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(Article begins on next page)

Developmental bias in cleavage-stage mouse blastomeres

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Summary

Introduction

The cleavage stage mouse embryo is composed of superficially equivalent blastomeres that will generate both the embryonic inner cell mass (ICM) and the supportive trophectoderm (TE). However, it remains unsettled whether the contribution of each blastomere to these two lineages can be accounted for by chance. Addressing the question of blastomere cell fate may be of practical importance, as preimplantation genetic diagnosis (PGD) requires removal of blastomeres from the early human embryo. To determine if blastomere allocation to the two earliest lineages is random, we developed and utilized a recombination-mediated, non-invasive combinatorial fluorescent labeling method for embryonic lineage tracing.

Results

When we induced recombination at cleavage stages, we observed a statistically significant bias in the contribution of the resulting labeled clones to the trophectoderm or the inner cell mass in a subset of embryos. Surprisingly, we did not find a correlation between localization of clones in the embryonic and abembryonic hemispheres of the late blastocyst and their allocation to the TE and ICM, suggesting that TE-ICM bias arises separately from embryonic-abembryonic bias. Rainbow lineage tracing also allowed us to demonstrate that the bias observed in the blastocyst persists into post-implantation stages, and therefore has relevance for subsequent development.

Discussion

The “rainbow” transgenic mouse we describe here has allowed us to detect lineage-dependent bias in early development. It should also enable assessment of the developmental equivalence of mammalian progenitor cells in a variety of tissues.

Highlights

- A mouse allowing rainbow lineage tracing in diverse tissues and developmental stages.
- A fraction of blastocysts exhibited biased clone contribution to the TE/ICM
- TE/ICM bias was uncorrelated with embryonic/abembryonic distribution of clones
- Post-implantation embryos displayed skewed clone contribution to different lineages

Introduction

During the cleavage stages of preimplantation development, the embryo undergoes cell divisions to produce 2, 4, and then 8 seemingly identical cells called blastomeres. Three additional cell divisions later, the embryo will have formed a spherical structure known as the blastocyst. The blastocyst consists of two distinct cell populations: the epithelialized trophectoderm (TE), and the inner cell mass (ICM). The TE comprises the majority of the blastocyst and will become the placenta. The ICM will give rise to the embryo proper and supportive tissues of the primitive endoderm (for review [1-2]).

Groundbreaking early studies using radioactive tracers and dyes to label blastomeres showed that they can contribute to both the TE and ICM [3-4]. A later study utilizing microinjection of a plasmid encoding Cre recombinase into single blastomeres of embryos containing a Cre-dependent lacZ reporter gene found two distinct patterns of contribution to embryonic and extra-embryonic lineages in postimplantation embryos [5]. However, no bias was observed in contribution of blastomere daughters to different regions of blastocysts [5]. All the methods described above could only be used to label one blastomere per embryo, and therefore, the interaction of multiple blastomere daughters within an intact embryo could not be examined [3-4]. Perturbations resulting from invasive labeling procedures could also affect subsequent behavior of blastomere-derived daughter cells [6-7], and prevent findings from being applicable to intact embryos. For instance, an early observation that the earliest dividing 4-cell blastomere contributes disproportionately to the ICM [4] was not confirmed by later experiments using live imaging [8].

Less-invasive markers, such as membrane labels, and intrinsic features of the embryo [9-11], sometimes combined with time-lapse imaging [10, 12], have also been used to assess cell fate. These studies focused on the contribution of daughters of 2-cell stage blastomeres to the embryonic region (Em, containing the ICM and the overlying polar TE) or the abembryonic region (Ab, containing the mural TE) of the blastocyst. The second polar body was initially observed to localize to the Em-Ab boundary of the blastocyst, suggesting that different regions of the zygote have distinct fates [9]. Transplantation of the animal cytoplasm to an ectopic location was observed to alter the orientation of the first cleavage [11]. However, embryos lacking either animal or a vegetal cytoplasm are able to develop to term, suggesting that neither is necessary for development [13]. Experiments utilizing time lapse imaging indicated that bias can be observed only in embryos with an intact zona pellucida (ZP), and disappears when the ZP is removed. Thus, it may be a result of the geometric constraints imposed by the ZP [12, 14]. The location of the sperm entry point has also been proposed to influence Em-Ab orientation of the blastocyst, suggesting that factors intrinsic to distinct locations within the embryo may be involved in bias [6]. All these experiments necessitated pooling results from multiple embryos for statistical analysis, making it possible a subpopulation of embryos displaying significant bias was not detected [6-7, 10-12, 14-16].

More recent work used time-lapse imaging of fluorescently labeled nuclei to track blastomere daughters to the early blastocyst stage and assess their Em-Ab contribution [8, 17-18]. Some observers suggested that certain orientations of cleavage divisions from the 2-cell stage to the 4-cell stage may produce blastomere daughters with bias to contribute to the Em

region [8]. A different experiment analyzing this relationship found that the observed bias could be eliminated by removal of the ZP, and may therefore be a property of embryonic geometry as opposed to intrinsic differences between cells [17].

Subsequent studies focused on detecting intrinsic differences between blastomeres. Higher levels of histone 3 arginine 26 methylation (H3R26m) in 4-cell blastomeres have been proposed to cause increased contribution of daughter cells to the ICM and polar TE [19-20]. Certain orientations of cell division from the 2-cell to the 4-cell stage appeared correlated to differences in H3R26m levels in the resulting blastomeres [19-20]. Fluorescence decay after photoactivation (FDAP) reveled differences between 4-cell blastomeres with regard to accessibility of nuclear DNA for binding by the ICM marker and transcription factor Oct4 [21]. Blastomeres where Oct4 bound to DNA more strongly displayed a tendency to divide asymmetrically, which would result in their daughter cells contributing to the ICM [21-22].

Despite a large number of studies examining the issue of blastomere bias, several questions remain to be addressed. It is still unclear whether the pattern of contribution of blastomere daughters to different regions of the blastocyst can be accounted for by random allocation. Furthermore, the exact nature of the relationship between Em-Ab and TE-ICM contribution still needs to be determined and described. Finally, it is important to ascertain what relevance, if any, the bias observed at preimplantation stages has for the postimplantation embryo. We used a non-invasive, heritable, multi-color lineage tracing approach to address these questions. Our observations are consistent with the proposal that blastomeres arising from

certain cell division patterns may inherit epigenetic factors predisposing them either to an ICM or a TE cell fate.

Results

Construction and characterization of the rainbow mouse

Multi-color lineage tracing was achieved through modification of a combinatorial fluorescent labeling method originally developed for tracing neurons, called brainbow [23]. In brainbow mice, stochastic action of Cre recombinase on sets of target sites results in expression of varying combinations of fluorescent proteins, creating unique color labels for individual cells (Figure S1A). Greater numbers of brainbow loci within the genome result in increased numbers of possible color combinations (Figure S1B).

To achieve multi-color lineage tracing in many organs at various stages of development, we constructed a transgene in which the broadly-expressed CAGGS promoter [24-26] was placed upstream of the brainbow1.0 construct [23](Figure 1A). After validating the multicolor labeling efficacy of our construct in a heterologous expression system (Figure 1B), we used pronuclear injection to generate transgenic mice. We obtained 3 founder animals with strong, ubiquitous red flourescent protein (RFP) expression (Rainbow1-3; Figure 1C and Figure S2).

We observed robust expression of RFP in almost all tissues of adult rainbow2 animals (Figure S2A). Flow cytometry analysis demonstrated that more than 80% of splenocytes and 90% of thymocytes, including B- and T-cells, also expressed RFP (Figure S2B). RFP was also observed in transgenic embryos at E3, E4.5, E6.5, E9.5, E14.5 and in neonates (Figure 1C, 2A, 3B).

To test whether recombination can be induced postimplantation, we crossed rainbow animals to CAGGS:CreER^{TM2} (CreER) mice that allow for temporal control of Cre activity through exposure to 4-hydroxytamoxifen (4-OHT), which is the active metabolite of the drug tamoxifen [27]**.** When pregnant females were treated with tamoxifen at E13.5, and the embryos isolated and sectioned 2 days later, we observed many distinct colors in various tissues (Figure 2A).

To test recombination efficiency in preimplantation embryos, we isolated zygotes from rainbow2 females crossed to CreER males, cultured these embryos to the morula stage, exposed them to 4-OHT, and then quantified the number of colors in the resulting blastocysts (Figure 2B, Figure S6A). Recombination was undetectable in the absence of CreER, and extremely rare without 4-OHT exposure (4/1157 recombined cells in 12 blastocysts) (Figure 3B and 6B, Figure S2 and S6). In striking contrast, embryos treated with 4-OHT were observed to contain cells with multiple distinct color combinations (Figure 2B).

Redundant recombination events leading to multiple cells being labeled with the same color are a concern in these experiments, as the progeny of such cells would be indistinguishable from each other. Increasing the number of possible color combinations would decrease the probability of such events. When we quantified the ratios of CFP, YFP, and RFP in rainbow2 embryos we observed at least 21 distinct color combinations. For the rainbow3 and rainbow1 lines, 10 and 6 distinct color combinations could be observed, respectively. Although the utility of each rainbow mouse line is likely to be different in different contexts, the large number of colors produced by rainbow2 mice made them the best choice for our experiments.

Results of recombination induction at the cleavage stages

We next tested whether the rainbow system could be used to label blastomere daughters. We isolated Rainbow2; CreER zygotes and induced recombination by 4-OHT treatment at the 4-cell stage (48 hours after administration of human chorionic gonadotrophin, abbreviated HCG). Following in vitro culture to the expanded blastocyst stage, confocal image analysis of the resulting blastocysts revealed groups of cells of distinct colors, which appeared to be clones (Figure 3C, Figure S3). When relative ratios of red, blue, and yellow fluorescence in each of these cells were plotted on a 3-dimensional scatterplot, they segregated naturally into distinct groups, confirming the accuracy of color assignments that had been performed by eye (Figure S1 D).

Most clones observed in these embryos comprised 1/4 or 1/8 of the blastocyst (24.8±6.3% of embryo for 24/33 clones; 11.0±2.0% for 7/33 clones; Figure 3C, 4C, Figure S3). Cell divisions in the preimplantation mouse embryo up to the 6^{th} cleavage (when our analysis was performed) have been reported to be relatively synchronized [28-29]. Therefore, these clones likely arose as a result of recombination in 4- and 8-cell blastomeres. We tested this assumption by inducing recombination at the 4-cell stage and arresting the embryos at the 8-cell stage with the DNA synthesis inhibitor aphidicolin, to allow accumulation of fluorescent proteins (Figure 4A, B). When analyzed, 91% of the resulting 8-cell embryos (10/11) were observed to contain 2 cells of the same color, indicating that many temporally stable 4-cell stage recombination events had occurred (Figure 4A, B).

As we had already observed that clone sizes in embryos treated at the morula stage are considerably smaller then in those treated at the 4-cell stage (Figure 2, Figure S2), it seemed likely that the size of the clones observed in the blastocyst was dependent on the timing of 4-OHT treatment. To test this further, we quantified clone sizes in blastocysts that had been pulsed with 4-OHT at the late 4-cell/early 8-cell stage (59 hours post HCG), (Figure 3A, D, Figure 4C, Figure S6B). This later pulse would be expected to label 8-cell and 16-cell stage blastomeres and result in clone sizes intermediate between the morula and the 4-cell treatment. Indeed, 12/31 clones comprised 12±2.5% of the total cells in the embryo, while 11/31 clones comprised 5.8±1.1%. Only 3/31 clones comprised 1/4 (on average 21.1±2.9%) of these blastocyst; Figure 3D and 4C; Figure S6). On average, these clones comprised 9±6% of the blastocyst, as opposed to 21±11% for the 4-cell stage treatment. Thus, the contribution of these uniquely labeled clones to the blastocyst was consistent with most recombination events occurring during the same cleavage division as the 4-OHT pulse (Figure 4C).

As we seemed to be able to label blastomere daughters, we formulated a statistical model designed to test for random allocation of labeled cells to the ICM and TE. If cells have no bias to contribute to either lineage, then each clone would be expected comprise a portion ICM and TE that is proportional to that clone's prevalence within the blastocyst. Under this model, some clones would be expected to contribute disproportionately to the TE simply by chance, as the number of cells in the ICM is much smaller than the number of cells in the TE. In contrast, significant and reproducible deviation from proportional contribution to the TE and

ICM would indicate that daughters of different blastomeres are non-randomly allocated to these two lineages.

The null hypothesis in our analysis was that the probability that any given cell will contribute to the ICM is independent of its color. To test this null hypothesis, we used Fisher's test, which is suitable for analysis of small samples. Unrecombined (red) cells were excluded from our analysis, as we could not readily determine their provenance. Importantly, we were able to assign cells to the TE and ICM with 99% accuracy (456/461 cells of 6 embryos, Figure S6).

We first used this statistical model to test the hypothesis that blastomere daughters are randomly allocated to the TE and ICM in embryos treated with 4-OHT at the 8-cell stage. We observed a statistically significant bias in 1 of 5 embryos examined (n=31 clones from 5 embryos; $p<0.05$ by Fisher's test using independence of color to ICM contribution as the null hypothesis; Figure 3D, Figure S6 and Table S1).

As our analysis included several embryos, each of which represented a separate statistical test, we would expect to observe some bias simply by chance, as a result of testing multiple samples. Therefore, we performed a Monte-Carlo simulation to determine the likelihood that our results were obtained entirely by chance and to compute an aggregate p-value for the data set. The aggregate p-value as computed by Monte-Carlo simulation for embryos treated with 4-OHT at the 8-cell stage was 1.22×10^{-3}. Thus, it is unlikely that bias we observed in this data set could be explained by chance, allowing us to reject the null hypothesis of independence between color and ICM contribution.

It is important to note that embryos containing dead blastomeres were excluded from our analysis (Figure S4C), and that the relative proportion of ICM to TE cells remained unchanged, even after prolonged 4-OHT treatment (Figure S4A-B). Thus, our observation of bias could not be explained by either selective death of blastomeres or biological effects of the 4-OHT pulse.

We next performed a similar analysis on embryos that had been treated with 4-OHT at the 4-cell stage. In this data set, we observed a significant deviation from random allocation in 30% of the embryos assayed (n=62 clones from 21 embryos; Figure 3C, Figure S3 and Table S2;). The aggregate p-value for this data set was 3×10^{-9}, indicating that our results were highly unlikely to be accounted for by chance. The increased proportion of biased embryos observed after 4-cell 4-OHT treatment, as opposed to the 8-cell 4-OHT treatment, is most likely a result of our enhanced ability to detect bias in the 4-cell treated data set. The earlier 4-OHT treatment produced larger clones, resulting in greater statistical power for our analysis in this data set.

Importantly, embryos with detectable bias were comparable to unbiased embryos with regard to total number of cells (biased: 73±15, unbiased: 72±28), and the percentage of total cells that contributed to the ICM (biased:12%±4%, unbiased: 16%±3%). As we observed a large number of colors that occurred with approximately equal frequency (Tables S3 and 4, Figure S1) it is likely that most recombination events that we analyzed were unique. Our results suggest that many 4-cell embryos contain developmentally non-equivalent blastomeres, but previous studies indicate that they are unlikely to be lineage restricted[30], unlike progenitors in other eukaryotes, such as Drosophila imaginal disks[31].

Relationship between spatial localization of clones and their TE-ICM contribution

It has recently been proposed that differences between 4-cell blastomeres can result in distinct propensities to contribute to either Em, or Ab hemispheres of the blastocyst [7, 12, 15, 17, 28, 32-36]. While the ICM is by definition always in the Em hemisphere, Em-Ab localization is a description of spatial allocation, as opposed to cell fate (Figure 5B). Thus, analyzing the relationship between ICM-TE and Em-Ab localization of blastomere descendants could indicate whether the orientation of the blastocoel cavity is dependent on the localization of clones with bias to contribute to the ICM (Figure 5C, Figure S5C-F). If this were the case, we would expect to see a strong correlation between clone contribution to the ICM and the Em region of the blastocyst. In contrast, if ICM-TE bias is not a result of Em-Ab bias, we would expect that Em contribution and ICM contribution of labeled cells to be uncorrelated (Figure 5C). When we analyzed the relationship between Em-Ab and TE-ICM contribution in blastocyst treated with 4-OHT at the 4-cell stage, the resulting correlations were either small or undetectable (Figure 5D-G, Figure S5C-F). Our results are consistent with a model where the orientation of the clone(s) biased to contribute to the ICM does not impact the orientation of the blastocoel cavity, at least at the expanded blastocyst stage (Figure 5C). It is therefore possible that Em contribution is independent from ICM contribution starting from the time that the embryo initiates cavitation. Alternately, morphological changes occurring during blastocyst development could weaken the relationship between Em and ICM contribution at later stages.

In the three-dimensional reconstructions of rainbow embryos treated with 4-OHT at the 4-cell stage, more mixing among cells of different colors was observed in the ICM than the TE. To determine whether adhesive or migratory properties of cells descending from different

blastomeres account for differences in their contribution to the ICM, we attempted to detect a correlation between the amount of cell mixing and ICM contribution. No correlation was observed (R= -0.07), suggesting that differential clone adhesion and migration are not the major mechanism by which non-random lineage allocation occurs (Figure S5B).

Maintenance of blastomere bias post-implantation

To analyze multiple clones post-implantation, we transferred blastocysts that had been pulsed with 4-OHT at the 4-cell stage into pseudopregnant recipient females. We re-isolated and imaged these embryos at E7.5. Three of four embryos contained 4 or fewer large clones of distinct colors, indicating that unique labeling of 4-cell blastomeres had likely occurred. A considerably smaller fifth clone in one embryo appeared to have arisen from a later recombination event (Figure 6B).

To test for bias in blastomere contribution to postimplantation embryos, we quantified the number of cells of each color in the embryonic domain (primarily derived from the ICM) and the abembryonic domain (primarily derived from the TE) of each embryo (Figure 6A). We observed clones that were highly skewed in their contribution to either the embryonic or abembryonic domains in 3 of 4 embryos (embryo I.1 blue clone, embryo I.2 green and blue clones, and embryo I.3 purple and green clones, Figure 6B). Other clones in these embryos did not appear biased (embryo I.1 purple clone, embryo I.2 purple clone, Figure 6B).

When 4-OHT treatment was performed at the late 8-cell stage, and these embryos were isolated and analyzed at E7.5, we also observed increased skewing in lineage contribution,

including a clone that was specific to the epiblast (Figure S6C). Importantly, no recombination was detectable in control embryos that had not been exposed to 4-OHT (Figure 6B). Thus, biased contribution of clones to different lineages is detectable postimplantation.

Discussion

We have developed a multicolor lineage tracing method that can be used to assess the contribution of mammalian progenitor cells to many different tissues (Figure S2). The rainbow method, like all retrospective lineage analyses, suffers from limitations resulting from lack of prospective information, including a limited ability to detect ectopic and redundant recombination events. However, unlike single-color lineage tracing, analysis of multiple clones within the same lineage can be carried out, as multiple recombination events to the same color would be expected to be rare. This property considerably increases the information content of the data set. Systems using 4-color labeling have proven to be useful in tissues with limited cell mixing [37-38]. However, stochastic labeling using 4 colors in our experiments would have resulted in a considerable number of cases where two or more blastomeres are labeled with the same color. For instance, in a system with completely stochastic recombination and 4 possible colors, the probability of obtaining two cells of the same color after 3 independent recombination events is 87%. With 10, 20, or 30 possible colors this probability would decrease to 28%,15%, and 10% respectively. Thus, the nature of our experimental system necessitated the use of combinatorial multi-color labeling. The ability to control the rate of recombination by varying the concentration of 4-OHT, and the relatively synchronized cell divisions during

preimplantation development [28-29] also allowed us to screen out redundant recombination events based on clone size.

Using the rainbow system, we examined the contribution of uniquely labeled clones derived from cleavage stage blastomeres to TE and ICM in individual embryos. We used statistical analysis to show that a subset of blastomere daughters can display significant bias in contribution to the TE and ICM. In a previous study [34], it was observed that 42% of embryos within a population exhibit the division pattern that causes 4-cell blastomeres to acquire lineage bias. A subsequent analysis by the same group showed that certain blastomeres derived from these cell divisions exhibit distinct levels of H3R26me, which in turn can predispose some of them to contribute to the TE and others to the ICM [20]. While the correlation between cell division and TE-ICM contribution observed in these studies was indirect, it is interesting that in our experiments, a comparable 30% of embryos treated with 4-OHT at the 4-cell stage displayed significant bias. It is important to note that cleavage pattern frequencies may be variable depending on mouse strains and other factors, and that there is no reason to expect that the proportion of biased embryos observed would be precisely identical between studies performed by different groups. The proportion of biased embryos observed in our study is therefore consistent with the proposal that certain orientations of cleavage divisions can confer lineage bias onto 4-cell blastomeres [19-20, 33-34]. It has yet to be determined how these variations in cleavage patterns result in epigenetic differences, such as varying levels of H3R26me [20, 35] and differing Oct4 DNA-binding dynamics at the 4-cell stage [21].

One striking finding from our study is the lack of detectable correlation between Em and ICM contribution in clones derived from 4-OHT treatment. The only significant relationship to emerge from our analysis was a weak negative correlation between the contribution of a given clone to the Ab region and its contribution to the ICM. This would be expected, as the Em region by definition contains the ICM (Figure S5). However, if bias to contribute to the ICM were the result of preferential contribution to the Em region, we would also expect a strong positive correlation between ICM contribution and Em contribution. Such a correlation was not observed, suggesting that independent mechanisms may drive blastomere contribution to the Em and ICM at the expanded blastocyst stage. From a mathematical perspective, this lack of correlation is possible because the ICM comprises a small percent of the embryo relative to the contribution of clones derived from 4-OHT treatment at the 4-cell stage.

Our observation that only a small subset of clones contributes exclusively either to the Em or Ab regions of the blastocyst is similar to the results obtained from an earlier live imaging study [17], where it was observed that Em-Ab distribution of the daughters of 2-cell blastomeres was strongly influenced by the orientation of the ZP [17]. This interpretation has been contested[8]. The lack of correlation between Em and ICM contribution in our study suggests that the orientation of the ZP, even if it is a determinant the Em-Ab axis, cannot fully account for all the bias observed in the expanded blastocyst [17]. It may be possible that the analysis of the Em-Ab distribution based on different parameters than those used by us, such as volume of cytoplasm as opposed to nuclear position, may lead to different results. Alternately, the orientation of the ICM within the blastocyst could change as the blastocoel cavity expands,

causing an earlier correlation between ICM and Em contributions to become undetectable at later blastocyst stages, when our analysis was performed. The mechanisms that generate various patterns of TE-ICM and Em-Ab contribution at different stages of development would need to be examined more carefully, in order to fully understand the rearrangements that the blastocyst undergoes during blastocoel expansion.

The genetic labeling inherent in the rainbow system allowed us to confirm a prior observation that 4-cell blastomeres display skewed contribution to embryonic and abembryonic lineages post-implantation [5]. In addition, we were able to demonstrate that individual embryos can contain blastomeres exhibiting biased and unbiased contribution patterns to the TE, ICM and their derivatives. It would be important to elucidate the consequences that this bias, and the epigenetic differences underlying it, could have for later development. For instance, the epigenetic differences between daughters of different blastomeres could also influence their survival in the postimplantation embryo, and skew contribution to various fetal lineages. This could impact the viability and development of the fetus. As studies of blastomere non-equivalence advance into post-implantation development, we have an opportunity to better understand effects of blastomere bias on the resulting organism. This understanding could help improve outcomes for patient undergoing fertility treatments and PGD, as well as the health of children born as a result of these procedures.

Experimental Procedures

The Rainbow construct was generated by ligating the brainbow1.0 construct into the pCAGEN plasmid (Adgene). Mice were generated by pronuclear injection with linearized

Rainbow plasmid into BDF2 embryos at the Harvard Genome Modification facility. To isolate preimplantation embryos, mice were superovulated with HCG and PMS, and mated to males. Embryos were dissected from oviducts in HCZB media, and cultured in KSOM media under mineral oil at 5% CO_2, 37°C. To induce recombination at preimplantation stages, embryos were pulsed for 6 hrs with 0.2 μM 4-OHT. For aphidicolin arrest, embryos were cultured in KSOM supplemented with 0.05 μg/mL aphidicolin. For imaging, embryos were mounted in HCZB supplemented with 20 μM DRAQ5 far-red nuclear dye. Embryos were imaged live wholemount on Olympus FV1000 and Zeiss LSM710 microscopes with 440, 515, 568, and 633 lasers. For cell membrane staining, zona-denuded blastocysts were incubated overnight in Vybrant DiO cell labeling solution (Invitrogen), diluted 1:100 in KSOM at 37°C, 5% CO_2. For postimplantation analysis, E3.5 blastocysts were transferred into uteri of pseudopregnant females 2.5 dpc. Embryos were dissected from the uterus at E7.5, fixed in 4% paraformaldehyde, and imaged. For recombination at E13.5, tamoxifen was administered orally to Rainbow3 females previously mated to CreER males. Embryos were recovered 2 days later, cryosectioned and imaged. Most image analysis was performed using the Imaris 6.0 image processing software (Bitplane). Nuclei were detected by far-red staining, and independently assigned a color and a position within the blastocyst. Image J was used for quantification of relative flourescence intensities. For statistical analysis, each embryo was studied as a contingency table with two outcomes but potentially varied counts of differentiable colors. Since counts sizes did not guarantee all expected clones large enough to support a Pearson's Chi-squared tests, a Fisher's p-value was

calculated using R-function fisher.test() to test against the hypothesis of independence between color and outcome. Pearson's r values were for embryonic-ICM correlation were calculated using Microsoft Excel.

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Figure legends

Figure 1: A mouse for Rainbow lineage tracing.
(A) The Rainbow construct. (B) Rainbow recombination after co-transfection with Cre into HEK cells. (C) dTomato expression at E6.5, E9.5, and in P1 Rainbow2 pups. See also Figure S1.

Figure 2: Expression and recombination of the rainbow transgene. (A) Sagital section through an E14.5 Rainbow3; CreER embryo that had been treated with 4-OHT at E12.5. Scale bar = 1 mm. Top inset: dorsal root ganglia. Middle inset: lung Bottom insert: heart muscle. (B) Wholemount confocal image of a Rainbow2; CreER blastocyst that had been treated with 4-OHT at the morula stage. Scale bar 20 um. See also Figure S2.

Figure 3: Skewed contribution of cleavage stage blastomeres to TE and ICM lineages.
(A) Experimental outline: Embryos were treated with 4-OHT at different stages of development, and contribution of each recombined clone to the ICM and TE was quantified. Images are confocal projections of whole-mount embryos (B) An untreated Rainbow2; CreER blastocyst, where only dTomato expression can be detected. The structure in the lower right corner is cells hatching from the ZP. (C-D) Representative Rainbow2; CreER embryos treated with 4-OHT at the 4-cell (C) and 8-cell (D) stages. Embryos are labeled as biased or unbiased. Scale bars 20 um. P = Fisher probability value for each embryo. Pie charts represent proportional contribution of cells in each clone to the overall embryo, ICM, or TE. The number of cells in

each population is listed on the corresponding slice of the pie chart. See also Figure S3 and Tables S1-S4.

Figure 4: Correlation between the timing of recombination and administration of 4-OHT (a) Representative confocal optical slices through a pharmacologically arrested 8-cell Rainbow2 embryo that had been treated with 4-OHT at the 4-cell stage. Slice thickness 3 um, scale bars 20 um (b) Distribution of clone sizes in embryos experimentally arrested at the 8-cell stage. N=44 clones. (c) Relationship between clone size in the blastocyst and timing of 4-OHT treatment. See also Figures S4 and S6 and Tables S3 and S4.

Figure 5: Comparison between embryonic-abembryonic contribution and TE-ICM bias.

(a) Analysis of contribution of clones to embryonic and abembryonic regions of a representative Rainbow embryo, showing colors, nuclei, designation of differently colored spots, designation of the ICM and the embryonic-abembryonic axis, and contribution of different clones to the embryonic and abembryonic halves of the embryo. (b) an illustration of the difference between TE/ICM and Em-AB designations (c) Model: Biased contribution of blastomeres to the ICM could be uncoupled from hemisphere bias if the blastocoel cavity forms independently of the location of a clone that preferentially contributes to the ICM. The number of cells in each population is listed on the corresponding slice of the pie chart. (d-g) A comparison of ICM versus embryonic contribution in embryos that had been treated with 4-OHT at the 4-cell stage. "Emb" indicates proportion of contribution to the embryonic hemisphere of the blastocyst. See also Figure S5.

Figure 6: Preferential contribution of clones resulting from 4-OHT treatment at the 4-cell stage to different regions of postimplantation embryos

(a) Experimental outline: Embryos were induced to undergo recombination at the 4-cell stage, transferred into pseudopregnant recipients, and re-isolated at E7.5 for analysis. (b) Slices and 3D reconstructions of resulting embryos show skewed contribution of clones to either embryonic or abembryonic lineages. Asterisks indicate location of ectoplacental cone. Scale bars=100 um. The number of cells in each population is listed on the corresponding slice of the pie chart. See also Figure S6.

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