Developmental Biology 355 (2011) 239–249

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Lineage mapping the pre-implantation mouse embryo by two-photon microscopy, new insights into the segregation of cell fates

Katie McDole [a,c,1], Yuan Xiong [b,1], Pablo A. Iglesias [b,*], Yixian Zheng [a,c,**]

[a] Department of Biology, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218, USA
[b] Department of Electrical and Computer Engineering, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218, USA
[c] Department of Embryology, Howard Hughes Medical Institute, Carnegie Institute for Science, 3520 San Martin Dr., Baltimore, MD 21218, USA

ARTICLE INFO

Article history:
Received for publication 27 August 2010
Revised 14 April 2011
Accepted 18 April 2011
Available online 22 April 2011

Keywords:
Two-photon microscopy
Pre-implantation
Asymmetric division
Cell-fate determination
Lineage tracing
Trophectoderm
Inner cell mass

ABSTRACT

The first lineage segregation in the pre-implantation mouse embryo gives rise to cells of the inner cell mass and the trophectoderm. Segregation into these two lineages during the 8-cell to 32-cell stages is accompanied by a significant amount of cell displacement, and as such it has been difficult to accurately track cellular behavior using conventional imaging techniques. Consequently, how cellular behaviors correlate with cell fate choices is still not fully understood. To achieve the high spatial and temporal resolution necessary for tracking individual cell lineages, we utilized two-photon light-scanning microscopy (TPLSM) to visualize and follow every cell in the embryo using fluorescent markers. We found that cells undergoing asymmetric cell fate divisions originate from a unique population of cells that have been previously classified as either outer or inner cells. This imaging technique coupled with a tracking algorithm we developed allows us to show that these cells, which we refer to as intermediate cells, share features of inner cells but exhibit different dynamic behaviors and a tendency to expose their cell surface in the mouse embryo between the fourth and fifth cleavages. We provide an accurate description of the correlation between cell division order and cell fate, and demonstrate that cell cleavage angle is a more accurate indicator of cellular polarity than cell fate. Our studies demonstrate the utility of two-photon imaging in answering questions in the pre-implantation field that have previously been difficult or impossible to address. Our studies provide a framework for the future use of specific markers to track cell fate molecularly and with high accuracy.

Introduction

The mouse pre-implantation embryo has been a useful model in which to study how cell fate determinations are made. After five rounds of division, the 32-cell embryo possesses the first committed cell lineages, the inner cell mass (ICM) and the trophectoderm (TE). The ICM contains apolar cells that further segregate into the epiblast (EPI) and primitive endoderm (PE), while the outer, polar cells form the TE. During and prior to the 8-cell stage all cells have a large amount of exposed surface as well as areas of contact with neighboring cells. It is only during the transition from an 8-cell to a 16-cell embryo that some cells become completely enclosed by other cells (Johnson and McConnell, 2004; Marikawa and Alarcon, 2009; Zernicka-Goetz, 2006). Although these initial inside cells are biased toward the ICM and outside cells toward the TE, their identities are not yet fixed.

Much effort has been devoted to study how cells in the pre-implantation embryo gradually sort into distinct and committed lineages. However, the origin of outer and inner cells found in the 32-cell embryo still remains a subject of debate. Depending on the methods and criteria used, previous studies have found anywhere from 6–8 (Fleming, 1987; Suwinska et al., 2008) to only 1–2 (Dietrich and Hiiragi, 2007) inner cells at the 16-cell stage. Additionally, to what extent the outer cells at the 16-cell stage can give rise to inner cells is unclear. Some studies have reported that asymmetric cell divisions generally occur perpendicular to the embryo surface and give rise to inner and outer cells, whereas symmetric divisions that occur tangential to the embryo surface only result in outer cells (Bischoff et al., 2008; Zernicka-Goetz, 2005). Yet other studies did not find such a correlation (Dard et al., 2009b). Resolving these controversies will rely on live-imaging techniques that allow for the tracking of individual cells in the entire embryo at much higher spatial and temporal resolutions than has previously been achieved.

Imaging the pre-implantation mouse embryo is challenging however, as it is very sensitive to prolonged periods of light exposure. To ensure proper development, 15–30 min time intervals have been used to acquire time-lapse movies in various studies (Dard et al., 2009a; Jedrusik et al., 2008; Morris et al., 2010). Furthermore, given

* Correspondence to: P. Iglesias, Department of Electrical and Computer Engineering, Johns Hopkins University, 3400 N. Charles St., Baltimore MD, 21218. Fax: +1 410 516 5566.
** Correspondence to: Y. Zheng, Department of Embryology, Carnegie Institution, 3520 San Martin Dr., Baltimore, MD 21218, USA. Fax: +1 410 243 6311.
E-mail addresses: pi@jhu.edu (P.A. Iglesias), zheng@ciwemb.edu (Y. Zheng).

0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2011.04.024

the depth (~100 μm) of the mouse embryo and dense cytoplasmic content, conventional confocal microscopy techniques suffer considerably in their ability to obtain a consistent signal throughout the entire depth of the embryo. This poses a significant problem when trying to reconstruct and track individual cells along with their lineages and fates, and do so for every cell in the embryo. Additionally, the sensitivity and dense nature of the pre-implantation embryo make it difficult to track endogenous fluorescent markers that have insufficient brightness, while the use of micro-injected dyes or markers has the potential to compromise developmental integrity.

Here we report the use of two-photon light scanning microscopy (TPLSM) to achieve superior time-resolution, complete depth of penetration, high viability, and non-invasive imaging of the pre-implantation mouse embryo. Coupled with tracking algorithms specifically tailored for the dividing mouse embryo we are able to extract detailed behaviors and create a complete lineage map for every cell in the embryo, providing new insights into how cell position is tied to cell fate, and definitively assign the origins of inner and outer cells.

Results

Two-photon imaging maintains viability and developmental competency

We chose to study the period of development between the 3rd and 6th cleavages as these are the stages in which the embryo segregates into inner and outer cell layers, and the first cell fate decisions are made. To maintain the developmental integrity and viability of embryos in culture while still being able to track individual cells we created a transgenic mouse model expressing Histone-2B-GFP (see Materials and methods). Embryos possessing this transgene begin to express Histone-2B-GFP at a 4-cell stage, allowing us to trace cell lineages from their early origins.

Embryos were collected at 1.5 days post coitum (d.p.c.) at a 2-cell or 4-cell stage and allowed to develop overnight in a tissue-culture incubator before being placed on the stage the next morning after reaching the 8-cell stage. While GFP has a broad two-photon spectrum with several excitation peaks we chose to image with a wavelength of 820 nm, as this provided the highest signal intensity with the least amount of noise on our particular system. Time-lapse movies were taken every 6–7 min for a full z-stack with 2 μm sections (~53 sections total) until the embryos developed to the blastocyst stage. This allowed us to resolve individual nuclei in every cell with great clarity and accuracy (Fig. 1A, Supplementary Movie 1). We observed that time intervals longer than 10 min lead to difficulties in tracking and inaccuracies in calculating the cell division angle as cellular movements during mitosis are too dynamic to capture accurately with long time intervals (data not shown). Embryos averaged a delay of 6–10 h to reach the blastocyst stage compared to those cultured without laser irradiation. Notably, embryos that did not express the H2B-GFP transgene but were imaged alongside their fluorescent litter-mates often reached the blastocyst stage faster and in some cases showed almost no delay compared to non-irradiated culture.

To test the developmental competency of embryos exposed to these imaging conditions, we transplanted imaged embryos into a pseudo-pregnant CD1 wild-type female. Pups carrying the H2B-GFP transgene were born naturally 18 days later and reared to P12 with normal appearance and behavior (Fig. 1B). Additionally, we repeated our results on a second TPLSM system with an identical Ti:Sapphire laser using the same growth conditions. This second system allowed for acquisition of a z-series at more than twice the speed of the first, and under these conditions embryos experienced negligible delay compared to non-irradiated in vitro culture (data not shown). This demonstrates that TPLSM provides superior temporal and spatial resolution as well as high viability for studying pre-implantation development.

Cells giving rise to both outer and ICM cell fates occupy unique positions

We reconstructed time-lapse movies into 3D using IMARIS software (Bitplane, AG), which allowed us to clearly visualize and follow embryo development over the entire course of the time-series. Additionally, by using the Surfaces function coupled with image overlays from the bright-field channel we were able to model an estimated projection of the embryo surface. While this is not a completely accurate or quantitative prediction of the embryo surface it allowed us to visually estimate the position of a cell's nucleus relative to the embryo surface and to construct lineages trees from the 8-cell to 32-cell stage. We defined the outer cells as those whose nuclei are closest to the outer surface of the embryos. Using this criterion, we found that at the 16-cell stage 72.3% of cells clearly localized to the outer layer and would contribute to the extra-embryonic lineages. Of these outer cells 81.9% underwent symmetric cell division to only give rise to TE cells. Interestingly, while the progeny from the remaining 18.1% of 16-cell outer parents initially localized to the outer surface of the embryo one daughter from these parents would suddenly fall inward and re-localize to the inside of the embryo just prior to or during cavitation of the 32-cell embryo (Fig. 2A and Supplemental Movie 2). Typically only 1–2 outer cells in the 32-cell stage embryo experienced this internalization. Since these relocated cells appear at the surface of the ICM facing the blastocoel cavity, they are likely giving rise to the primitive endoderm (PE) lineages. We will refer to these cells as transient-outer cells to distinguish them from the TE cells. However, longer-term imaging beyond the 32-cell stage using genetic markers is needed to define their cell fate.

We defined inner cells as those that have their nuclei clearly surrounded by the nuclei of their neighbors. By this criterion, we found that only 6.3% of cells occupied this position in 16-cell embryos, and that these cells only gave rise to ICM progenies (Fig. 2A). This is consistent with previous reports that found only 1–2 inner cells in the 16-cell embryo (Dietrich and Hiiragi, 2007). We found that the remaining 21.4% of cell nuclei occupied a position between the inner cells and the outer cells as defined above, and that while they were predicted to expose at least some of their cell surface during the 16-cell stage they were located more inward compared to nuclei of the solely outer lineages. Of these cells, 68.2% underwent asymmetric divisions to produce both TE and ICM daughters, whereas the remainder underwent symmetric divisions to give rise to two ICM daughters (Fig. 2A). Importantly, we found that none of these cells produced two symmetric outer daughters. These analyses suggest that in the 16-cell stage embryo cells that give rise to both inner and outer progeny occupy a unique intermediate position between the inner and outer cells.

Development of a cell-tracking algorithm to analyze dynamic nuclear positions in the embryo

The high temporal and spatial resolutions provided by TPLSM allowed us to more quantitatively analyze dynamic cell behaviors from the 8-cell to 32-cell stages using nuclei positions. We reconstructed the time-lapse images into 3D movies using IMARIS software and marked nuclei positions using the Spot Identification Function. Each embryo was then manually checked to ensure proper identification of nuclei for every frame in the movie. Coordinates for each nuclear position at each frame were then exported for tracking. To trace and calculate the position of each individual nucleus within an embryo for each frame, we developed an automated tracking algorithm and convex hull model, which is used to predict the embryo surface and calculate the center of the embryo at each frame (see Materials and methods for details). This allowed us to not only trace cell lineages but to also analyze the dynamics of individual cell positioning based on their nuclear positions. Tracking and