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Mini Review

Embryo-derived stem cells -a system is emerging

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In mammals, major progress has recently been made with the dissection of early embryonic cell specification, the isolation of stem cells from early embryos, and the production of embryonic-like stem cells from adult cells. These studies have overcome long-standing species barriers for stem cell isolation, have revealed a deeper than expected similarity of embryo cell types across species, and have led to a better understanding of the lineage identities of embryo-derived stem cells, most notably of mouse and human embryonic stem (ES) cells. Thus, it has now become possible to propose a species-overarching classification of embryo stem cells, which are defined here as pre- to early post-implantation conceptus-derived stem cell types that maintain embryonic lineage identities *in vitro*. The present article gives an overview of these cells and discusses their relationships with each other and the conceptus. Consequently, it is debated whether further embryo stem cell types await isolation, and the study of the earliest extraembryonically committed stem cells is identified as a promising new research field. [BMB reports 2009; 42(2): 72-80]

Potency, self-renewal, and the preimplantation embryo

Developmental potency

Mammalian development is the process which, starting with a single cell (the zygote), forms a multi-cellular organism through the hierarchical specification of cellular identities, proper spatial arrangements of the specified cells, and their functional differentiation (1, 2). The participating cells have different developmental potentials that generally decrease in the process. That is, while the zygote can give rise to a complete conceptus (= "totipotency"), the cells just a few divisions down the line (at the morula and blastocyst stages) are already developmentally restricted (2): The morula cells (blastomeres) first lose the ability to individually give rise to a complete conceptus but remain able to contribute to all its parts (such cells are also often considered totipotent); then the blastomeres and subsequently blastocyst cells become restricted to forming either a few extraembryonic cell types ("oligopotency") or all of the 200 or so cell types that constitute the fetus ("pluripotency"). After implantation of the conceptus into the uterine wall, the pluripotent cells become "multipotent", i.e. restricted to form all or many cell types of any one of the three embryonic germ layers (ectoderm, endoderm, mesoderm), or they become unipotent precursors of the gametes (oocytes, sperm cells).

Stem cells and embryos

In addition to its potency, each cell can be characterized by whether it can produce an identical copy of itself ("self-renewal"). This property is not identical with the ability to proliferate, since proliferation can go along with differentiation ("transit amplifying cells"). Fully differentiated cells are not normally able to proliferate, not to mention self-renew, but certain immature stages can self-renew, at least *in vitro*, thus providing a way to generate unlimited amounts of differentiated and, if desired, genetically manipulated, cells. The combination of a differentiation potential with the ability to self-renew is the hallmark of *stem cells* (3). As a consequence of this definition, only a stage showing active transcription of its identity-defining genes can be a stem cell, i.e. the zygote would not be a stem cell, and morula cells may also not be stem cells. In fact, when insisting on self-renewal as part of the stem cell definition, it is not obvious that normal pre-implantation embryos (i.e., morulae and blastocysts) contain stem cells: Since pre-implantation embryos are constantly changing, it is not clear that the lineage-defining transcriptional profile of any of their transiently existing cell types is identical to that of its progeny. Regardless, however, of whether early embryos *actually* contain stem cells in the strict meaning of the current definition (3), it is becoming increasingly apparent that at least some cells of pre- and peri-implantation embryos can give rise to stem cells that maintain the original lineage identity and developmental potential *in vitro*. In these cases, the ability to self-renew is evidenced most convincingly by maintenance in the form of permanent cell lines *in vitro*, while the potency is evidenced either by re-integration into an embryo similar to the originating one (gold standard, not applicable in humans) or by differentiation in ectopic sites or *in vitro* (second choices). In view of the dynamics of early development and the small number of cells involved, the possibility to "freeze" and expand transient embryonic cell types in the form

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of such immortal cell lines remains remarkable - more astonishing indeed than the existence of so-called "adult stem cells" that maintain tissues throughout postnatal life. In the present article, embryo (or conceptus)-derived stem cells that show a similar developmental potential as their presumed embryonic counterparts, i.e. that appear to preserve an embryonic identity *in vitro*, will be called *embryo stem cells*. Note that "embryo stem cells" are not necessarily identical with "embryonic stem cells" (ES cells), a term that - for historic reasons - refers to only a few types of embryo stem cells (see further below).

Goal of article

The present mini-review was prompted by three lines of progress that occurred mainly during the last three years: (i) in the dissection of early cell specification; (ii) in the isolation of new stem cell types from early embryos; and (iii) in overcoming species barriers for stem cell isolation. The merger of these lines is now leading to a quantum leap in our understanding of the nature and relationships of early conceptus-derived stem cells, most notably of the identity of ES cells. Because of their therapeutic potential, these latter cells are causing enormous interest, which has been boosted further by the recent production of ES cells and ES-like "induced pluripotent stem cells" (iPS cells) from adult cells through artificial reprogramming approaches such as nuclear transfer and the transfection of master regulatory genes (4, 5). This medical prospect is the main, albeit not the only, reason, why the elucidation of the identities and relationships of early embryo-derived stem cells is of practical relevance. In the present article, it will be argued that a number of the known pre-implantation embryo cell types can now be represented by embryo stem cells *in vitro*, and it is discussed whether/which of the remaining pre-implantation embryo cell types have the capacity to generate embryo stem cells as well. In addition, it is argued that the embryo stem cells from human and rodent embryos are principally similar and that more research emphasis should be put on the earliest committed stages of the extraembryonic lineages.

Recent progress in understanding the identities of early embryonic cell types

The existence of embryo stem cells cannot be claimed without knowing the identities of the presumed embryonic equivalents. Here, early development is illustrated in the mouse, the best-studied mammal (2). Cleavage of the zygote gives rise to the morula, which is composed of blastomeres. The blastomeres are initially morphologically uniform and equipotent, but upon compaction (8-cell stage) and continued cleavage they differentiate into a polarized outer layer (the trophectoderm, TE = precursor of the placental trophoblast) and an inner group of cells. At the 32-cell stage, an acentrically positioned cavity becomes conspicuous that displaces the inner cells (now called Inner Cell Mass, or ICM) to the side, thus dividing the TE into a mural zone (facing the cavity) and a polar zone (facing the ICM) and transforming the morula into a blastocyst (Fig. 1). Based on morphology, it used to be thought that the ICM consists of a uniform group of cells that later splits into a mural layer (the primitive endoderm, or PrE) and an inner layer (the epiblast, or EPI). However, we have now learned (6-8) that at least in the mouse, the ICM is heterogeneous already during the morula-to-blastocyst transition, i.e. consists by the ~64-cell stage (mid blastocyst) of two intermingled, morphologically indistinguishable cell populations: the committed EPI precursor (EPI-P) and the committed extraembryonic endoderm precursor (ExEn-P). Next (80-120-cell stage = late blastocyst), the ExEn-P cells move to the blastocoelic surface of

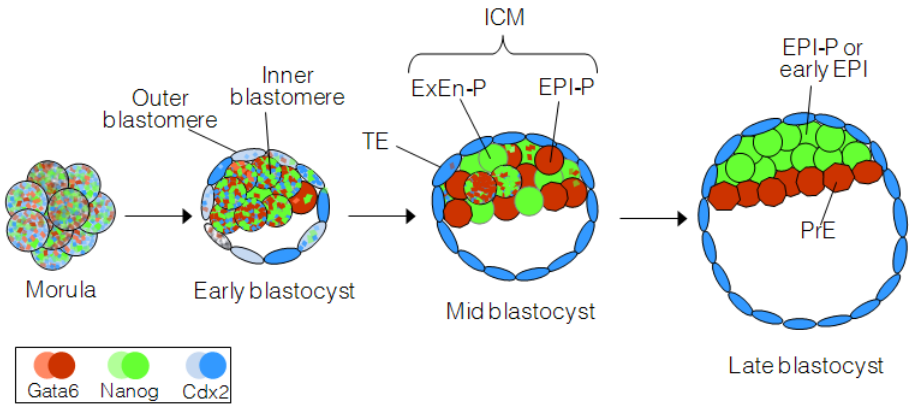

Fig. 1. Cell types of pre-implantation embryos. Colors indicate expression of lineage determinants Cdx2, Gata6, and Nanog. The lineage determinants are not yet segregated at the morula stage, but are initially expressed at low levels stochastically and independently; eventually one of them prevails in a given cell (see ref. 8 for a more detailed presentation). This simplified schematic also does not show that ExEn-P cells start differentiating to PrE already while they are moving to the surface of the ICM (9). However, an analogous difference is not known regarding EPI-P vs. pre-implantation EPI; therefore cellular identity may be the same). Abbreviations: ExEn-P, commited extraembryonic endoderm precursor; EPI-P, committed epiblast precursor; ICM, inner cell mass; PrE, primitive endoderm; TE, trophectoderm.

the ICM; during this sorting process, they commence molecular differentiation towards the PrE (9), which becomes morphologically distinct after sorting. Around implantation, the PrE then gives rise to parietal endoderm (PE) and visceral endoderm (VE) that later constitute the extraembryonic endoderm (ExEn), i.e. the yolk sac parenchyma. In contrast to the ExEn lineage, where sorting ends with a molecularly and morphologically distinct layer (the PrE), the remaining ICM cells (now called EPI) do not seem to be significantly different from the pre-sorting EPI-P cells (10). Therefore, early pre-implantation EPI and EPI-P will not be distinguished here. Sharply around implantation, however, the EPI cells show changes in gene expression that define an intermediate stage (10), and shortly after implantation, changes in gene expression occur again, now along with massive proliferation and epithelization (10). Up to this point, the EPI is pluripotent and expresses a common set of "pluripotency factors", foremost among them Oct4 (10). Eventually, the EPI gives rise to amnion, extraembryonic mesoderm, and the fetus (2).

Embryo stem cells

Rodent ES cells

Mouse ES cells - the first ES cells to be isolated - were described in 1981 (11, 12). These cells not only show pluripotency *in vitro* and in tumor (teratocarcinoma) assays but also can give rise to a complete, fertile mouse (13). However, microinjected ES cells do not efficiently contribute to TE or ExEn *in vivo* (14). By contrast, ES cells easily give rise to ExEn-like cells *in vitro* (15), and in fact ExEn-like cells are the first cell type to be formed in the standard *in vitro* differentiation protocol, which involves the formation of "embryoid bodies" via ES cell aggregation (16). In addition, ES cells can convert into trophoblast cells *in vitro* (17). Interestingly, the proliferative self-renewal of mouse ES cells *in vitro* requires leukemia inhibitory factor (LIF), a cytokine that is not normally needed for early embryonic development (18, 19); this emphasizes that self-renewal may not necessarily be a feature of any *in vivo* ES cell counterpart during normal development. Until very recently, the mouse has been the only species from which true (i. e., pluripotent AND germline competent) ES cells have been obtained. However, after decades of failures, rat ES cells have finally been isolated, and their key features (pluripotency, germline competence, morphology, molecular signature, LIF dependence) are not significantly different from those of mouse ES cells (20, 21). Hence, the association of pluripotency and germline competence in the same cell type is not a peculiarity of one species.

It has been much discussed which resident embryonic cell type, if any, ES cells represent (e.g., 22, 23). Since unlike the ICM, blastocyst-injected ES cells give rise to EPI but not ExEn (14), and also since ES cells can be more easily derived from epiblasts of implanting embryos than from the ICM (24), some scientists tended to see ES cells as EPI-like. Others saw ES cells closer to the ICM before PrE formation, since ES cells can easily produce ExEn-like cells *in vitro* (15, 16) and the ability of the ICM cells to give rise to chimerism drops dramatically around implantation, i.e. when the EPI forms (22). Also, gene expression seems to put the ES cells closer to the pre-EPI stage rather than the EPI (10). Because of this discrepancy, yet other investigators saw ES cells as a unique *in vitro* entity, a viewpoint that appeared to be supported by the fact that LIF signaling is not required within the normal early conceptus (18, 19). However, it was discovered that in a specific physiological situation called diapause, the ICM expands and this does require the LIF co-receptor protein gp130 (25). Diapause might hence be a state containing true stem cells and in fact, ES cells are derived more easily from such embryos (24). However, the key to end the confusion regarding the *in vivo* correlate of mouse ES cells was probably the observation (6-8) that the ICM is not a homogeneous precursor of both EPI and ExEn as traditionally thought but rather a mix of EPI-P and ExEn-P, as explained in the previous section (Fig. 1). If we see ES cells as equivalents of EPI-P (including early pre-implantation EPI as discussed above), rather than of ICM *per se* or of (peri/post-implantation) EPI, it becomes understandable why they express pre-EPI-stage markers and easily contribute to coat color chimeras (like ICM, unlike EPI) but not to ExEn (unlike ICM, like EPI). This concept can be easily reconciled with the more facile isolation of ES cells from peri-implantation EPI than from whole pre-implantation ICM (24), since the peri-implantation stage EPI appears to be able to revert to the ICM (i.e., EPI-P) stage in suitable culture conditions (comp. refs. 10 and 26) yet is free from the differentiating influences of ExEn (27) that are present when isolating ES cells from whole ICMs. Likewise, the observed switch of the outer layer of ES cells to ExEn-like cells during embryoid body formation *in vitro* (15, 16) can be explained by plasticity exhibited in face of environmental change *in vitro*, which is discussed further below. In summary, ES cells seem to correspond to the EPI-P/early pre-implantation EPI (Fig. 2). In light of the foregoing discussion, it would seem appropriate to revise the EPI nomenclature such as to more clearly distinguish early pre-implantation EPI (which may be similar to EPI-P), peri-implantation EPI, and post-implantation EPI.

Human ES cells

Human ES cells were first described in 1998 (28) and have been characterized by *in vitro* differentiation and teratoma assays (chimerism and germline transmission cannot be tested for ethical reasons). These assays revealed a degree of pluripotency comparable with that of mouse ES cells, although the gene expression profile and the growth requirements of human ES cells are different from those of mouse ES cells and notably do not include LIF. The lack of a strict developmental *in vivo* assay for human ES cells prevents a direct developmental comparison with mouse ES cells and may explain why it took years to realize that the differences between these two cell isolates

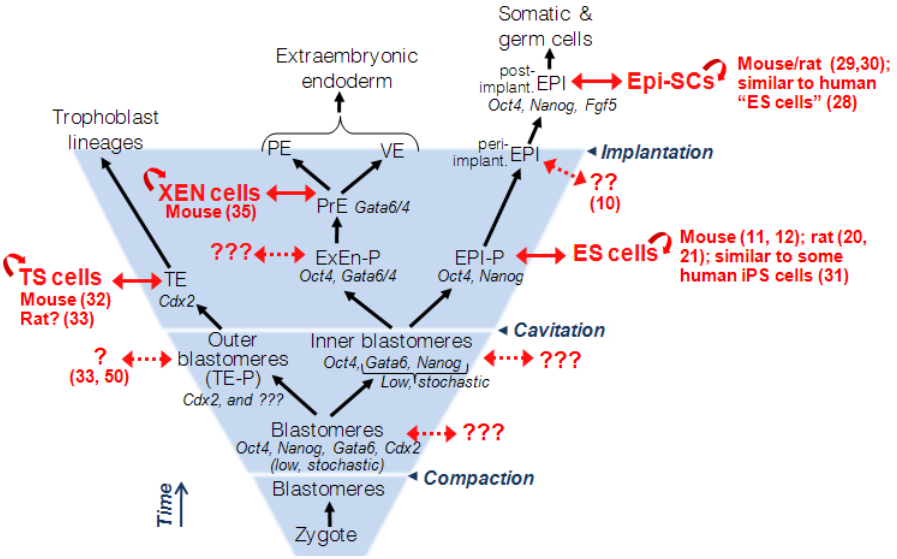

Fig. 2. Putative embryo stem cell lines in rodents and humans. The schematic indicates proposed relationships between conceptus-derived stem cell lines (red) and the conceptus (black). Drawn-out red arrows show well-supported relationships of established cell lines; dotted red arrows with question marks are used where no candidate stem cell lines exist or where evidence is very weak. Self-renewal of stem cells is indicated with curved red arrows. Abbreviations of cells of the conceptus: EPI, epiblast; EPI-P, committed EPI precursor; ExEn, extraembryonic endoderm; ExEn-P, committed ExEn precursor; ICM, inner cell mass; PE, parietal endoderm; PrE, primitive endoderm; TE, trophectoderm; TE-P, committed TE precursor (hypothetical) ; VE, visceral endoderm. Abbreviations of stem cell lines: Epi-SC, epiblast stem cells; ES, embryonic stem; TS, trophoblast stem; XEN, extraembryonic endoderm-like cell line.

are less likely to be species differences rather than a difference in the developmental stage represented by these cells (see next paragraph).

Rodent epiblast stem cells (EpiSCs)

Like the discovery of EPI-P and ExEn-P cells (6, 7), the recent isolation of EpiScs (29, 30) from early post-implantation EPIs has greatly advanced our understanding of the lineage identity of ES cells, in this case human ES cells. EpiSCs, which were isolated both from mouse and rat, show a remarkable similarity of their molecular profile with EPI and human ES cells; also like EPI, they do not efficiently contribute to chimerism after injection into blastocysts (29, 30). The isolation of murine EPI-like stem cell lines (EpiSCs) distinct from mouse ES cells but similar to human ES cells in gene expression, growth requirements, and *in vitro* differentiation potential, appears to have solved the riddle of whether mouse and human ES cells represent the same developmental stage. That is, human ES cells appear to be closer to the post-implantation EPI stage, while mouse ES cells are closer to the EPI-P/pre-implantation EPI stage (as already discussed). Hence, strictly speaking, the term "ES cells" may have to be dropped for either mouse or human ES cells or better, should be dropped altogether since it is not specific enough and ignores that several other stem cell types have meanwhile been isolated from the same embryonic stages. Irrespective of terminology, if human ES cells and mouse ES cells do not represent the same developmental stage, the question arises whether there is a human cell line equivalent to the stage represented by mouse ES cells (i.e. EPI-P); these would then be the "real" human ES cells if one prioritizes the "mouse terminology". It is therefore of great interest that human iPS cells were recently obtained that display striking similarities with, and no obvious difference from, mouse ES cells (31), although a detailed comparison has yet to be made. It will be very interesting to see whether in the same medium, cell lines that are similar to rodent ES cells can also be derived from human blastocysts. Note that since the original human ES cells (28) were isolated from blastocysts using a different medium, this new finding indicates that culture conditions can change the original embryonic cell type (EPI-P) into a related embryonic cell type (post-implantation EPI), i.e. the specific outcome of an embryo stem cell isolation attempt depends on species, embryonic stage, and culture conditions. Perhaps this culture-modulated plasticity explains also why all EPI-like cell lines (i.e., both human ES cells and mouse and rat EpiSCs) can give rise to extraembryonic cell types *in vitro* - something that is not believed to occur with EPI *in vivo*. In any case, the similarity of human ES cells and rodent EpiSCs, as well as the isolation of human iPS cells with mouse ES-like characteristics indicate a strong fundamental similarity of early embryonic cell types among evolutionarily divergent mammals.

Trophoblast stem (TS) cells

Using mouse blastocysts and post-implantation embryos as

starting material, TS cells were first described in 1998 (32), and similar rat cells seem to have been produced in 2003 but were not characterized (33). When injected into blastocysts, these cells contributed to trophoblast tissues but no other tissue (32), and they were able to rescue a placental defect of the host conceptus (34). However, it remains an unanswered question whether the TS cells really represent the *earliest* committed precursor stage for the trophoblast lineage. As will be argued further below, to qualify as the earliest committed precursor, a cell may have to have the ability to revert to the original (non-committed) stage (i.e. to a morula-like stage in this case) by environmental (as opposed to genetic) manipulation. No evidence of such a conversion exists for TS cells.

Extraembryonic endoderm cells (XEN cells)

XEN cells have been isolated many years ago, but were first characterized in 2005 (35). When injected into blastocysts, they give rise to mainly the parietal ExEn (PE), although an isolated case of visceral ExEn (VE) contribution was observed. Hence, the XEN cells do not seem to represent the earliest committed stage of the ExEn lineage. Just like with TS cells, no environmental condition that may return XEN cells to a non-committed stage cells has been found so far.

The relationship of known embryo stem cell lines to their originating cells

From the above, it appears that at least some embryo-derived stem cell lines faithfully maintain the principal identity of their originating cell type or assume the features of a closely related cell type that also exists *in vivo*. Paradoxically, such embryo stem cell lines might embody the original better than the original itself. In the early embryo, a newly committed cell type may still contain products of genes that have been already switched off while already differentiating according to the new commitment. Hence, the brief commitment step may be difficult or impossible to observe in "clean" form *in vivo*. By contrast, embryo stem cells are - by definition of self-renewal (3)- free of both pre-commitment and differentiation products. Thus, cell lines such as ES, TS, and XEN cells may provide an advantage over original embryonic cells not only in terms of amount and accessibility, but also of quality. On the downside, there may be adaptations to the culture environment, but overall, genetically unaltered embryo-derived stem cell lines seem to "gravitate" remarkably well towards *in vivo*-like identities.

Missing embryo stem cell types and embryonic plasticity

Have all possible embryo stem cell types been isolated from the preimplantation embryo? Here it is argued that one or two, or perhaps even more, embryo stem cell types remain to be discovered.

Are there totipotent embryo stem cells?

In the mouse, embryonic gene transcription starts at the two-cell stage, long before the cells arise that are represented by the currently known embryo stem cell types. Is it possible that before the specification of TE or before the specification of the inner morula cells into EPI-P or ExEN-P lineages, an epigenetic state/transcriptional profile becomes established that could sustain itself through self renewal? One could argue that the larger size of the early blastomeres and presence of maternally-encoded products create a unique identity that cannot self-renew based only on embryonic gene transcription. From this perspective, it does not surprise that cell line derivations from morulae have so far ended not with blastomere-like cells, but rather with the same cell types that were isolated from blastocysts, notably ES cells (36). ES cells are of course different from blastomeres since they cannot efficiently contribute to all lineages when injected into or aggregated with morulae (13, 37). On the other hand, however, ES cells clearly maintain a close developmental relationship with the blastomere state since they have the ability to produce all cell lineages (13, 15, 17) - they just cannot do that *in vivo*. Although it is of course impossible that a *single* embryo stem cell could directly act like a zygote, it remains conceivable that blastomere-like embryo stem cells could contribute to all tissues after aggregation with a morula or could form a morula. In fact, even ES cells are able to generate blastocyst-like structures *in vitro*, although this may have occurred through oocyte formation and parthenogenesis rather than de-differentiation (38).

Part of the problem with obtaining totipotent stem cells may be that blastomeres are in an unstable transitional state characterized by stochastic expression and/or unresolved antagonisms of early lineage determinants (8, 39, 40). Morula cells co-express some or all of the transcription factors thought to define different early lineage identities (8, 39, 40; see also Fig. 1). For example, Cdx2 and Oct4 may be engaged in a specific lineage-deciding antagonism; Cdx2 wins over Oct4 in the outer morula cells, which develop into the TE, while Oct4 wins over Cdx2 in the inner cells, which become the ICM. This functional antagonism was proposed based on genetic experiments in which manipulations of Cdx2 and Oct4 altered the fate of morula cells: Inhibition of Cdx2 prevented down-regulation of Oct4 and Nanog and hence formation of a TE layer (40); knockout of Oct4 prevented formation of an ICM and rather lead to trophoblast (41). In turn, the ICM cells fall into two groups, one in which prevails Nanog, which becomes EPI-P, and one in which prevails Gata6, which becomes XEN-P (6-8); a manipulation that prevents upregulation of Gata6 maintains Nanog (6). These functional antagonisms are further supported by experiments in ES cells (see next paragraph). Although these antagonisms are still being debated, and probably are only part of the mechanisms that decide the fate of blastomeres, they illustrate that, should it become possible to derive blastomere-like, totipotent stem cells, the re-

spective culture environment may need to maintain a delicate and very unstable balance of antagonistic lineage-determining mechanisms.

The plasticity of ES cells

In fact, the transcription factor antagonisms just mentioned are maintained in (at least mouse) ES cells, albeit not in the undecided state that seems to be characteristic of earlier blastomeres. Nevertheless, the functionality of the antagonisms is maintained, which actually appears to be the basis of the ability of ES cells to easily cancel their epiblastic commitment. Indeed, both the overexpression of Cdx2 (42) and the reduction of Oct4 (43) switch ES cells towards a TE fate (TS cells or differentiated trophoblast cells, depending on the presence of fibroblast growth factor). Importantly, this lineage switch can also be operated by environmental manipulation (17). Likewise, reduction of Nanog (44) and forced expression of Gata6 or Gata4 (45) can switch ES cells to an ExEn-like fate, which (as already mentioned above) is also achievable by environmental treatment (aggregation to embryoid bodies) (15, 16). Conversely, Nanog suppresses the Gata6 gene by binding to its promoter (46). Taken together, the experiments indicate that although ES cells have already passed the first two lineage decisions (TE vs. ICM, EPI-P vs. ExEn-P), these decisions are only preliminary, i.e. can be easily revised. In other words, ES cells appear to maintain the regulatory capacity typical of a blastomere, the difference being that in ES cells the regulatory balance has been tipped - but the decision is not yet epigenetically fixed. In line with this interpretation, the lineage-determining switches appear to be still fluctuating in cultured ES cells, probably in reflection of similar events in morula and ICM (47). This phenotypic flexibility of ES cells is probably based on their unique epigenetic state characterized by an open chromatin conformation in most loci (4). By contrast, TS and XEN cells appear to be irreversibly committed; no environmental condition is known to reverse their choice (i.e. go back to the morula level), i.e. the teeter-totter is locked. At least in part, this occurs by epigenetic fixation, as indicated, for example, by the observation that the *Oct4* and *Nanog* genes become methylated in TS cells (48, 49).

Putative trophectoderm and XEN precursor cells

Provided that ES cells appear to maintain functionality of the lineage switches characteristic of the morula stage, yet are preliminarily committed towards a somatic fate, the question arises: Are there analogous self-renewable states in which the lineage switches have been set *preliminarily* towards *extraembryonic* fates (i.e., TE or ExEn)? From rat blastocysts, two laboratories isolated cell lines that are different from all known embryo stem cells (33, 50). These cells, which can be isolated easily, rapidly, and reproducibly from different strains (hence represent a relatively stable state), show an ES-like morphology and other ES-like features such as expression of SSEA1 and alkaline phosphatase and LIF-stimulated growth. However, these cells lack the pluripotency markers Oct4 and Nanog and instead express the TE marker Cdx2, and they can give rise to giant trophoblast cells (29, and our unpubl. observations). Importantly, these cells do not seem to represent the rat version of TS cells, which can be isolated separately (33). Interestingly, these "extraembryonic stem cells" appear to be able to also convert into ExEn-like cells (XEN cells) although this was not verified at the clonal level (33). Furthermore, although blastocyst injection experiments failed to show contribution to somatic tissues, these cells appeared to contribute to blood lineages when injected into the livers of adult allogeneic rats (50). Although the exact identity of the "extraembryonic stem cells" remains unclear, the known facts allow the hypothesis that they represent a preliminarily committed TE precursor (TE-P) that can switch fates under suitable environmental circumstances, just like ES cells can. Clearly, these cells deserve further study. Another extraembryonic cell type, also from rat, has recently been isolated in our laboratory (Debeb et al, submitted). These cells show ICM markers (SSEA1, Oct4) along with ExEn commitment (Gata6) and can give rise to differentiated ExEn, just like the ExEn-P. It will be interesting to determine whether the putative TE-P and ExEn-P-like cells maintain the morula-type lineage switch mechanisms in a similar manner as the EPI-P-like ES cells. Given the increasingly strong evidence for principal similarity of rat and mouse embryology, such as isolation of rat ES and EpiScs (see foregoing discussion) and interspecies chimerism (51), it seems likely that these putative TE and ExEN precursors will also be found in the mouse.

FGF/Activin/Bio stem cells (FAB-SCs)

Recently, a new stem cell type was isolated by applying Epi-SC culture conditions to mouse blastocysts (52). These "FAB-SCs" showed an overlapping ES-like and Epi-SC-like gene expression pattern (including Oct4, Nanog, Sox2, Ssea1), but they were not pluripotent. However, by treating them with LIF the FAB-SCs could be converted into pluripotent cells that *de facto* were not distinguishable from ES cells (52); moreover, even a transient treatment with LIF converted them into pluripotent cells (i.e., cells that could form teratomas and contribute to chimeras). Thus, the FAB-SCs may be called near-pluripotent. At this time, it is unclear whether they have an *in vivo* equivalent, but the fact that they were obtained without genetic or other overtly artificial treatments distinguishes them from other near-pluripotent cell lines such as pre-iPS cells (53) that are unlikely to reflect a natural epigenetic state. At this point, it remains generally unclear how many self-renewable states that do NOT have *in vivo* correlates can be obtained through simple environmental manipulations (substrata, growth factors) from early embryos, and how many pluripotent *in vivo* states (10) can be cultured in self-renewable form.

Conclusion and prospects

Thanks to the recent discovery of ICM heterogeneity (6, 7), the recent isolation of EpiSCs (29, 30), and the recent isolation of rat ES cells (20, 21) and human iPS cells that are similar to mouse ES cells (31), a consistent, species-overarching classification of early embryo stem cells is now emerging (Fig. 2). Thus, almost 30 years after the initial isolation of ES cells (11, 12), and 10 years after initial isolation of human ES cells (28), the different phenotypes of the most important *cultured* embryo-derived stem cells finally appear to be falling into *natural* places. It is also becoming apparent that some of these phenotypes are plastic and can be modulated and converted into each other by manipulation of environmental signals and hence signal transduction. The two specific results that deserve to be emphasized in particular are that (i) with the rat, we now have a second species known to deliver cells that fit the strictest ES cell definition (self-renewal, pluripotency, AND germline competence) and (ii) that mouse and human ES cells most likely represent different embryonic stages rather than species variations of the same cell type. The challenge for the future is to fill the remaining gaps in the emerging embryo stem cell classification and to determine the common epigenetic and transcriptional features that appear to distinguish the plastic group of early stem cells from their closest non-plastic, i.e. irreversibly committed relatives. This will require in particular the detailed analysis of the earliest committed stages of the extra-embryonic lineages (ExEn-P and hypothetical TE-P cells), i.e. cell types that have not been studied so far, but also of other peri-pluripotent states; and their comparison with ES cells. Without doubt, such analysis will prove rewarding not only from the academic point of view but also facilitate practical procedures such as the isolation of ES and other embryo stem cells, the directed differentiation of these cells, and the production of embryo stem cell-like cells through reprogramming.

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