly and Müller 2001). For our discussion, it is of interest that in two-thirds of these cases human monozygotic twins are monochorial. This means that they must have originated from a process of spontaneous splitting of the group of cells forming the embryo proper into two, at a stage after formation of the blastocyst (see, for example, O'Rahilly and Müller 2001). Therefore the embryoblast (inner cell mass) of the blastocyst, or (more rarely) the epiblast after formation of the amniotic cavity, is still able to split into two or more individual embryonic anlagen and to regulate the processes of early embryonic pattern (axis) formation in such a way that two harmonious bodies develop. If this separation occurs late, at the stage of the epiblast (after formation of the amnionic cavity, i.e. monochorial/monoamniotic twins), it is possible that this separation may remain incomplete and that Siamese twins will be formed.

Experimentally it is easy to produce monozygotic twins by separating blastomeres from each other in cleavage stages. However, experimentation is more difficult in stages after formation of the blastocyst where spontaneous twinning occurs most often (see above). Thus hardly any data on these processes are available. This is a disadvantage for our discussion of the developmental potential of ESCs, since the latter are usually derived from the embryoblast of blastocysts and may even represent slightly later stages (epiblast) as discussed later, i.e. the stages in which spontaneous twinning occurs most often. Observations concerning twinning at such relatively late stages have mostly been made only incidentally. Thus in the human the blastocyst stage seems to be sensitive to disturbance by various noxes in vitro; one possible result that has been described is the separation of the embryoblast into two cell groups in the sense of monozygotic twinning (da Costa et al. 2001; Milki et al. 2003). Under even more artificial conditions, if mouse blastocysts are allowed to attach in vitro, the resulting abnormal steric conditions in such an outgrowth system relatively frequently (1%) cause a separation of cells of the embryoblast into two groups (Hsu and Gonda 1980). Interestingly those authors have in this context referred to the cells of the embryoblast as "totipotent" cells. The formation of mono-amniotic twins in vivo has been observed in a study on the effects of vincristin in the mouse (Kaufman and O'Shea 1978). The sensitive phase was found to lie between the late egg-cylinder and the early head-process stage, i.e. a period during which the equivalents of the Spemann-Mangold organizer are formed and are acting (see detailed discussion below). Since vincristin is a cytostatic drug, the mode of action here is probably through induced changes of cell proliferation kinetics in the epiblast, resulting in the (complete or incomplete) separation of two cell groups that subsequently develop one organizer each.

A fascinating experiment of nature is the formation of monozygotic quadruplets in the nine-banded armadillo (see Fig. 1.9 in McLaren 1982). This process, called polyembryony, is even more extreme in the twelve-banded armadillo, where as many as eight monozygotic young are produced in this way. Enders (2002a) has recently described the morphological details of the separation of the epiblast in the former case. Interestingly, the prospective four individual embryonic anlagen can be recognized early in the epiblast on the basis of the formation of four distinct cranio-caudal (anterior-posterior) axes that are identifiable due to the regionally differing height of the epiblast epithelium.

An observation made by chance in the rhesus monkey shows that in primates a separation of the embryonic anlage into two parts still appears to be possible at a relatively late stage (embryonic disc, day 15) but may remain incomplete then, possibly leading to the formation of Siamese twins (Enders 2002b).

Axis determination and formation of the basic body plan in vertebrate embryos

A central element of embryonic development is pattern formation, in addition to the differentiation of the various cell types of which the body consists. A teratoma (a tumour which may consist of a mixture of all these cell types; see Sherman and Solter 1975; Denker 1983) never

acquires individuality (as defined as the ability for an independent life as a new entity). A basic body plan has to be laid down and organogenesis has to proceed in a structured way in order to develop a new individuum that is able to conduct such an independent life as an autonomous new entity.

The Spemann-Mangold organizer is of central importance in laying down the basic body plan (and therefore in individuation) in vertebrate embryos. The application of molecular approaches to developmental biology has in recent years led to an impressive gain in our knowledge of the mode of action of the organizer, its origin and its functional significance during formation of the basic body plan (Gerhart 2001; Gilbert 2001; Joubin and Stern 2001; De Robertis et al. 2001). This knowledge has predominantly been gained from studies on non-mammalian vertebrates (the classical models such as amphibia, chick and, more recently, zebra fish). However, accumulating data suggest that the basic mechanisms have been conserved in evolution and do also apply to mammals. The organizer is itself induced in amphibia by the Nieuwkoop centre, whose position defines the future body axes. This also appears to be generally valid for the mammalian system.

The organizer is defined as a source of important global information for development, in particular of axial organs, via a small set of spatially coherent signals. The organizer is structured in itself. In addition to providing inductive signals, the cells of the organizer also have the ability to perform morphogenetic movements during gastrulation and to coordinate these in neighbouring cells. In the combination of these properties, the organizer provides information about time, place, scale, and orientation for development of the large groups of multipotent competent cells in its surroundings. Without an organizer the embryo does not develop its phylotypic basic body plan (Körpergrundgestalt, according to Seidel 1960a): notochord and branchial apparatus, central nervous system, and post-anal tail. The products of appropriate deletion experiments are similar to teratomas (Gerhart 2001, see his Fig. 4).

Since the Spemann-Mangold organizer has this central role in the formation of the basic body plan, the question arises whether processes leading to formation of an organizer can also take place spontaneously in colonies of ESCs in vitro. We therefore briefly review below some basic principles involved in the formation of the organizer in vivo.

Axis development, Nieuwkoop centre and the Spemann-Mangold organizer in amphibian development

In amphibia, processes that are relevant for the determination of the future axial organization of the body start long before gastrulation, i.e. during oogenesis. Growing oocytes develop a cytoplasmic polarity which in amphibia is quite obvious due to the unequal distribution of yolk. This cytoplasmic polarity is also easily recognizable by the eccentric formation of the polar bodies and defines the animal-vegetal (A-V) axis of the oocyte (see Fig. 3, step 1). Most relevant for embryonic development is that not only paraplastic structures relevant for energy production but also morphogenetic substances are distributed unequally in the oocyte cytoplasm and will later on regulate the differential gene activation cascades in the various blastomeres to which they are segregated during cleavage. In the following section we briefly outline the principles of main processes involved (for a more detailed review,

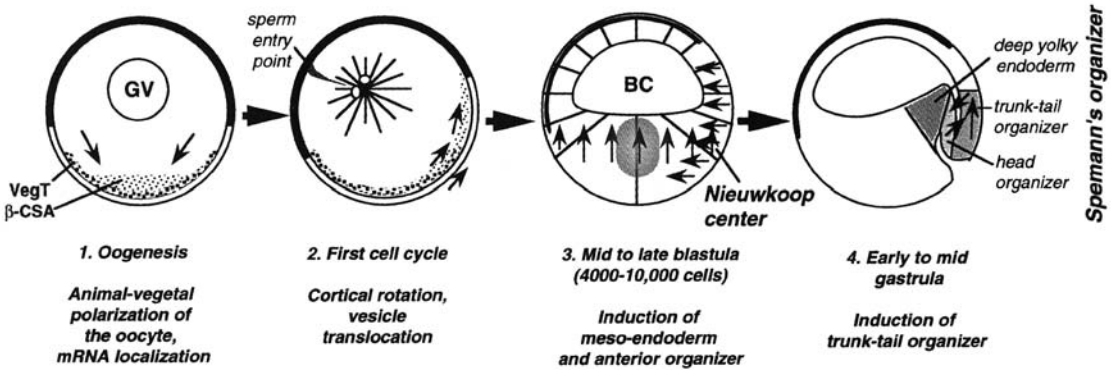


Fig. 3 Major processes involved in formation of the Spemann-Mangold organizer in amphibia. *Step 1* Oogenesis: translocation of β-catenin stabilizing agents (β-CSAs, *fine dots*) and of VegT mRNA (*coarse dots*) to the cortical cytoplasm at the vegetal pole. *Step 2* First cell cycle after fertilization: due to the cordical rotation initiated by sperm entry, parallel tracks of microtubules are formed close to the cortex and cause the β-CSA-containing vesicles to move to the future dorsal site opposite to the sperm entry point. The Nieuwkoop centre and the organizer will later form here. The maternal VegT mRNA, in contrast, remains symmetrically distributed around the vegetal pole. *Step 3* Mid- to late blastula period: zygotic gene expression starts and meso-endoderm induction begins, mediated by secreted Nodal signals from cells containing maternal VegT. Cells containing high β-catenin secrete (e.g.) Xnr3. Induction of the head organizer, dependent on the Nieuwkoop centre, the organizer of the organizer. *Step 4* Early to mid-gastrula: completion of organizer formation. Cells of the dorsal band of the marginal zone mesoderm are recruited into the trunk-tail organizer by a spreading induction about which little is known. The head organizer may initiate the recruitment; *GV* germinal vesicle of the oocyte, *BC* blastocoel (after Gerhart 2001, with permission of the author and UBC Press, Bilbao)

see Gerhart 2001; for the signalling pathways involved, see Xanthos et al. 2002). One very relevant process is that factors which stabilize high levels of β-catenin (here referred to using the general term β-catenin-stabilizing agents, β-CSAs) are concentrated in a narrow region of the egg cortex around the vegetal pole. This is notably the protein "dishevelled" (an inhibitor for glycogen synthase kinase 3, GSK-3, which itself indirectly accelerates β-catenin degradation). Other factors, Vg1 and VegT mRNA, are also translocated to the vegetal cortex over a broad area. These processes of ordered translocation are complex and are being studied intensively (see, e.g., Etkin 1997). The main aspect relevant to our discussion is that these processes result in morphogenetic information encoded differentially in the various regions of the cytoplasm, and that this cytoplasmic organization develops during oogenesis, long before fertilization, and is therefore dependent exclusively on maternal genes and on the interaction with somatic cells of the follicle.

The asymmetry of the amphibian oocyte thus established appears to be essentially radial around the animal-vegetal (A-V) axis. During fertilization, this radial symmetry is transformed into a bilateral one. The penetration of the sperm elicits a characteristic process of cytoplasmic movements called cortical rotation (Fig. 3, step 2). In connection with the cortical rotation, microtubules near the cortex are arranged as parallel tracks with their plus ends pointing towards the site opposite to the sperm entry point (SEP), thus defining which cytoplasmic area will become dorsal. β-CSAs (dishevelled, see above), are transported along these tracks from the vegetal pole to this future dorsal site. The penetration of the sperm is indeed instrumental here: in addition to egg activation (completion of meiosis, metabolic activation, pronucleus formation followed by the first mitosis), it therefore also provides axis information by restructuring the egg cytoplasm. The cytoplasmic region opposite to the sperm entry point thus acquires special properties and in this way a new axis (dorso-ventral, D-V) is added to the A-V axis so that the original radial symmetry of the oocyte around the A-V axis is transformed into a bilateral symmetry in the zygote. In the dorsal vegetal zone, the Nieuwkoop centre, the "organizer of the organizer", will form. Subsequently this Nieuwkoop centre will induce the Spemann-Mangold organizer in the area directly adjacent in the animal direction. In the region where the β-CSA is deposited, high β-catenin levels persist until the mid-blastula stage, while in the other regions the concentration decreases due to continuous turnover. When expression of the zygote genome starts, in the mid- to late-blastula stage, β-catenin is translocated into the nuclei in the dorsal region initiating differential gene expression (see Fig. 3, step 3). The developing Nieuwkoop centre consists of subregions with differential inductive properties and different potential for self-differentiation. This architecture of the Nieuwkoop centre is brought about by locally differing concentrations of, on one hand, VegT and Vg1, that are found in the individual vegetal cells and, on the other hand, β-catenin that, as described above, is concentrated in the dorsal region. Thus two gradients of gene expression regulating agents, an A-V (VegT/Vg1) gradient and a second one which is positioned in an angle to it (β-CSAs, β-catenin) overlap in the forming blastomeres resulting in differential gene expression, specifically of nodal-related proteins, Xnr 1–4, in the individual cells. These Xnrs in turn determine the type and arrangement of the various parts of the mesoderm differentiating here: the Xnrs activate the gene goosecoid, which in turn activates several organizer-specific genes.

For the molecular basis of the function of the organizer, an antagonism can be observed in the interaction between the dorsal and the ventral zone [inhibitory interaction of organizer molecules such as chordin and noggin with BMP4 in the marginal zone ("anti-organizer"); for references see Gilbert 2000]). After the complete organizer has been induced, the complex morphogenetic movements of gastrulation and subsequent processes of induction have to follow in order to achieve the definitive positioning of the body axes and of the various differentiating cell types and organ anlagen. The Spemann-Mangold organizer is of central importance for initiating these interconnected processes. The organizer itself has a substructure (head organizer, trunk-tail organizer). As described above for the Nieuwkoop centre, differential gene activations (as compared to other parts of the meso-endoderm) take place also in the organizer, and here again the specification involves specific combinations of activators. The number of involved genes/gene products increases with progressing development, and the system becomes more and more complex. The topographic interrelationships between the cells of the three forming germ layers change continuously during gastrulation, as do in consequence the possibilities for inductive interactions and for the triggering of new gene activation cascades. Inductions occur in part in a planar manner (within the same layer) and in part vertically (like the classical neural induction). The combination of the ordered morphogenetic movements with these induction cascades finally results in the typical arrangement of groups of different cells along the definitive body axes (cranio-caudal = anterior-posterior, A-P; dorso-ventral, D-V) so that the basic body plan (Körpergrundgestalt, see Seidel 1960a) and in this way the main structural requirement for future independent life as a new entity (individuum) is achieved.

My intention in giving this abbreviated outline of the complex processes of early development is to illustrate that the final structural and functional complexity of the organism starts developing with reading information out of simple asymmetries, i.e. the unequal distribution of relatively few components. These are translated into differential gene expression in the various cells of the developing organism on the basis of processes primarily involving segregation, morphogenetic movements and inductive processes. Pattern development makes use originally of a simple single axis (A-V) already present in the uncleaved egg cell cytoplasm, but a second axis is added by the penetration of the sperm, and its oblique

position in relation to the A-V axis is finally read into the definitive cranio-caudal (= anterior-posterior, A-P) and dorso-ventral (D-V) axes. The asymmetric distribution of certain cytoplasmic components before the onset of cleavage determines, via a process of segregation, the positioning (and substructure) of the Niewkoop centre, which in turn determines the position of the organizer. The principal way in which such hierarchically interdependent processes may lead to pattern formation can be shown, in reductionistic computer models, to require as a minimum only a few simple components and biophysical processes (activation/amplification, inhibition, diffusion) (Meinhardt 2001). When the physico-chemical properties of the components are adequate, not much basic spatial information is necessary in order to initiate the formation of patterns of the discussed type. Even slight inhomogeneities (as can arise spontaneously due to stochastic processes) in the distribution of the few components of the initial, simple system can suffice in order to start the pattern formation process. However, not all types of cells or cell groups have the appropriate machinery (potential). This aspect is of importance in our final considerations concerning the developmental potential of ESCs.

Principles of axis determination in fishes, birds and mammals

Recently an increasing number of data suggest that the principles of axis determination and of the formation of the basic body plan that we have described above for amphibia are in many respects also at work in fishes, birds and mammals. There are differences in detail, in part due to the different degrees of development of extra-embryonic cells and tissues. However, it seems that in all these systems we can find equivalents for the Nieuwkoop centre and the Spemann-Mangold organizer. A very useful comparative overview (also including molecular aspects of involved signal transduction processes) has been given by Joubin and Stern (2001).

In the ***zebra fish***, induction of mesoderm and of the organizer seem to be quite similar to the situation in amphibia. An equivalent for the Nieuwkoop centre seems to lie in the dorsal yolk syncytium, the equivalent of the Spemann-Mangold organizer in the shield (a part of the dorsal mesendoderm that forms predominantly prechordal plate and notochord). Also a subdivision seems again to be demonstrable in the Nieuwkoop centre equivalent as well as in the organizer that it induces (Kudoh and Dawid 2001). The orientation of the subpattern within the Nieuwkoop centre seems to depend on asymmetries within the yolk, which in turn are determined by the location of the egg nucleus and the cytoplasmic streaming around it (for references, see Joubin and Stern 2001). This is reminiscent of the role of cytoplasmic movements during cortical rotation in amphibia (see above).

In the ***chick***, the hypoblast, Koller's (Rauber's) sickle and the posterior marginal zone have been considered as equivalents of a Nieuwkoop centre. Although all three of these regions can induce a primitive streak in appropriate experiments, only the posterior marginal zone does indeed fulfil the criteria for an equivalent of a Nieuwkoop centre, i.e. to act before gastrulation and to induce an organizer without contributing directly to the formation of axial organs. Criteria for a chick equivalent of a Spemann-Mangold organizer are best fulfilled by Hensen's node (part of the primitive streak) (Waddington and Schmidt 1933; for a recent review, see Boettger et al. 2001). At least two groups of cells with somewhat different position are considered to provide cellular material for the forming organizer; one being located in the middle layer, associated with the inner face of Koller's sickle, and the other one in the epiblast just above the anterior face of Koller's sickle at stage X (Joubin and Stern 2001). Both groups finally meet in the centre of the embryo and form the complete organizer. With respect to the signalling pathways involved in the formation of the organizer, there seem to be considerable similarities to the amphibia. In the chick we also find a locally increased transcription (in the prospective caudal region) of genes that are related to those involved in organizer induction in amphibia (Tbx-6L, a VegT homologue; cVg1; CMIX), and also the overlap with the nuclear translocation of β-catenin and the Wnt signalling pathway seem to play a role (Seleiro et al. 1996; Boettger et al. 2001). cVg1 and Wnt8c (which are later expressed in the middle of the primitive streak) can induce an ectopic organizer in the chick (Joubin and Stern 2001).

Of particular interest for our discussion is that the flat embryonic disc of the chick has originally, in the blastoderm stage, no rigid axis determination: All parts of the blastoderm have a potential for primitive streak formation. Therefore, experimental twin formation is possible in these pre-streak stages, as shown in the classical experiments by Lutz and by Spratt and Haas (for references see Joubin and Stern 2001). A simple experimental subdivision of the blastoderm is sufficient. Primitive streaks can in fact form at any region of the embryonic disc (most frequently at the presumptive posterior end). A central role of the posterior marginal zone for induction of an organizer (and subsequently the primitive streak) is shown by the classical experiments of Kochav and Eyal-Giladi (see Fig. 4; Gilbert 1997). An inhibitory activity, originating from the forming primitive streak, which seems to depend on members of the BMP and ADMP family, seems to ensure that normally only one primitive streak forms. The computer models already referred to above (Meinhardt 2001) impressively simulate these interactions as well as possible conditions for the formation of more than one primitive streak.

The posterior marginal zone of the chick embryonic disc (blastoderm) can be recognized due to the fact that formation of the hypoblast starts here. What, however, determines its position in the circumference of the round blastodermal disc (and consequently the future location of the organizer and the primitive streak, i.e. the position of the A-P axis)? In the chick this is achieved by a cooperation between gravitation and the rotation of the

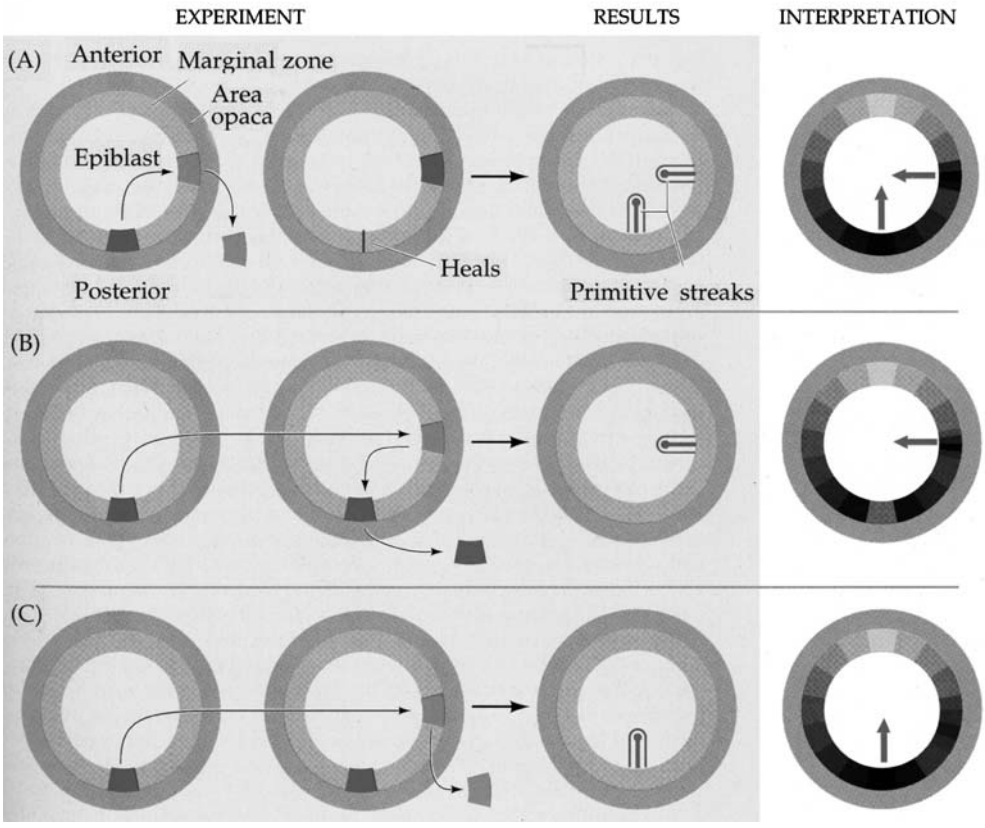


Fig. 4A–C Experiments illustrating the role of the posterior marginal zone of the chick germinal disc as an equivalent for a Nieuwkoop centre. Note that in contrast to the previous figures not sections but full-face views of the flat germinal disc are shown here. **A** Part of the posterior marginal zone is transplanted to the lateral marginal zone of the same embryo. A primitive streak is now formed here, at the non-typical location, showing the organizer-inducing potential of the transplanted material. If the defect in the posterior marginal zone heals and is regulated, a primitive streak is also formed there (at the typical location) in addition. **B** If a posterior region is reciprocally transposed with a lateral region, only one primitive streak forms, namely where the originally posterior cell material is located now. **C** Part of a posterior marginal zone is transplanted from one embryo to the lateral margin area of another embryo that retains its own posterior margin. No primitive streak is formed in the lateral region but rather only one at the host's original posterior margin which dominates. These experiments show that organizer/primitive streak-inducing activity resides in the posterior marginal zone, and that an inhibitory activity originates from here which normally prevents the formation of supernumerary primitive streaks (after Khaner and Eyal-Giladi, from Gilbert 1997, by permission of the author and of Sinauer Associates)

egg during its transport through the genital tract. This is basically known already from the classical observations made by K.E. von Baer (von Baer's rule, see Starck 1975; Boettger et al. 2001) and has been proven experimentally by Kochav and Eyal-Giladi (for references see Gilbert 1997; Boettger et al. 2001): The rotation of the egg in the genital tract forces the blastoderm into an oblique position relative to the yolk gradient, since the heavy yolk tends to return to the vertical. Due to the oblique position, the prospective posterior pole of the blastoderm is exposed to egg cytoplasm whose composition is different from that at the opposite pole. That part of the embryonic disc which takes the highest position will become the posterior pole, where the Nieuwkoop centre, the organizer and the primitive streak will form. In amphibia, in contrast, the transformation from the radial to the bilateral symmetry (and therefore the determination of the definitive body axes) is achieved by the penetration of the sperm, as we have seen. The chick may have to use a different type of mechanism (gravitation), since in this case fertilization is not monosperm but polysperm.

With respect to axes determination in ***mammals***, many authors have assumed during the last 30 years that, analogous to the chick, some type of external signal must also operate here, since no clear indications had been found for a bilateral symmetry in oocytes, zygotes and early blastocysts. Therefore it was proposed that the uterus provides the missing axis information during implantation of the blastocyst and imposes polarity onto the embryonic disc or its equivalents (Smith 1980, 1985; Viebahn et al. 1995). This view has also played a role in certain arguments put forward in connection with the debate on ethical aspects of the status of the human embryo and of ESCs (see, e.g., Kummer 1999a, 1999b; but compare and contrast Kummer 2000, 2002). However, this view is being drastically revised in recent times: indications have been found that in mammals the axis of bilateral symmetry is indeed determined (although at first

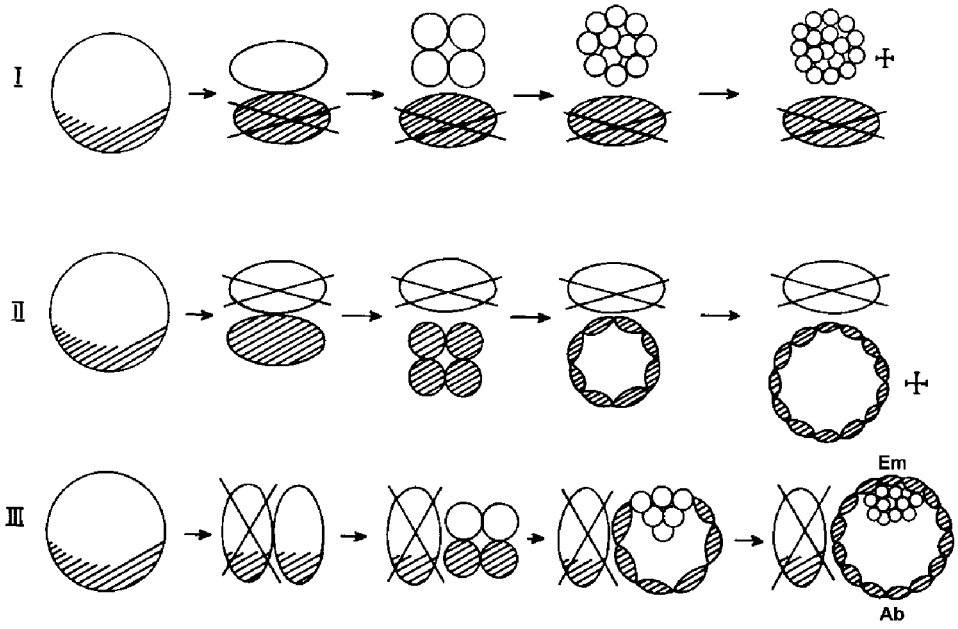


Fig. 5 Seidel's (1960b) experiment in the rabbit redrawn assuming a cytoplasmic field of factors in the zygote that is trophoblast-determining. (Alternatively, the factors could be embryoblast-determining.) The first cleavage furrow can lie in different planes and either restrict this field to one of two blastomeres (*I*, *II*) or divide it (*III*). For descriptive purposes, the situation is oversimplified in this diagram illustrating mosaic-type reactions (although in fact mammalian embryos are highly regulatory): half-embryos which consist only of "trophoblast-factor cytoplasm" form only trophoblast (*II*), half-embryos without it form only embryoblast (*I*), half-embryos with both types of cytoplasm form both types of cells, i.e. a whole blastocyst (*III*). Note that in addition to cell fate (embryoblast vs trophoblast), the position of the embryonal-abembryonal (Em-Ab) axis is also thought to be determined by the distribution of the cytoplasmic determinants in the uncleaved egg. Recent insight into the orientation of cleavage planes in relation to the A-V axis and the sperm entrance point (Fig. 7) are omitted from this diagram (from Denker 1976, by permission of Springer, Berlin Heidelberg New York)

in a labile way) by sperm penetration, as in amphibia. Bilateral symmetry can already be detected in the early blastocyst and is not dependent on implantation. We give a more detailed account of these findings below. Indeed arguments put forward for a postulated extrinsic determination of axes in mammalian embryos have never been very strong, and it appears strange that this view has nevertheless been held by many researchers during the last 30 years. So, for example, a normal basic body plan was shown to develop in vitro even without implantation (Hsu 1979, 1980; Hsu et al. 1974).The observation that primitive streak formation in the rabbit blastocyst in vitro can be stimulated by basic fibroblast growth factor (bFGF) (which in vivo seems to be provided by the endometrium), which has been taken as an argument for a role of extrinsic factors (Hrabé de Angelis and Kirchner 1993; Hrabé de Angelis et al. 1995), in fact cannot disprove any pre-existing functional polarity of the embryonic disc. Classical experiments have instead suggested that the early rabbit blastocyst, although possessing a high regulative capacity, does appear to show signs of a functional polarity (Seidel 1952, 1954). According to Seidel, the presumptive posterior zone should play a key role here, whereas Viebahn (1999) and Knoetgen et al. (2000) present arguments for a role of the anterior region.

Historically the reason why during the last 30 years a majority of investigators favoured the view that the early mammalian embryo does not possess any pre-existing axis-determining cues was that experiments had shown a very impressive regulative capacity of these embryos. This clearly disproved a strictly "mosaic" type of development, although it did not necessarily exclude a role for axis-determining asymmetry of the zygote that might at first be weak and which could be overridden by experimentation (reviewed by Denker 1976, 1983). Indeed, at an earlier timepoint the classical histochemical investigations by Dalcq and his associates (for references see Denker 1976; rat/mouse) as well as deletion experiments done by Seidel (1960b; rabbit) had led to the idea (dominating around the middle of last century and shortly thereafter) that mammalian zygotes, like those of amphibia, do possess cytoplasmic determinants that are segregated unequally to the various blastomeres during cleavage (reviewed by Denker 1976; Fig. 5). These views later received support from histochemical studies in the rabbit (Denker 1970) and, very recently, findings made with confocal laser scanning microscopy in the mouse and human (Antczak and van Blerkom 1997, 1999). Dalcq and Seidel discussed their findings in the first place not in relation to determination of the main body axes but with respect to the determination and differentiation of trophoblast vs embryoblast cells and the role that

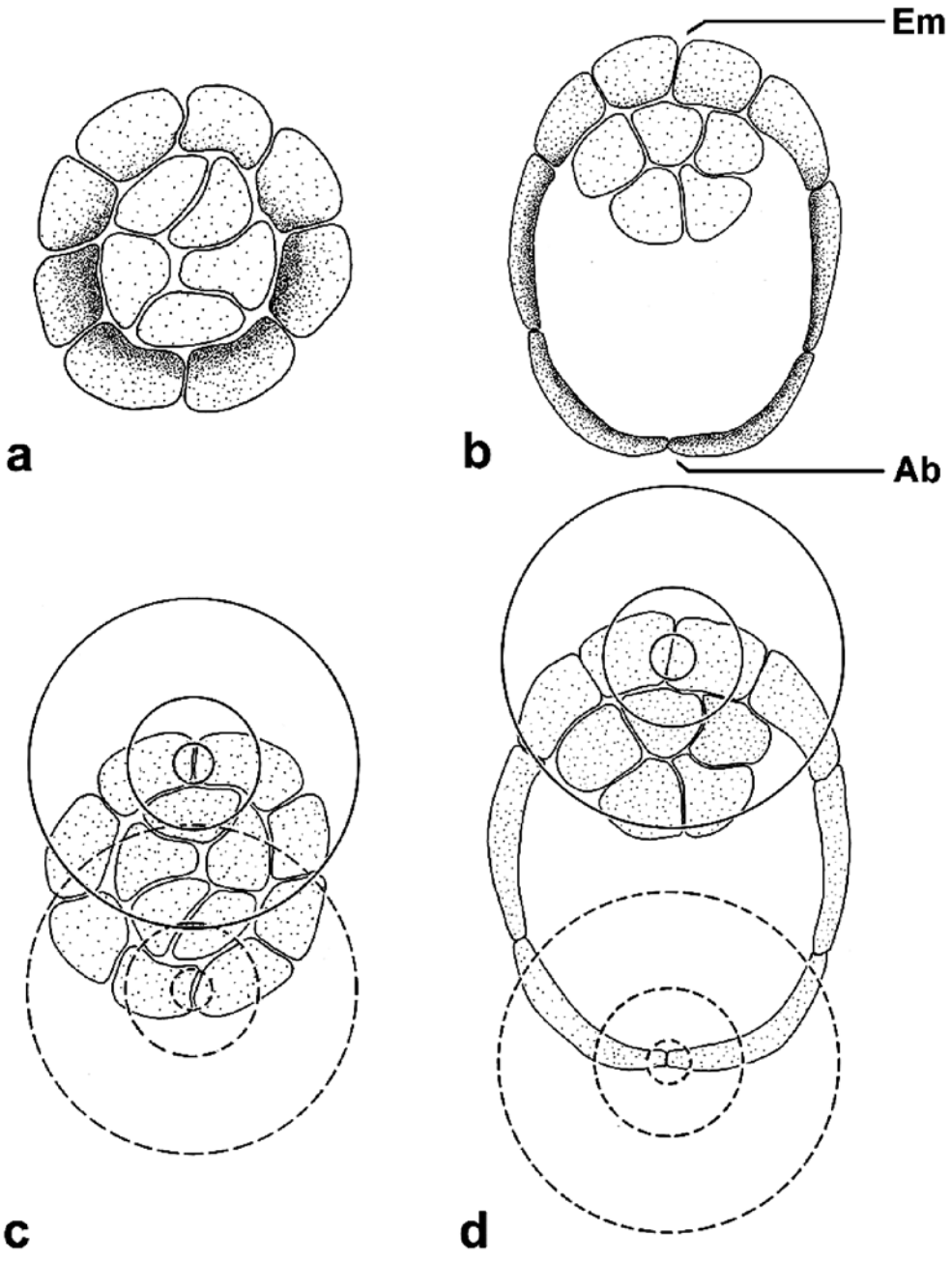


Fig. 6 Histochemical findings from some mammals suggest that the embryonal-abembryonal (Em-Ab) axis of the blastocyst does not form by chance but must be derived from zygotic axis information. This axis can already be detected in the cleavage stages due to the position of two groups of blastomeres which differ in cytoplasmic characteristics: *I* embryoblast and embryonic pole trophoblast (*light stippling*); *II* abembryonic and mural trophoblast (*dense stippling*). It was postulated that group I shows a higher proliferative activity (proliferative centre; *solid circles*) whereas group II represents a centre of early differentiation (trophoblast epithelium; *broken circles*). It was proposed that positional information (inside/outside position in the morula) and axial information derived from the zygote cooperate in forming these centres. This hypothesis, based primarily on histochemical findings, comes quite close to the recent views about axis determination (see Fig. 7) (from Denker 1983, by permission of S. Karger, Basel)

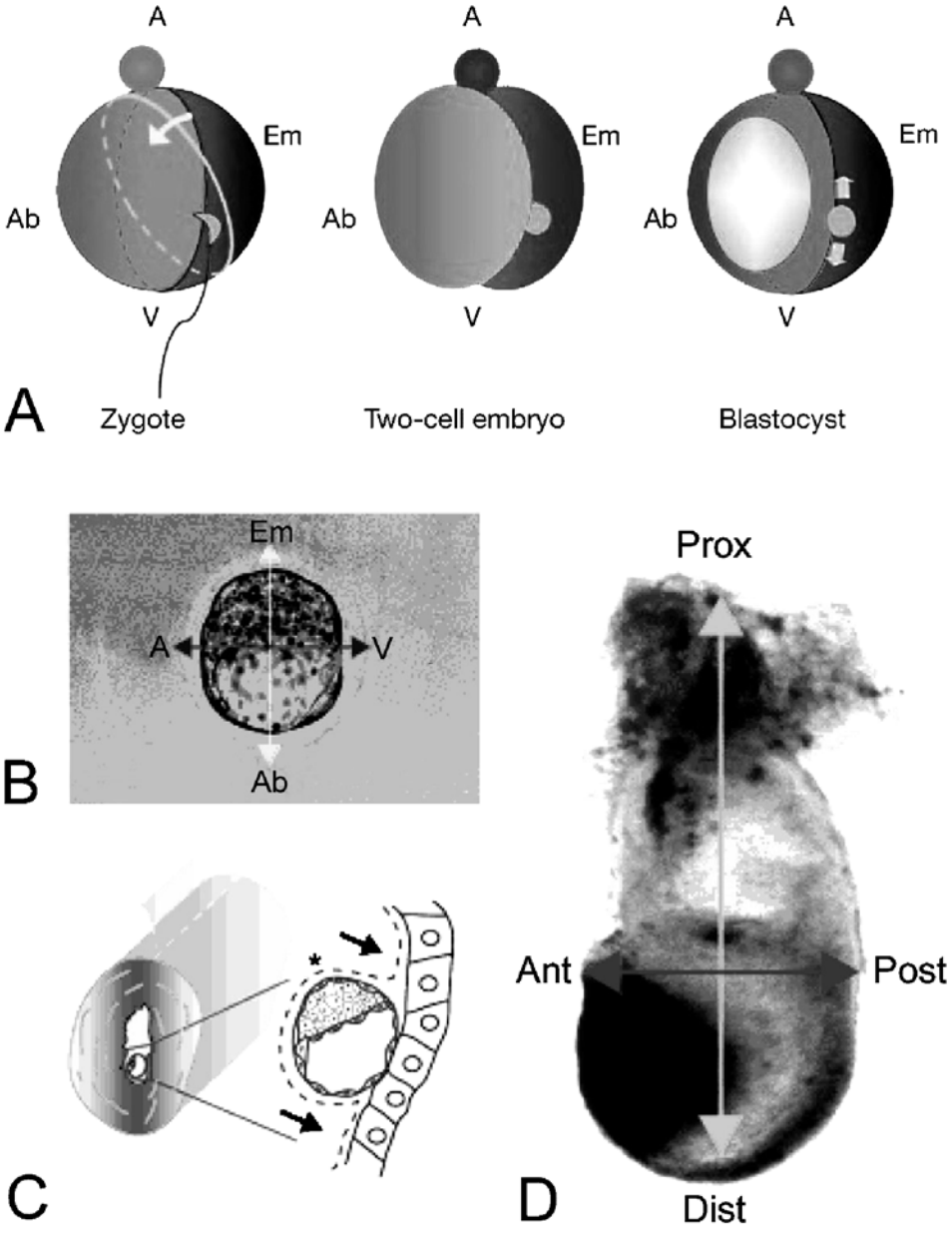
cytoplasmic determinants may play here. It was not particularly emphasized by those authors that (as known from lower animals) axis determination is usually connected with determination of cell fate by segregation of cytoplasmic determinants during cleavage. The terminology used (e.g. "dorsal" in Dalcq's publications) clearly shows, however, that this implication was indeed present (for references see Denker 1970, 1976, 1983).

Recent work, in particular by the groups of Gardner and Zernicka-Goetz, has seen the pendulum swing back closer to these classical ideas (De Smedt et al. 2000; Gardner 2001; Gardner and Davies 2002; Lu et al. 2001; Piotrowska and Zernicka-Goetz 2001, 2002; Plusa et al. 2002; Weber et al. 1999; Zernicka-Goetz 2002). Interestingly, this new trend has developed now in spite of the fact that data presented by one of these authors only a few years earlier (Zernicka-Goetz 1998) had still underlined, once again, the considerable plasticity of the mouse egg/early embryo, which was shown to be able to regulate experimental loss of animal or vegetal pole cytoplasm and to develop normally thereafter. The new focus does not only envisage the embryonic-abembryonic (Em-Ab) axis (with its importance for embryoblast vs trophoblast determination; see Figs. 5 and 6) but also the definitive body axes with their significance for the formation of the basic body plan. Magdalena Zernicka-Goetz describes the new focus very strikingly as follows: "Lewis Wolpert captivated our attention with the concept that 'It is not

birth, marriage or death, but gastrulation, which is truly the most important time in our life' (.....). Perhaps gastrulation was endowed with such importance because it implements the body plan, and it is so well conserved in evolution. However, (.....) the general strategy whereby the body layout is first drafted also now appears to have been strongly conserved. The major body axis of the mammalian embryo is not initiated, as was previously thought, at the onset of gastrulation, but can be recognized well before this process begins" (Zernicka-Goetz 2002). In brief, this present view sees the major events in early mammalian development as follows: probably as early as oogenesis an animal-vegetal (A-V) axis is laid down; at first it is, however, morphologically not recognizable in these eggs due to the lack of yolk but becomes apparent (or is formed?) during egg maturation (polar body: animal pole). However, it has not been possible to show a clear functional relevance of this axis, probably due to the high regulative capacity of the mammalian egg (Zernicka-Goetz 1998; Ciemerych et al. 2000). Nevertheless, the surprising regularity in developmental patterns as related to the final positioning of axes in later developmental stages had led investigators to postulate recently that this axis must have some function. Contrary to earlier reports it was found that mouse blastocysts are bilaterally symmetric, and their embryonal-abembryonal (Em-Ab) axis and their plane of bilateral symmetry are normally positioned at a right angle to the plane of the first cleavage division (two-cell stage) (Gardner 2001). Attempts are now being made to relate this bilateral symmetry of the blastocyst to, on one hand, the definitive axes of the basic body plan and, on the other hand, to asymmetries of the zygote. Interestingly evidence has been presented that, as in amphibia, sperm penetration adds a second axial formation to the already existing A-V axis of the oocyte (Piotrowska and Zernicka-Goetz 2001; Zernicka-Goetz 2002; Fig. 7). When sperm penetration is missing, i.e. in parthenogenetic egg activation, the development of definitive axial information is disturbed (Piotrowska and Zernicka-Goetz 2002). Davies and Gardner (2002) have questioned, for methodological reasons (lectin labelling possibly being inadequate for marking the sperm entrance point) whether it is indeed the sperm entrance point that determines the position of the plane of the first cleavage and the plane of bilateral symmetry of the embryo; however, they do maintain that the bilateral plane of the blastocyst is aligned with the animal-vegetal (A-V) axis of the zygote, and that its specification may depend on the intrinsic polarity of the oocyte or zygote. With an elegant long-lasting cell labelling method for studying cell fate (using the Cre-loxP system), Fujimori et al. (2003) have recently confirmed Gardner's and Zernicka-Goetz' observations concerning the regularity of the positioning of the Em-Ab axis as related to the plane of the first cleavage division. Their observations did not reveal any role of egg architecture for the A-P axis, however, but indicated drastic cell mixing at post-blastocyst stages in the mouse. Indeed, there are still many questions remaining with respect to details of how exactly the radial symmetry of the oocyte around the A-V axis and any bilateral symmetry of the zygote may finally be used in determining the site where an organizer and the primitive streak will form (and thus the basic body plan), but after asking the relevant questions appropriate investigations can now be done. In spite of all the impressive regulative capacity of the early mammalian embryo, similarities of involved mechanisms to what is known from amphibian development seems to be much more pronounced than was thought by many during the second half of the last century. There are also indications that these recent views, developed on the basis of investigations in the mouse, may indeed apply to early human development: most human blastocysts also have oval (bilaterally symmetric) inner cell masses, and such blastocysts, which do show this symmetry, seem to have a better chance for development after transfer in IVF programs (Richter et al. 2001). An asymmetry observed in the human egg cytoplasm (a half-moon-like zone of cytoplasm called the "halo effect") appears to play a role in embryonic development (Stalf et al. 2002) that could indeed be of significance here.

The mammalian equivalent for a Spemann-Mangold organizer appears to be the primitive node (Hensen's node) as in the chick (at least it represents a trunk organizer, while the head organizer function seems to depend also on the primitive endoderm/hypoblast, see below; Beddington and Robertson 1999; Knoetgen et al. 2000). It is not clear at present how exactly the asymmetries of the zygote and of the early blastocyst are finally translated into the localization of the Spemann-Mangold organizer and subsequently of the primitive streak in mammals. As in amphibia (see above), nodal (a protein of the TGFβ family) appears to be involved. In contrast to the amphibian models, a signal exchange between embryonic and extra-embryonic cells seems to play a role in mammals (Beddington and Robertson 1999; Brennan et al. 2001; Gardner and Davies 2002). Interestingly the determination of primordial germ cells also seems to depend on signal exchange between extra-embryonic and embryonic cells (Lawson et al. 1999). An important function in the context of axis determination seems to have a part of the extra-embryonic endoderm, the anterior visceral endoderm (AVE), which itself is in

Fig. 7A–D Novel aspects of axis development in mammalian eggs and embryos. Recent work has dramatically changed the views about early mammalian development prevailing during the last 30 years or so, and it is now suggested that, somewhat similar to amphibia, the cytoplasmic architecture of the zygote provides basic information, partly derived from the oocyte but considerably changed and specified by sperm penetration (compare with Fig. 3). **A** The first axis present, the animal-vegetal axis (A-V), is defined by the position of the polar body (*above*) and has provided a radial symmetry; the first cleavage division (two blastomeres, light and dark grey) is meridional. Which of the possible meridians is selected, however, is determined by the penetration of the sperm (sperm entrance point, *grey disc*). The selected cleavage plane comes to lie close to both the polar body and the sperm entrance



point. The embryonic-abembryonic (Em-Ab) axis of the blastocyst (*right hand illustration*) lies approximately orthogonal to the A-V axis. The polarity of the Em-Ab axis (at which end Em is), however, appears to be determined again by the sperm entry point: That blastomere which inherits the sperm entrance point tends to cleave ahead and to form cells of the embryonic hemisphere. Note that there are interesting parallels to the older hypothesis concerning a proliferative centre and a centre of differentiation present already in the cleavage stages and anticipating the Em-Ab axis (see Fig. 6). The positioning of the A-P axis of the embryo proper (and of the organizer) is not directly dealt with by any of these hypotheses but it appears possible that the necessary asymmetries may likewise be derived from those of the zygote and might be read in analogy to the amphibian system (see Fig. 3), for instance from the angle between the axis set by the sperm entrance point and the A-V axis, transferred to later stages via an asymmetry imprinted on the blastocyst. **B–D** The asymmetry of the mouse blastocyst and its translation into the axial organization of the egg cylinder stage. **B** The Em-Ab axis is orthogonal to the animal-vegetal axis (A-V); the latter lies roughly parallel to the border between embryoblast (inner cell mass) and blastocyst cavity, but not exactly: the inner cell mass is in an oblique position (and its circumference is also not spherical). In some blastocysts the polar body persists up to this stage and then still marks the animal pole. **C** Due to its asymmetric shape, the blastocyst starts implanting in an oblique position, and it is not the uterus which imposes asymmetry on the blastocyst. **D** Post-implantation stage (advanced egg cylinder stage, gastrula): due to the germ layer inversion of the mouse the topographic situation is more complex than in the human; the proximo-distal axis (Prox-Dist) indicated here does not exist in the same way in species without germ layer inversion. Structures approximately above the Ant-Post (anterior-posterior) line are extra-embryonic tissues (e.g. ectoplacental cone = trophoblast). In the embryo proper (below Ant-Post) the cranio-caudal (A-P) axis can now be recognized by the location of the primitive streak at the posterior end (between Dist and Post, stained here due to in situ hybridization of T-gene expression) (**A** from Piotrowska and Zernicka-Goetz 2001, by permission of the authors and of Nature/McMillan Magazines; **B–D** from Tam et al. 2001, by permission of the authors and of Wiley Liss)

active signal exchange with the neighbouring trophoblast ("extra-embryonic ectoderm" in the mouse) (Beddington and Robertson 1999; Bielinska et al. 1999; Brennan et al. 2001; concerning the "anterior marginal crescent" of which the hypoblast part may be an equivalent for the AVE of the mouse in species with a flat embryonic disc, see Viebahn 1999). Possibly this pattern depends on the asymmetry that is already found in the early blastocyst and that, in the mouse, manifests itself in a unilaterally dominating migration of trophoblast from the embryonic towards the abembryonic pole (Gardner and Davies 2002). Presumably extra-embryonic cells like the AVE, in interaction with parts of the trophoblast (that are close to the embryoblast) and with neighbouring parts of the epiblast, are the equivalent for a Nieuwkoop centre and may have a central role in induction of an equivalent for a Spemann-Mangold organizer, in particular its anterior part (head organizer). The molecular biology of the involved signalling processes is currently under active investigation (Bielinska et al. 1999; Jin et al. 2001; Kimura et al. 2001; Kinder et al. 2001; Perea-Gomez et al. 2001; Tam et al. 2001). A fascinating open question, arising from recent research on cloning by nuclear transfer to oocytes and on the determination of germ line cells, is what role epigenetic phenomena (methylation patterns, imprinting) may play in the regulation of the differential gene expression in embryoblast and extra-embryonic cells, and consequently in the discussed pattern formation processes (Boiani et al. 2002; Kato et al. 1999; McLaren and Durcova-Hills 2001).

Conclusion: Regulative capacity of early embryos, individuation and the status of embryonic stem cells

We have seen that the Spemann-Mangold organizer, which is of central importance in regulating individuation, is formed during a series of hierarchically arranged, vectorial events that at many points allow for regulative processes. Axis information is apparently derived from very simple asymmetries, starting with cytoplasmic asymmetries of the oocyte, modified by the penetration of the sperm (in the chick by gravitation and egg rotation). This seems to apply generally also to mammals. In a cascade of developmental processes (segregation, proliferation, morphogenetic movements, induction processes) these simple asymmetries are translated into the formation of ordered patterns. The zygote already has all the information necessary to run this developmental programme. However, axis information is first laid down in the form of a pre-pattern that at first is not rigid but still allows for some modification. The system leaves room for regulative processes on many levels as long as the patterns have not yet been specified in every detail (Zernicka-Goetz 2002; Joubin and Stern 2001). In the blastocyst or embryonic disc stage, the mammalian embryo appears to develop an equivalent for a Nieuwkoop centre (whose localization most probably depends on the simple asymmetries derived from the zygote) through which subsequently an organizer is induced. Similar to the situation in amphibian and chick development, the system still possesses quite a considerable regulative capacity at these stages. This is shown by the "experiment of nature", twinning (see above). Obviously the asymmetries which are normally derived from the zygote via cleavage divisions and via the asymmetry of the embryonic disc can be changed and/or replaced by other asymmetries.

Since ESCs are derived from inner cell mass cells and also appear to largely maintain the properties of embryoblast/epiblast in vitro, we must ask to what degree they may also maintain the potential for early embryonic pattern formation. ESCs show their morphogenetic potential in the teratoma model and in chimeras. This is particularly striking in the "tetraploid complementation" experiment according to Nagy et al. (1993): in this case a whole mouse is finally formed exclusively by ESCs (whereas the complementing tetraploid helper cells end up in extra-embryonic tissues only; see discussion by Denker 2002). As discussed above, there is good reason to believe that embryoblast cells receive important axial information (for a Nieuwkoop centre and organizer) by interaction with extra-embryonic cells (AVE, trophoblast). In contrast to the mouse, primate and human ESCs have the potential to differentiate in vitro abundant cells of these types (trophoblast, extra-embryonic endoderm; for a literature review, see Denker 2002; Xu et al. 2002). Locally differing densities of such extra-embryonic cells and of their signals, which may give important axial information, must be expected to form spontaneously by stochastic processes in vitro, thus potentially replacing what is derived in vivo from the (in this case strictly ordered) asymmetries of the blastocyst.

Lability of axis development in early embryos in vitro and at ectopic sites

Why then do ESCs in culture normally fail to form harmonious embryonic anlagen but rather show (as a rule) a chaotic mixture of differentiating cells, as in a teratoma? Based on what we have discussed above, we must assume that this is due to lack of formation of a single and normally structured Spemann-Mangold organizer. Indeed structures comparable to teratomas are formed from an originally normal embryo if a normally positioned and structured organizer is missing, as convincingly shown by dissociation and reaggregation experiments in amphibia (Nieuwkoop 1992).

Colonies of ESCs forming in vitro must be expected to lack, as a rule, the simple but ordered asymmetries of the embryonic disc of normal embryos that are derived from the asymmetries of the egg and zygote system. As previously discussed, these asymmetries may normally be transferred from the zygote to the blastocyst and finally to the embryonic disc via an intermediate stage where the asymmetry is manifested in extra-embryonic groups of cells (trophoblast cell proliferation and migration; see

Gardner and Davies 2002; AVE, Beddington and Robertson 1999; Viebahn 1999). One should not be surprised if such an ordered pattern (1) does not form and (2) if it ever forms incidentally is not maintained, during in vitro culture of ESCs. It will at least be disturbed each time the cultures are passaged. This involves (more or less complete) dissociation of cells and growth of new groups of cells (colonies) developing new cell–cell interactions. In such a system, stochastic processes must be expected to dominate, and processes of cell proliferation and migration will be largely determined by the physical conditions of the culturing system. On the other hand, new asymmetries will arise here (local differences in the density of cell groups and of the extracellular matrix, influenced by the geometry of the substratum). Indeed such parameters are signals that can be read by early embryonic cells and embryos so that it must be assumed that extrinsic asymmetry fields can easily perturb the (primarily weak) axis formation cues of an early embryo. Even a normal blastocyst of the mouse responds to explantation into a flat in vitro culture by showing considerable disturbance of the normal pattern formation processes, particularly obvious first in the extra-embryonic parts (Wiley and Pedersen 1977). If normal embryos are transplanted to ectopic sites within a mouse they do not develop a normal basic body plan but form a teratoma (as mentioned above) obviously again due to the abnormal environmental conditions. This shows just how vulnerable early morphogenesis is and that there are limits to regulative processes even in originally normal embryos. While this may sound trivial, it appears to be very relevant for our main question, since this is the same type of conditions (in vitro culture; explantation to ectopic sites) under which (1) the derangement of development of a normal embryo to a teratoma occurs and (2) formation of teratomas out of ESCs are observed. This means that the lack of formation of harmonious embryos by ESCs under these conditions can logically not be used as an argument against any possible potential of ESCs to initiate highly ordered morphogenetic processes under other, more appropriate, conditions. Unfortunately this illogical argument (non-totipotency) has often been used in the ethics debate. As we discuss below, the formation of a harmonious embryonic anlage by ESCs should be considered a possibility and can at least never be excluded not to occur as a rare event, in vitro. This must be seen as a serious point of concern. More than that, it does not appear impossible that appropriate culturing conditions can be found under which such processes can be observed more frequently and predictably.

Early embryonic pattern formation processes in stem cell cultures?

As discussed above, the patterning events that result in the formation of a Nieuwkoop centre, a Spemann-Mangold organizer and a basic body plan are based on a peculiar property of the early embryonic system, i.e. the ability to respond to the presence of simple asymmetries (in the distribution of, for example, only a few types of compounds or locally differing cell density) by initiating complex pattern formation cascades, and that this, due to the hierarchic interdependence of the cascades, will continue to reach higher complexity if not disturbed. The computer models mentioned above (Meinhardt 2001) visualize impressively how stochastic processes can, in such systems, lead to the formation of additional centres of asymmetry that can give origin to a doubling/multiplication of such patterns (twinning). Computer models also suggest that such pattern formation processes do not strictly depend on a preformed axial information/asymmetry, but that indeed stochastic inhomogeneities suffice to allow the system to initiate a pattern formation process if positive feedback (autocatalytic) processes are involved. While this potential can lead to twinning in originally normal embryos, it can nevertheless be overridden by environmental disturbances such as probably predominate in the situation of ectopically transplanted embryos (teratoma formation) and the usual in vitro culture systems.

The mechanisms that we have discussed above for twinning in the embryonic disc of the chick can probably be essentially transferred to the human. Can these views also be applied to ESC colonies in vitro, and to what extent can these be compared with the system of epiblast, trophoblast and extra-embryonic endoderm of a mammalian embryo in the embryonic disc stage in vivo? Can local inhomogeneities that stochastically arise in both systems suffice to serve as centres of asymmetry, initiating an ordered formation of a Nieuwkoop centre, an organizer and subsequently a basic body plan? Is the asymmetry that is normally derived from the zygote completely dispensable in an ESC colony in vitro, and what can replace it, if anything? It appears reasonable to tackle such questions by the use of systematic experimentation. Obviously these questions are complex, and so is the system to be analysed, in spite of the original morphological simplicity it presents. Such investigations will probably benefit from making use of concepts of systems biology, since they finally aim at elucidating the difficult phenomenon of "wholeness" or "Ganzheit" (Seidel 1960b, 1969; Gilbert and Sarkar 2000).

Non-human primate ESCs could provide a very attractive model system for studies on these pattern formation processes in primates, where such investigations will probably never be feasible with the use of normal embryos. This has already been suggested by Thomson et al. (1996) and Thomson and Marshall (1998) and such studies have been proposed by those authors on the basis of their findings from the marmoset monkey referred to above. To perform such experiments with human ESCs, however, must be considered to be totally unacceptable for ethical reasons, since the formation of an embryonic anlage would mean that a process of reproductive or research cloning had been initiated. These considerations touch upon a very important point: according to our theoretical arguments (and in agreement

with the observations made with marmoset monkey ESCs) it cannot be excluded that a process of early embryonic morphogenesis in this sense can start spontaneously as a rare event even in standard cultures of human ESCs. Experimental investigations aiming at verifying this point can logically be defended only in case of non-human primate ESCs which are hopefully close enough to the human to yield results that are transferable by extrapolation and that do not suffer from the drawbacks of the mouse model already discussed above. Initial experiments along these lines suggest that the concern expressed is well founded: colonies of rhesus monkey ESCs do express, in vitro, genes that play a central role in the formation and functioning of Spemann-Mangold organizer equivalents (Behr et al. 2003).

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References

Amit M, Carpenter MK, Inokuma MS, Chio C-P, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA (2000) Clonally derived human stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227:271–287

Amit M, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, Itskovitz-Eldor J (2003) Human feeder layers for human embryonic stem cells. Biol Reprod 68:2150–2156

Andrews PW (2002) From teratocarcinomas to embryonic stem cells. Philos Trans R Soc Lond B 357:405–417

Antczak M, Blerkom J van (1997) Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the pre-implantation stage embryo. Mol Hum Reprod 3:1067–1086

Antczak M, Blerkom J van (1999) Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. Hum Reprod 14:429–447

Beddington RSP, Robertson EJ (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development 105:733–737

Beddington RSP, Robertson EJ (1999) Axis development and early asymmetry in mammals. Cell 96:195–209

Behr R, Thie M, Bruckmann E, Kromberg I, Denker H-W (2003) Rhesus embryonic stem cell colonies express marker genes for early embryonic patterning. Verh Anat Ges 98. Anat Anz/Ann Anat 185 (Suppl.):224

Bielinska M, Narita N, Wilson DB (1999) Distinct roles for visceral endoderm during embryonic mouse development. Int J Dev Biol 43:183–205

Boettger T, Knoetgen H, Wittler L, Kessel M (2001) The avian organizer. Int J Dev Biol 45:281–287

Boiani M, Eckardt S, Schöler HR, McLaughlin KJ (2002) Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 16:1209–1219

Brennan J, Lu CC, Norris DP, Rodriguez TA, Beddington RSP, Robertson EJ (2001) Nodal signalling in the epiblast patterns the early mouse embryo. Nature 411:965–969

Burdsal CA, Damsky CH, Pedersen RA (1993) The role of E-cadherin and integrins in mesoderm differentiation and migration at the mammalian primitive streak. Development 118:829–844

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113:643–655

Ciemerych MA, Mesnard D, Zernicka-Goetz M (2000) Animal and vegetal poles of the mouse egg predict the polarity of the embryonic axis, yet are nonessential for development. Development 127:3467–3474

Cole RJ, Edwards RG, Paul J (1966) Cytodifferentiation and embryogenesis in cell colonies and tissue cultures derived from ova and blastocysts of the rabbit. Dev Biol 13:385–407

Costa ALE da, Abdelmassih S, Oliveira FG de, Abdelmassih V, Abdelmassih R, Nagy ZP, Balmaceda JP (2001) Monozygotic twins and transfer at the blastocyst stage after ICSI. Hum Reprod 16:333–336

Damjanov I, Solter D (1974) Experimental teratoma. In: Grundmann E, Kirsten WH (eds) Current topics in pathology (Ergebnisse der Pathologie) vol 59. Springer, Berlin Heidelberg New York, pp 69–130

Davies TJ, Gardner RL (2002) The plane of first cleavage is not related to the distribution of sperm components in the mouse. Hum Reprod 17:2368–2379

Denker H-W (1970) Topochemie hochmolekularer Kohlenhydratsubstanzen in Frühentwicklung und Implantation des Kaninchens. I. Allgemeine Lokalisierung und Charakterisierung hochmolekularer Kohlenhydratsubstanzen in frühen Embryonalstadien. II. Beiträge zu entwicklungsphysiologischen Fragestellungen. Zool Jb Physiol 75:141–308

Denker H-W (1976) Formation of the blastocyst: determination of trophoblast and embryonic knot. In: Gropp A, Benirschke K (eds) Developmental biology and pathology. [Current topics in pathology (Ergebnisse der Pathologie), Grundmann E, Kirsten WH (Ser.-eds) vol 62] Springer, Berlin Heidelberg New York, pp 59–79

Denker H-W (1983) Cell lineage, determination and differentiation in earliest developmental stages in mammals. In: Hilscher W (ed) Problems of the Keimbahn: new work on mammalian germ cell lineage. (Bibliotheca anat 24) Karger, Basel, pp 22–58

Denker H-W (1997) Nature and status of the human embryo and its sanctity of life: biomedical points of view. Third German-Israeli symposium on human dignity and sanctity of life in biomedical ethics, Ramat-Gan (Israel) 20–21 May, 1997 (cited in: Institut für Wissenschaft und Ethik, Bonn: IWE-Informationsbrief 1/1999, pp 7–9)

Denker H-W (1999) Embryonic stem cells: an exciting field for basic research and tissue engineering, but also an ethical dilemma? Cells Tissues Organs 165:246–249

Denker H-W (2000) Embryonale Stammzellen und ihre ethische Wertigkeit: Aspekte des Totipotenz-Problems. In: Honnefelder L, Streffer C (eds) Jahrbuch für Wissenschaft und Ethik, vol 5. de Gruyter, Berlin, pp 291–304

Denker H-W (2002) Forschung an embryonalen Stammzellen: eine Diskussion der Begriffe Totipotenz und Pluripotenz. In: Oduncu F, Schroth U, Vossenkuhl W (eds) Stammzellenforschung und therapeutisches Klonen. Vandenhoeck and Ruprecht, Göttingen, pp 19–35

Denker H-W (2003) Embryonale Stammzellen als entwicklungsbiologisches Modell: Frühembryonale Musterbildung und Totipotenz. In: Rager G, Holderegger A (eds) Die Frühphase der Entwicklung des Menschen: Embryologische und ethische Aspekte. (Reihe: Herausforderung und Besinnung, vol 19) Fribourg University Press, Switzerland, pp 23–71

De Robertis EM, Aréchaga J (eds) (2001) The Spemann-Mangold organizer: 75 years on. Int J Dev Biol 45:1–378

De Robertis EM, Wessely O, Oelschläger M, Brizuela B, Pera E, Larrain J, Abreu J, Bachiller D. (2001) Molecular mechanisms of cell–cell signalling by the Spemann-Mangold organizer. Int J Dev Biol 45:189–197

De Smedt V, Szöllösi D, Kloc M (2000) The Balbiani body: asymmetry in the mammalian oocyte. Genesis 26:208–212

Enders AC (2002a) Implantation in the nine-banded armadillo: how does a single blastocyst form four embryos? Placenta 23:71–85

Enders AC (2002b) Formation of monozygotic twins: when does it occur? Placenta 23:236–238

Etkin LD (1997) A new face for the endoplasmic reticulum: RNA localization. Science 276:1092–1093

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

Fujimori T, Kurotaki Y, Miyazaki J, Nabeshima Y (2003) Analysis of cell lineage in two- and four-cell mouse embryos. Development 130:5113–5122

Gardner RL (2001) Specification of embryonic axes begins before cleavage in normal mouse development. Development 128:839–847

Gardner RL, Brook FA (1997) Reflections on the biology of embryonic stem cells. Int J Dev Biol 41:235–243

Gardner RL, Davies TJ (2002) Trophectoderm growth and bilateral symmetry of the blastocyst in the mouse. Hum Reprod 17:1839–1845

Gerhart J (2001) Evolution of the organizer and the chordate body plan. Int J Dev Biol 45:133–153

Gilbert SF (1997) Developmental biology, 5th edn. Sinauer, Sunderland, Mass.

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

Gilbert SF (2001) Continuity and change: paradigm shifts in neural induction. Int J Dev Biol 45:155–164

Gilbert SF, Sarkar S (2000) Embracing complexity: organicism for the 21st century. Dev Dynam 219:1–9

Grabel L, Becker S, Lock L, Maye P, Zanders T (1998) Using EC and ES cell culture to study early development: recent observations on Indian hedgehog and BMPs. Int J Dev Biol 42:917–925

Hay ED (1995) An overview of epithelio-mesenchymal transformation. Acta Anat 154:8–20

Hemberger M, Nozaki T, Winterhager E, Yamamoto H, Nakagama H, Kamada N, Suzuki H, Ohta T, Ohki M, Masutani M, Cross JC (2003) Parp1-deficiency induces differentiation of ES cells into trophoblast derivatives. Dev Biol 257:371–381

Hrabé de Angelis M, Kirchner C (1993) Fibroblast growth factor induces primitive streak formation in rabbit pre-implantation embryos in vitro. Anat Embryol 187:269–273

Hrabé de Angelis M, Gründker C, Herrmann BG, Kispert A, Kirchner C (1995) Promotion of gastrulation by maternal growth factor in cultured rabbit blastocysts. Cell Tissue Res 282:147–154

Hsu Y-C (1979) In vitro development of individually cultured whole mouse embryos from blastocyst to early somite stage. Dev Biol 68:453–461

Hsu Y-C (1980) Embryo growth and differentiation factors in embryonic sera of mammals. Dev Biol 76:465–474

Hsu Y-C, Gonda MA (1980) Monozygotic twin formation in mouse embryos in vitro. Science 209:605–606

Hsu Y-C, Baskar J, Stevens LC, Rash JE (1974) Development in vitro of mouse embryos from the two-cell egg stage to the early somite stage. J Embryol Exp Morphol 31:235–245

Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF III, Boiani M, Schöler HR (2003) Derivation of oocytes from mouse embryonic stem cells. Science 300:1251–1256

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N (2000) Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol Med 6:88–95

Jin O, Harpal K, Ang S-L, Rossant J (2001) Otx2 and HNF3β genetically interact in anterior patterning. Int J Dev Biol 45:357–365

Joubin K, Stern CD (2001) Formation and maintenance of the organizer among the vertebrates. Int J Dev Biol 45:165–175

Kaufman MH, O'Shea KS (1978) Induction of monozygotic twinning in the mouse. Nature 276:707–708

Kato Y, Rideout WM III, Hilton K, Barton SC, Tsunoda Y, Surani MA (1999) Developmental potential of mouse primordial germ cells. Development 126:1823–1832

Kimura C, Shen MM, Takeda N, Aizawa S, Matsuo I (2001) Complementary functions of Otx2 and Cripto in initial patterning of mouse epiblast. Dev Biol 235:12–32

Kinder SJ, Tsang TE, Ang SL, Behringer RR, Tam PPL (2001) Defects of the body plan of mutant embryos lacking Lim1, Otx2 or Hnf3β activity. Int J Dev Biol 45:347–355

Knoetgen H, Teichmann U, Wittler L, Viebahn C, Kessel M (2000) Anterior neural induction by nodes from rabbits and mice. Dev Biol 225:370–380

Kudoh T, Dawid IB (2001) Role of the iroquois3 homeobox gene in organizer formation. Proc Natl Acad Sci USA 98:7852–7857

Kummer C (1999a) Biomedizinkonvention und Embryonenforschung. Wieviel Schutz des menschlichen Lebensbeginns ist biologisch "angemessen"? In: Eser A (ed) Biomedizin und Menschenrechte. Die Menschenrechtskonvention des Europarates zur Biomedizin – Dokumentation und Kommentare. Knecht, Frankfurt am Main, pp 59–78

Kummer C (1999b) Was man aus Embryonen machen kann. Über Wert und Verwertung menschlicher Stammzellen. Stimmen der Zeit H 3:172–182

Kummer C (2000) Stammzellkulturen – ein brisantes Entwicklungspotential. Stimmen der Zeit H 8:547–554

Kummer C (2002) Läßt sich ein Zeitpunkt für den Beginn des personalen Menschseins angeben? In: Oduncu F, Schroth U, Vossenkuhl W (eds) Stammzellenforschung und therapeutisches Klonen. Vandenhoeck and Ruprecht, Göttingen, pp 148–162

Lake J-A, Rathjen J, Remiszewski J, Rathjen PD (2000) Reversible programming of pluripotent cell differentiation. J Cell Sci 113:555–566

Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL (1999) Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13:424–436

Leahy A, Xiong JW, Kuhnert F, Stuhlmann H (1999) Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. J Exp Zool 284:67–81

Lu CC, Brennan J, Robertson EJ (2001) From fertilization to gastrulation: axis formation in the mouse embryo. Curr Opin Genet Dev 11:384–392

Maye P, Becker S, Kasameyer E, Byrd N, Grabel L (2000) Indian hedgehog signaling in extra-embryonic endoderm and ectoderm differentiation in ES embryoid bodies. Mech Dev 94:117–132

McLaren A (1982) The embryo. In: Austin CR, Short RV (eds) Reproduction in mammals (2nd edn). vol 2. Embryonic and fetal development. Cambridge University Press, Cambridge, pp 1–25

McLaren A, Durcova-Hills G (2001) Germ cells and pluripotent stem cells in the mouse. Reprod Fertil Dev 13:661–664

Meinhardt H (2001) Organizer and axis formation as a self-organizing process. Int J Dev Biol 45:177–188

Milki AA, Jun SH, Hinckley MD, Behr B, Giudice LC, Westphal LM (2003) Incidence of monozygotic twinning with blastocyst transfer compared to cleavage-stage transfer. Fertil Steril 79:503–506

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642

Murray P, Edgar D (2001) The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF). Differentiation 68:227–234

Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci USA 90:8424–8428

National Bioethics Advisory Commission (1999) Ethical issues in human stem cell research. Vol I: Report and recommendations of the National Bioethics Advisory Commission. Rockville, Md., September 1999 (http://www.bioethics.gov). Executive summary in: Honnefelder L, Streffer C (eds) Jahrbuch für Wissenschaft und Ethik, vol 5. de Gruyter, Berlin, pp 425–437

Nichols J, Chambers I, Taga T, Smith A (2001) Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. Development 128:2333–2339

Nieuwkoop PD (1992) The formation of the mesoderm in urodelean amphibians. VI. The self-organizing capacity of the induced meso-endoderm. Roux Arch Dev Biol 201:18–29

O'Rahilly R, Müller F (1987) Developmental stages in human embryos: including a revision of Streeter's "Horizons" and a survey of the Carnegie Collection. Carnegie Institution of Washington, Publication 637

O'Rahilly R, Müller F (2001) Human embryology and teratology, 3rd edn. Wiley-Liss, New York

Pelton TA, Sharma S, Schulz TC, Rathjen J, Rathjen PD (2002) Transient pluripotent cell populations during primitive ectoderm formation: correlation of in vivo and in vitro pluripotent cell development. J Cell Sci 115:329–339

Pera MF (2001) Scientific considerations relating to the ethics of the use of human embryonic stem cells in research and medicine. Reprod Fertil Dev 13:23–29

Perea-Gomez A, Rhinn M, Ang S-L (2001) Role of the anterior visceral endoderm in restricting posterior signals in the mouse embryo. Int J Dev Biol 45:311–320

Piotrowska K, Zernicka-Goetz M (2001) Role for sperm in spatial patterning of the early mouse embryo. Nature 409:517–521

Piotrowska K, Zernicka-Goetz M (2002) Early patterning of the mouse embryo: contributions of sperm and egg. Development 129:5803–5813

Plusa B, Grabarek JB, Piotrowska K, Glover DM, Zernicka-Goetz M (2002) Site of the previous meiotic division defines cleavage orientation in the mouse embryo. Nature Cell Biology 4:811–815

Prelle K, Vassiliev IM, Vassilieva SG, Wolf E, Wobus AM (1999) Establishment of pluripotent cell lines from vertebrate species: present status and future prospects. Cells Tissues Organs 165:220–236

Rathjen J, Lake JA, Bettess MD, Washington JM, Chapman G, Rathjen PD (1999) Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. J Cell Sci 112:601–612

Reubinoff BE, Pera MF, Fong C-Y, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18:399–404, 559

Richards M, Fong C-Y, Chan W-K, Wong P-C, Bongso A (2002) Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 20:933–936

Richter KS, Harris DC, Daneshmand ST, Shapiro BS (2001) Quantitative grading of a human blastocyst: optimal inner cell mass size and shape. Fertil Steril 76:1157–1167

Robertson EJ (1987) Embryo-derived stem cell lines. In: Robertson EJ (ed) Teratocarcinomas and embryonic stem cells: a practical approach. IRL, Oxford, pp 71–112

Rohwedel J, Guan G, Wobus AM (1999) Induction of cellular differentiation by retinoic acid in vitro. Cells Tissues Organs 165:125–256

Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N (2000) Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci 97:11307–11312

Seidel F (1952) Regulationsbefähigung der embryonalen Säugetierkeimscheibe nach Ausschaltung von Blastemteilen mit einem UV-Strahlenstichapparat. Naturwissenschaften 39:553–554

Seidel F (1954) Das entwicklungsphysiologische Verhalten des Säugerkeimes beim Beginn seiner Uteruswanderung. Verh Dtsch Zool Ges, Tübingen, pp 371–380

Seidel F (1960a) Körpergrundgestalt und Keimstruktur. Eine Erörterung über die Grundlagen der vergleichenden und experimentellen Embryologie und deren Gültigkeit bei phylogenetischen Überlegungen. Zool Anz 164:245–305

Seidel F (1960b) Die Entwicklungsfähigkeiten isolierter Furchungszellen aus dem Ei des Kaninchens *Oryctolagus cuniculus*. Wilhelm Roux Arch Entwicklungsmech Org 152:43–130

Seidel F (1969) Klassische Aspekte der Entwicklungsbiologie. Naturwiss Rundsch 22/4:141–153

Seleiro EAP, Connolly DJ, Cooke J (1996) Early developmental expression and experimental axis determination by the chicken Vg1 gene. Curr Biol 6:1476–1486

Sherman MI, Solter D (eds) (1975) Teratomas and differentiation. Academic Press, New York

Smith AG (2001) Embryonic stem cells. In: Marshall DR, Gardner RL, Gottleib D (eds) Stem cell biology, vol 40, Cold Spring Harbor Laboratory Press, New York, pp 205–230

Smith LJ (1980) Embryonic axis orientation in the mouse and its correlation with blastocyst relationships to the uterus. Part 1. Relationships between 82 hours and 4 1/4 days. J Embryol Exp Morphol 55:257–277

Smith LJ (1985) Embryonic axis orientation in the mouse and its correlation with blastocyst relationships to the uterus. Part II. Relationships from 4 1/4 to 9 1/2 days. J Embryol Exp Morphol 89:15–35

Spemann H (1936) Experimentelle Beiträge zu einer Theorie der Entwicklung. Springer, Berlin Heidelberg New York

Spemann H, Mangold H (1924) Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. Arch Mikrosk Anat Entwicklungsmech 100:599–638

Stalf T, Herrero J, Mehnert C, Manolopoulos K, Lenhard A, Gips H (2002) Influence of polarization effects in ooplasma and pronuclei on embryo quality and implantation in an IVF program. J Assist Reprod Genet 19:355–362

Starck D (1975) Embryologie: ein Lehrbuch auf allgemein biologischer Grundlage. Georg Thieme, Stuttgart

Tam PPL, Gad JM, Kinder SJ, Tsang TE, Behringer RR (2001) Morphogenetic tissue movement and the establishment of body plan during development from blastocyst to gastrula in the mouse. BioEssays 23:508–517

Thomson JA (1998) Characteristics of primate embryonic stem cells. Society for the Study of Reproduction 1998 – 31st Annual Meeting, Texas, USA. Biology of Reproduction Vol. 58, Suppl. 1:25–26

Thomson JA, Marshall VS (1998) Primate embryonic stem cells. Current Topics in Developmental Biology 38:133–165

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP (1995) Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci (USA) 92:7844–7848

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP (1996) Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. Biology of Reproduction 55:254–259

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Viebahn C (1995) Epithelio-mesenchymal transformation during formation of the mesoderm in the mammalian embryo. Acta Anat 154:79–97

Viebahn C (1999) The anterior margin of the mammalian gastrula: comparative and phylogenetic aspects of its role in axis formation and head induction. Curr Top Dev Biol 46:63–103

Viebahn C, Mayer B, Hrabé de Angelis M (1995) Signs of the principal body axes prior to primitive streak formation in the rabbit embryo. Anat Embryol 192:159–169

Waddington CH, Schmidt GA (1933) Induction by heteroplastic grafts of the primitive streak in birds. Wilhelm Roux Arch Entwicklungsmech Org 128:522–563

Watt FM, Hogan BLM (2000) Out of Eden: stem cells and their niches. Science 287:1427–1430

Weber RJ, Pedersen RA, Wianny F, Evans MJ, Zernicka-Goetz M (1999) Polarity of the mouse embryo is anticipated before implantation. Development 126:5591–5598

Wiley LM, Pedersen RA (1977) Morphology of mouse egg cylinder development in vitro: a light and electron microscopic study. J Exp Zool 200:389–402

Wobus AM, Boheler KR (eds) (1999) Embryonic stem cells as a developmental model in vitro. Cells Tissues Organs 165:129–130

Xanthos JB, Kofron M, Tao Q, Schaible K, Wylie C, Heasman J (2002) The roles of three signalling pathways in the formation and function of the Spemann Organizer. Development 129:4027–4043

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001) Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19:971–974

Xu R-H, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA (2002) BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 20:1261–1264

Zernicka-Goetz M (1998) Fertile offspring derived from mammalian eggs lacking either animal or vegetal poles. Development 125:4803–4808

Zernicka-Goetz M (2002) Patterning of the embryo: the first spatial decisions in the life of a mouse. Development 129:815–829

Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA (2001) In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 19:1129 – 1133