Tracing blastomere fate choices of early embryos in single cell culture

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Blastomeres of early vertebrate embryos undergo numerous fate choices for division, motility, pluripotency maintenance and restriction culminating in various cell lineages. Tracing blastomere fate choices at the single cell level in vitro has not been possible because of the inability to isolate and cultivate early blastomeres as single cells. Here we report the establishment of single cell culture system in the fish medaka, enabling the isolation and cultivation of individual blastomeres from 16- to 64-cell embryos for fate tracing at the single cell level in vitro. Interestingly, these blastomeres immediately upon isolation exhibit motility, lose synchronous divisions and even stop dividing in ≥50% cases, suggesting that the widely accepted nucleocytoplasmic ratio controlling synchronous divisions in entire embryos does not operate on individual blastomeres. We even observed abortive division, endomitosis and cell fusion. Strikingly, ~5% of blastomeres in single cell culture generated extraembryonic yolk syncytial cells, embryonic stem cells and neural crest-derived pigment cells with timings mimicking their appearance in embryos. We revealed the maternal inheritance of key lineage regulators and their differential expression in cleavage embryos. Therefore, medaka blastomeres possess the accessibility for single cell culture, previously unidentified heterogeneity in motility, division, gene expression and intrinsic ability to generate major extraembryonic and embryonic lineages without positioning cues. Our data demonstrate the fidelity and potential of the single cell culture system for tracking blastomere fate decisions under defined conditions in vitro.

Numerous cell fate choices occur throughout the animal life. Early in development of diverse animal species, fertilization between a sperm and an egg leads to the totipotent zygote, which undergoes continuous divisions and lineage restrictions, producing an increasing cell number and creating many different cell types. A mammalian embryo undergoes two cell fate decisions until the blastocyst stage, generating two extraembryonic lineages, the trophectoderm (TE) and primitive endoderm (PE), and an embryonic lineage, the epiblast for the future body[1]. The epiblast is pluripotent and generates three germ layers (ectoderm, mesoderm and endoderm), neural crest (NC) and germline. It is a fundamental challenge in developmental biology when and how different cell fates are precisely determined and regulated. Fate tracing in vivo is powerful for analyzing cell fate choices in developing embryos and adult tissues[2]. Recently, in vitro fate tracing has been developed by using either whole embryos[3] or pooled cell populations[4-6]. In no organism has it so far been possible to culture single blastomeres for extended period to trace cell fate choices. Here we chose medaka (*Oryzias latipes*) to establish a single cell culture system to trace blastomere fate choices in vitro.

Medaka embryos are accessible for blastomere isolation

Medaka is an excellent lower vertebrate model for embryonic development[7], stem cell culture[2,8-13] and has a unique embryology favoring blastomere isolation. In many fish species including zebrafish, early blastomeres undergo meroblastic cleavages atop but not through the yolk[14], which are incomplete cell divisions and thus prevent blastomere isolation. In medaka, we found a seemingly intact yolk

membrane from the 2-cell stage onwards after in situ hybridization with antisense RNA probes against various genes such as *boule* and *dazl*[15], *mitf1* (Supplementary Figure 1a-c) and *vasa* (Supplementary Figure 1g and h), which is easily separable together with the blastoderm from the yolk (Supplementary Figure 1d-f and i). To determine when the yolk membrane establishes its intactness, a fluorescent dye was microinjected into different positions of freshly fertilized eggs or one cell of the 2-cell embryos and the dye distribution was monitored at the 64-cell stage. The dye injected into the yolk near the cytoplasm at the animal pole was transported to the future blastoderm (Figure 1a), whereas the dye injected at the vegetal yolk was not (Figure 1b), and the dye injected into one cell of 2-cell embryos was restricted to daughter cells of the injected blastomere (Figure 1c). Taken together, a prominent yolk membrane is formed and acquires its structural and physiological integrity at the first cleavage, which allows for blastomere isolation in subsequent stages.

Tracing fate choices of blastomere divisions in culture

We chose medaka strain HB32C for cell isolation and culture, a permissive strain for blastula cell culture in gelatin-coated multiwell plates[10]. We first determined the developmental stages for the possibility and efficiency of single cell isolation and cultivation. Isolated cleavage blastomeres were capable of survival and/or proliferation in single cell culture, producing an efficiency of 26%, 49% and 67% for 16-, 32- and 64-cell blastomeres, respectively (Table 1). Thus, blastomeres can reliably be isolated from 16- to 64-cell stages for single cell culture under defined conditions.

We began with the 16-cell stage (Figure 1e) to examine cell divisions as the first fate choice of blastomeres. In many animals, both invertebrates and vertebrates including Xenopus[16] and zebrafish[14], all blastomeres until the midblastula transition undergo 10~12 abbreviated cycles of rapid synchronous cell divisions without G1 and G2 phases and motility[16]. The cause and mechanisms for cell synchrony of abridged divisions have been a mystery since long. In both Xenopus and zebrafish, rapid cleavages have been thought of as being controlled by a cytoplasmic clock, which operates independently of the nucleus but dependent on the nucleocytoplasmic ratio[14,16]. We found that medaka 16-cell blastomeres displayed considerable differences in morphology, with their diameters ranging from 50 μm to 100 μm (Figure 1f and Supplementary Figure 2a). Strikingly, blastomeres immediately upon isolation lost synchronous cell divisions and even stopped dividing, and exhibited motility as evidenced by movements and pseudopodia (Supplementary Figure 2; Supplementary Movie 1). Notably, motility was seen also in actively diving blastomeres (Supplementary Figure 2; Supplementary Movies 1 and 2). Within the first 2 hour of culture, we observed abortive cell divisions (Supplementary Figure 2a; Supplementary Movie 1) and cell fusions between dividing and non-dividing blastomeres (Supplementary Figure 2e-h; Supplementary Movie 2). More importantly, we observed fast dividing, slow dividing and non-dividing blastomeres, with the fast dividing blastomere having completed four cycles of divisions within 2 h (Supplementary Figure 2e-h; Supplementary Movie 2), comparable to rapid cleavages in intact embryos[17]. Taken together, rapid division is maintained in one

or few blastomeres but lost in the remainder. The nucleocytoplasmic ratio controlling rapid synchronous divisions in entire cleavage embryos does not appear to operate on isolated single blastomeres in culture.

We then seeded individual blastomeres in gelatin-coated 96-well plates for single cell culture (Figure 1d) to tracking behaviors for extended periods of time at the single cell level. To observe nuclear behaviors in more detail, we explored zygotic microinjection of RNA encoding a fusion between the histone 2B and green fluorescent protein (H2Bgfp) to visualize nuclei. This led to the identification of three major classes of 16-cell blastomeres (Table 1): 74% belonged to class I, which ceased cell divisions even after completion of one nuclear division (Figure 1g), 6% was class II that underwent endomitosis (Supplementary Figure 3), and 20% fallen into class III, which divided three or more times within 24 h of culture and produced daughter cells of both heterogeneous (Figure 1h) and homogeneous sizes (Figure 1i and j). We also observed incomplete divisions (Supplementary Figure 3a). In particular, certain blastomeres continued rapid divisions and formed a cluster of ~100 cells at 24 h of culture (Figure 1j). Clearly, blastomeres have the heterogeneity in division, suggesting again the presence of novel mechanisms in medaka other than the nucleocytoplasmic ratio.

Tracing fate choices of blastomere lineage restriction in single cell culture

We tested the usefulness of single cell culture for tracing blastomere fate choices in lineage restriction to the yolk syncytial layer (YSL), pluripotent embryonic stem (ES) cells and pigment cells. A medaka embryo at the morula stage has two cell

populations, the envelope layer (EVL) and deep cells. The deep cells are equivalent to the inner cell mass in mouse, which is then separated into the deep cell layer (DCL) and YSL during blastulation[17]. Therefore, a medaka blastula embryo is similar to a mouse blastocyst embryo in having three blastula lineages: EVL, DCL and YSL, which are equivalent to the mouse TE, epiblast and PE, respectively (Fig. 2a). Around 6.5 h post fertilization at the early blastula stage with ~1000 cells, YSL is seen as 4-5 layers of nuclei[17], which became easily visible upon nuclear labeling by H2Bgfp RNA injection (Fig. 2b). The DCL contributes to the future embryo body and is capable of generating diploid and even haploid ES cell cultures[10,11,13]. Pigment cells originate from the NC, a transient population arising from the neural plate in vertebrate embryos, which migrate throughout the body to generate many other cell types[18]. NC is elusive for analyses of fate choices because of a transient and migratory nature. In medaka, pigment cells become visible at day 3 post fertilization[8,11,17], which comprise black-pigmented melanophore and other chromatophores including the ridophore[19], which in medaka is autofluorescent[8,11].

We found that daughter cells of certain 16-cell blastomeres were capable of generating YSL in single cell culture. As early as 4 h of culture, YSL precursors appeared as round and large-sized (~50 μm in diameter) cells, in which the syncytial cytoplasm was located peripherally and the nuclei were positioned centrally (Figure 2c). Subsequently, nuclei moved to the periphery and the cytoplasm moved to the center until 6 h of culture (Figure 2d). They developed into YSL cells until day 1, which had multiple prominent nuclei in the periphery

surrounding the centrally residing cytoplasm (Fig. 2e). The 16-cell blastomere-derived YSL cells were indistinguishable from those from midblastula embryos in culture (Figure 2f). Interestingly, the YSL cytoplasm actively formed pseudopodia (Supplementary Figure 4; Supplementary Movie 3). Moreover, 16-cell blastomeres produced a cluster of actively dividing cells at day 1 post culture (Figure 1j), which eventually developed into compacted cells at day 3 (Figure 2g), phenotypically resembling ES cells[10,13]. At day 3, melanocytes began to appear, which exhibited the characteristics of NC-derived pigment cells, including a flat shape, pigmented granules and dendritic processes (Figure 2g). At day 4 of culture, iridocytes became clearly visible by autofluorescence (Figure 2h). We obtained similar results with 32-cell blastomeres (Supplementary Figure 5) and 64-cell blastomeres (Supplementary Figure 6). Although the frequency of non-dividing blastomeres decreased to ~50% for the 32- and 64-cell stages, the proportion of blastomeres capable of lineage restriction remained 4~6% from 16- to 64-cell stages (Table 1). Collectively, single 16- to 64-cell blastomeres possess the ability to generate extraembryonic YSL cells, ES cells and NC-derived pigment cells.

In single cell culture, YSL cells appeared at day 1 and pigment cells appeared at day 3, reminiscent of the timing of their appearance in developing embryos. Interestingly, only proliferative blastomeres were capable of generating ES cells, YSL and pigment cells; In contrast, non-dividing blastomeres did survive for up to 5 days of culture without showing any sign of differentiation (Supplementary Movie 4). These results point to the fidelity of in vitro lineage

tracing in single cell culture. Convincingly, the ability to generate major blastula lineages and differentiated pigment cells of the NC origin is intrinsic to blastomeres and present already at the 16-cell stage, prior to the separation into the three blastula lineages until the 1000-cell stage and far before the organogenesis stage when pigment cells become visible.

Expression of key lineage regulators

In order to determine the molecular basis of the inherent potential for ES cells, YSL and pigment cells, we analyzed the RNA expression of *nanog, oct4 sox17* and microphthalmia-associated transcription factor (*mitf)*. *nanog* and *oct4* are key pluripotency regulators in mouse[20,21] and exhibit pluripotent expression in medaka[22-24]. Mouse *sox17* is a key regulator of PE development[25]. Fish *mitf* is a master regulator of pigment cells of NC lineage[26]. In mice, only *oct4* is maternally supplied[21], whereas *nanog*[20], *sox17*[25] and *mitf*[27] commence their expression at the 8-cell stage, 32-cell stage and around embryonic day 10, respectively. We found that the transcripts of medaka *oct4* and *nanog* were maternally supplied and expressed in cleavage embryos (Supplementary Figure 7), consistent with their reported expression[22-24] and the ES cell formation from cleavage blastomeres in single cell culture. Surprisingly, the transcripts of *sox17* and *mitf1* (one of the two medaka *mitf* genes due to an ancient whole genome duplication event in the fish lineage) were also maternal and persistent in cleavage embryos (Supplementary Figures 1 and 8). More intriguingly, *sox17* was predominant in central blastomeres of cleavage embryos (Supplementary Figure 8a and b), and became preferentially expressed in YSL cells when they are formed at the early

blastula stage (Supplementary Figure 8c) and marginal blastomeres that produce the embryonic endoderm[28], whereas *mitf1* distributed preferentially in peripheral blastomeres (Supplementary Figure 8d-f). Differences in temporospatial distribution becomes more evident after two-color fluorescent in situ hybridization (Figure 3), reinforcing the heterogeneity of blastomeres at the molecular level. Therefore, medaka 16-cell blastomeres already exhibit differential expression of lineage markers, in contrast to the mouse situation where all 16-cell blastomeres retain the ability to contribute to any of the three blastocyst lineages[29] and show a similar gene expression profile[30]. Collectively, the intrinsic potential of early blastomeres to produce YSL, ES cells and pigment cells accompanies the expression of respective lineage markers.

Discussion

In this study, the establishment of a single blastomere culture system and its exploitation in tracing cell fate choices lead to twelve important findings that are novel or different from previous results obtained in whole embryos. First, we show in medaka that embryonic cells as early as the 16-cell stage can be robustly isolated without losing their viability and developmental potential. Conceptually, meroblastic cleavages in fish are thought to be incomplete due to the lack of an intact yolk membrane and thus prevent isolation of intact and viable blastomeres. Three lines of evidence point to the intactness of yolk membrane in early medaka embryos, leading to our choice for this organism for single blastomere isolation. In situ hybridization reveals that maternal RNAs often do not completely enter the cellular blastodisc but leave a substantial level in the

yolk to form an area beneath the blastodisc, indicating the presence of a barrier between the yolk and blastodisc as early as the 2-cell stage. Furthermore, the yolk membrane together with the blastoderm is easily separable from the yolk. Convincingly, fluorescent dye injected in the vegetal yolk completely remains in the yolk, demonstrating the physiological integrity of the yolk membrane. Therefore, medaka is unique among organisms with meroblastic cleavages in its accessibility for blastomere isolation at the cleavage stages. In this regard, mammalian embryos must be also accessible for blastomere isolation because of complete cleavages. Second, we show that early blastomeres can survive and divide in single cell culture in the absence of any feeder cells. Third, we reveal that early blastomeres manifest motility upon isolation, in contrast to previously described absence of motility in blastomeres until the midblastula stage in many egg-laying species including Xenopus[31] and zebrafish[14]. Notably, only a subset of isolated 16-cell blastomeres exhibit pseudopod formation, while the remainder does not show this measure of motility during whole period of culture. It appears that isolation and cultivation allows for manifestation and easy detection of motility, rather than stimulating or inducing motility. Even after midblastula transition in zebrafish, pseudopodia appear in interphase but do not form during mitosis[14]. In medaka, pseudopodial formation has not been described before the midblastula stage. It is likely that rapid cleavage divisions and positioning in the embryo prevent pseudopodial formation and cell movement. In this study, even dividing blastomeres formed pseudopodia and moved round in culture, suggesting that mitotosis does not prevent cellular motility in vitro. Fourth, we

demonstrate the previously unidentified heterogeneity of blastomeres in morphology, motility, division, gene expression and more importantly, developmental potential in single cell culture. Fifth, our finding that ≥50% of 16~64-cell blastomeres maintain a large size but stop dividing immediately after isolation indicates the presence of unknown mechanisms that regulate synchronous blastomere cleavages. In invertebrates and lower vertebrates including fish, it is widely accepted that all embryonic cells undergo rapid and synchronous divisions until the midblastula stage of ~2000 cells when the midblastula transition begins, and cell synchrony of continuous divisions has widely been thought of as being determined by the nucleocytoplasmic ratio[14]. It remains unknown why this ratio does not operate on isolated blastomeres. It is likely that the synchrony of rapid divisions requires cell-cell communications. Intriguingly, there are certain blastomeres that continue rapid divisions in single cell culture. Possibly, such blastomeres may initiate and orchestrate synchronous cleavage divisions via cell-cell interactions. Sixth, we have also observed abortive division, endomitosis and cell fusion in isolated blastomeres, which have not yet been described in early cleavage embryos of lower vertebrates. All these processes may each lead to higher ploidy levels in certain embryonic cells. We have previously reported the invariant presence of ~20% tetraploid subpopulation in medaka diploid ES cells derived from fertilization midblastula embryos[10] and the presence of ~20% diploid subpopulation in medaka haploid ES cells derived from gynogenetic midblastula embryos[13]. Whether these variations are causative for the appearance of higher ploidy levels in ES cells remains unknown. Seventh,

we show in medaka that the extraembryonic YSL cells can intrinsically be specified from cleavage blastomeres. In mouse, the two earliest events leading to the separation of TE and PE have been found under the multifaceted regulation including cell polarity, position and gene expression[1,29]. Eighth, the single cell culture system revealed the dynamic process of YSL cell formation and the motility of YSL cytoplasm. In zebrafish, some marginal blastomeres are confluent with the yolk cell cytoplasm resulting from incomplete division collapse and deposit their nuclei and cytoplasm into the cytoplasmic cortex of the yolk cell, thereby forming the YSL, whose nuclei then undergo three to five rounds of endomitosis without cytokinesis[14,32]. In medaka, the YSL originates also from marginal blastomeres and its nuclei undergo endomitosis[17], but apparently in the absence of cell fusion with the yolk, because the yolk membrane acquires its integrity already at the 2-cell stage and prevents the direct interaction, as we have shown in this study. The YSL is elusive for study due to difficulties in interfering specifically with YSL formation and morphogenesis[32]. Our observations make medaka model for experimental analyses of YSL formation and its separation from the pluripotent DCL. In addition, YSL formation in single cell culture will provide an excellent system to elucidate the mechanisms underlying endomitosis and its role in cell fate specification. Ninth, we show that certain 16-cell blastomeres have the potential to generate pluripotent ES cells, far before the blastula stage when deep cells are formed. This is different from the situation in mouse, where a pluripotent ground state is established in the inner cell mass and epiblast until the blastocyst stage[33]. Tenth, the fact that medaka

blastomeres even at as early as the 16-cell stage already possess the intrinsic ability to specify pigment cells as the representative NC derivatives suggest the cell-autonomous NC specification in this organism, which is in contrast to the widely accepted notion that NC is induced from the neural tube by BMP and Wnt signalings[19,34]. Eleventh, we show that key lineage regulators are maternally supplied in medaka, in contrast to their zygotic expression in mouse[27]. Finally, our observations that lineage formation in single cell culture is dependent on cell divisions and timings similar to developmental programs of a developing embryo strongly suggest the fidelity of the single cell culture system to recapitulate blastomere fate choices in vivo.

Taken together, we have successfully developed a novel ability to isolate and cultivate individual blastomeres of early medaka embryos and investigated their fate choices. It is evident that the single cell culture system is ideal for fate tracing in vitro under defined culture conditions without an intact embryonic environment. Our success is ascribed to the blastomere accessibility for isolation and well-defined culture conditions[10,13,35]. We believe that this single cell culture system can be developed also in other organisms to trace the behaviors/fates at the single cell level in culture, offering invaluable lights on the mode, process and mechanisms of various cell fate choices.

METHODS

Embryo microinjection and cell culture. Embryos were microinjected with 50 ng of H2Bgfp RNA at the 1-cell stage as described[36]. Embryo manipulation, cell isolation and culture were done essentially as described[8,10-13]. Briefly, embryos

were treated with proteinase K (10 mg/ml) for 60 min at 28°C to remove the attachment filaments, rinsed twice in phosphate-buffered saline (PBS) and sterilized in PBS-0.1% bleach for 2 min, and rinsed 5 times in PBS. Embryos were incubated in PBS and monitored for developmental stages under aseptic conditions. The chorion was manually torn with a pair of fine forceps at the vegetal half. For embryos at 32- and 64-cell stages, cells were dissociated by gentle pipetting. For embryos at the 16-cell stage, cells were individually dissociated by using a fine forceps to prevent damage. Healthy cells were seeded by pipetting into gelatin-coated 96-well plates containing 150 μl of ES cell culture medium ESM2. Within 10 min of seeding, the plates were monitored under an invert microscope to ensure that one cell was present each well. Cell growth, attachment, proliferation and differentiation were monitored at regular intervals of culture at 28°C in air.

Procedures for fish maintenance, gene cloning, RNA synthesis and in situ hybridization were performed as described in Supplementary Methods.

Full methods and associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/.

Supplementary Information is linked to the online version of the paper at http://www.nature.com/naturecellbiology/.

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Author Contributions

M.L., N.H., Z.L., M.Y., R.L., H.Z., Y.Y. and Y.Y performed research; M.L. and N.H. analyzed the data; C.L., J,S. and Y.H. designed research, R.G. and Y.H. wrote the paper.

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20 **Figure legends**

Table 1 | Efficiency and differentiation of single blastomere culture

22 **Figure 1 | Single cell culture.** (a-c) Merged micrographs of live embryos. The physiological integrity of the yolk membrane (ym) is evidenced by the prevented
24 distribution of injected red fluorescent dye from yolk to the cell mass. Vg transgenic embryos were injected with red fluorescent dye at the positions
26 (arrows) and photographed at 32-cell stage. The GFP signal is from maternal expression from the vasa promoter. (a and b) Lateral views following yolk
28 injection 5 min after fertilization near the blastoderm (bd; a) or at the vegetal pole (b). (c) Top view of one-cell injection at the 2-cell stage, showing the restricted
30 dye distribution into descendents of the injected blastomere. ch, chorion; od, oil droplet; yk, yolk. An embryo is 1 mm in diameter. (d) Schematic illustration of
32 single cell isolation and culture. Shown here is a 16-cell embryo for manual dissociation, and cells are individually seeded into 96-well plate, one cell one well.

Cell behaviors are regularly monitored. (e) 16-cell blastoderm. (f) Isolated 16-cell blastomeres, showing differences in size and transparency. (g-j) Heterogeneity of 16-cell blastomeres. Embryos were microinjected with H2Bgfp RNA at the 1-cell stage for labeling nuclei (green). Three major classes of blastomeres are seen. Class I is yolky, actively forms pseudopodia (asterisks) without cell division (g). Class II undergoes endomitosis (see Supplementary Figure 3). Class III exhibits active divisions and produce daughter cells of heterogeneous sizes (h) and a homogeneous size (i and j) often capable of attachment (arrow) until day 1 of culture. Embryos are 1 mm in diameter. Scale bars, 50 μm.

Figure 2 | Lineage tracing in vitro. (a) Medaka blastula lineages and mouse blastocyst lineages. (b) Early medaka blastula following zygotic injection of H2Bgfp RNA, showing YSL nuclei (green). Oil droplets (od) depict the vegetal pole. (c) YSL precursor at 4 h of culture. (d) Developing YSL cells at 6 h of culture. (e) YSL cells at day 1 of culture. Three nuclei (nu) are seen in a large-sized cytoplasmic syncytium. (f) Control YSL cells from midblastula embryos at day 3 of culture. (g) ES cells and black pigmented melanocytes (mc) at day 3. (h) Yellow fluorescent iridocytes (ic). Scale bars, 50 μm.

Figure 3 | RNA Expression of key lineage regulators. After whole mount in situ hybridization with antisense RNA probes, the signals were detected by green (*oct4* and *mitf1*) and red fluorescence (*nanog* and *sox17*), and the blastoderm were separated from the yolk for photography. Nuclei were stained blue with DAPI. (a) *oct4* and *nanog* expression. (b-d) *mit1* and *sox17* expression at the 16~32-cell stage (b), 32-cell stage (c) and 64-cell stage (d). Scale bars, 50 μm.

Table 1 | Efficiency and differentiation of single blastomere culture[1)]

Stage	Blastomere, n (%)		Class, n (%)[3)]			Daughter cells, n(%)[4)]		
	Seeded	Survived[2)]	I	II	III	ES	Pig	YSL
16-cell	194	50 (26±10.7)	37 (74±12.9)	3 (6±3.9)	10 (26±10.9)	3 (6)	3 (6)	3 (6)
32-cell	206	100 (49±14.5)	50 (50±12.3)	4 (4±2.3)	46 (46±12.3)	5 (5)	4 (4)	4 (4)
64-cell	224	150 (67±12.4)	77 (51±16.7)	6 (4±4.8)	67 (45±13.8)	9 (6)	6 (4)	6 (4)

1) HB32 embryos with and without injection with H2Bgfp RNA at the 1-cell stage were dissociated for single cell culture at 16-, 32- and 64-cell stages. Data are presented as means ± s.d. from four independent experiments.

2) Survival rate at 1 day post culture was derived by comparison to the number of cells seeded.

3) Classes I, II and III are non-dividing, endomitotic and dividing blastomeres until 1 day post culture. Percentages are derived by comparisons to the number of cells survived.

4) Blastomeres that produced daughter cells containing embryonic stem (ES) cells, yolk syncytial layer (YSL) cells, and pigment cells (Pig; melanocytes and/or iridocytes) at 1-4 dpc. Percentages were derived by comparisons to the number of cells survived.

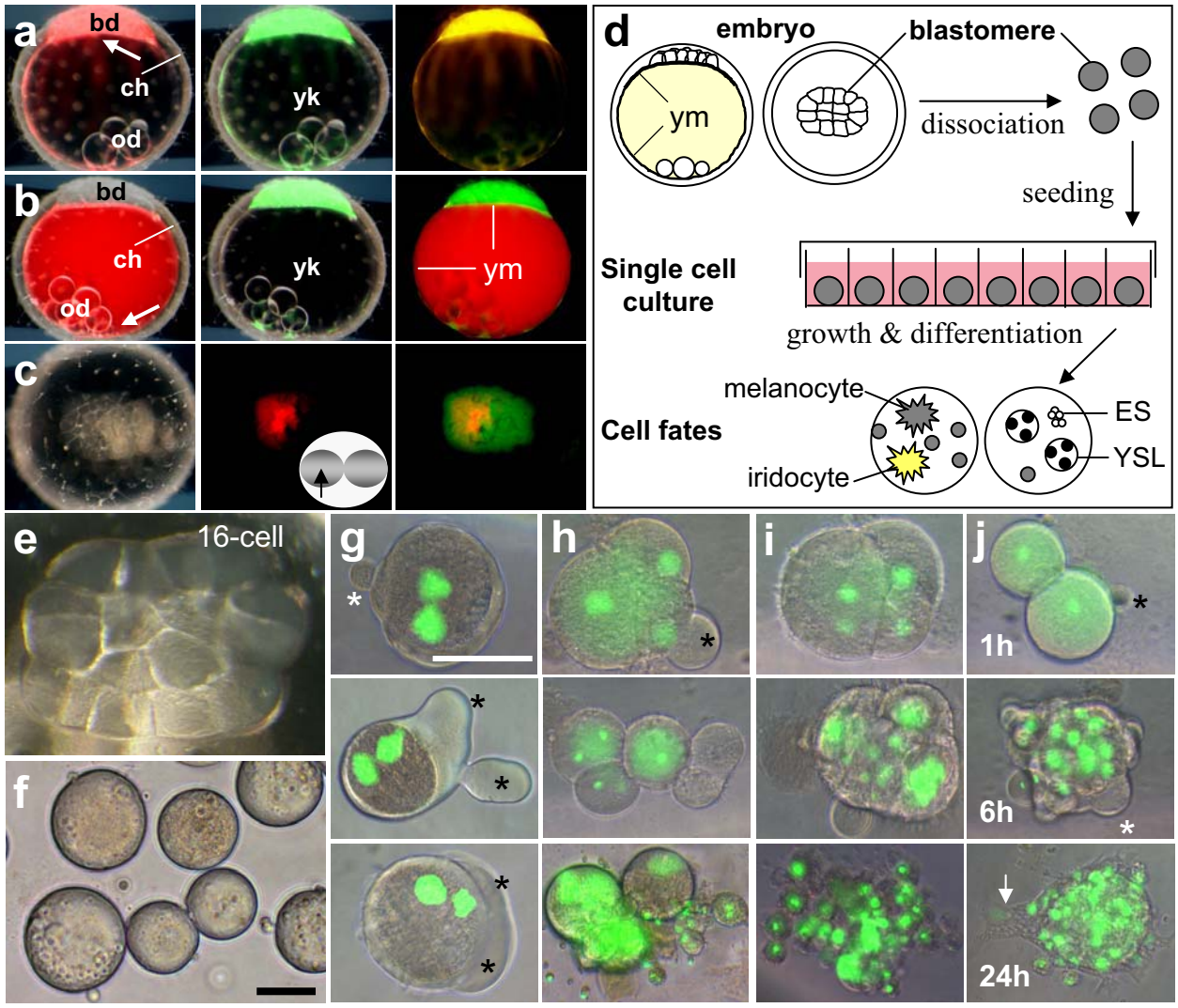

Figure 1 | Single cell culture.

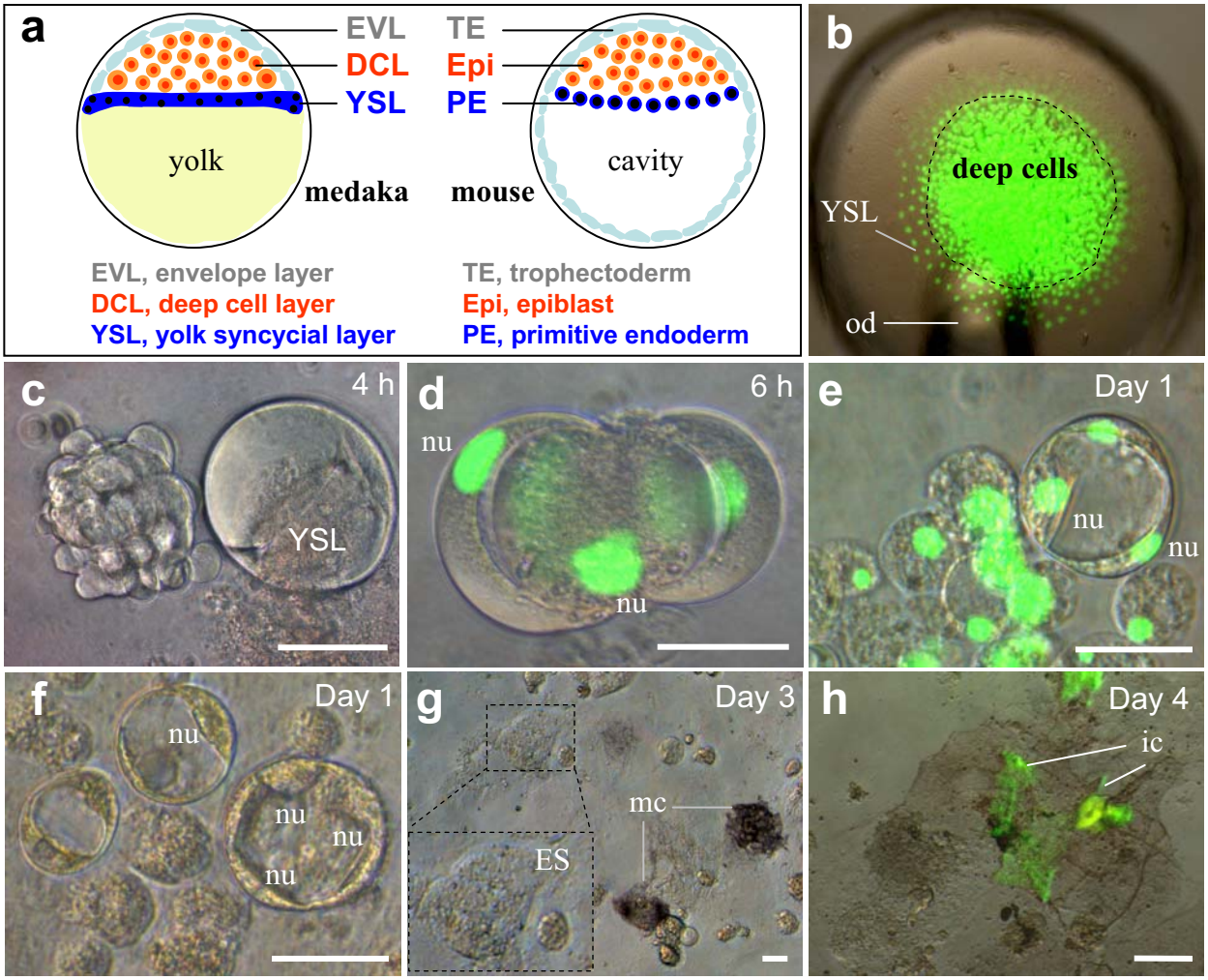

Figure 2 | Lineage tracing in vitro.

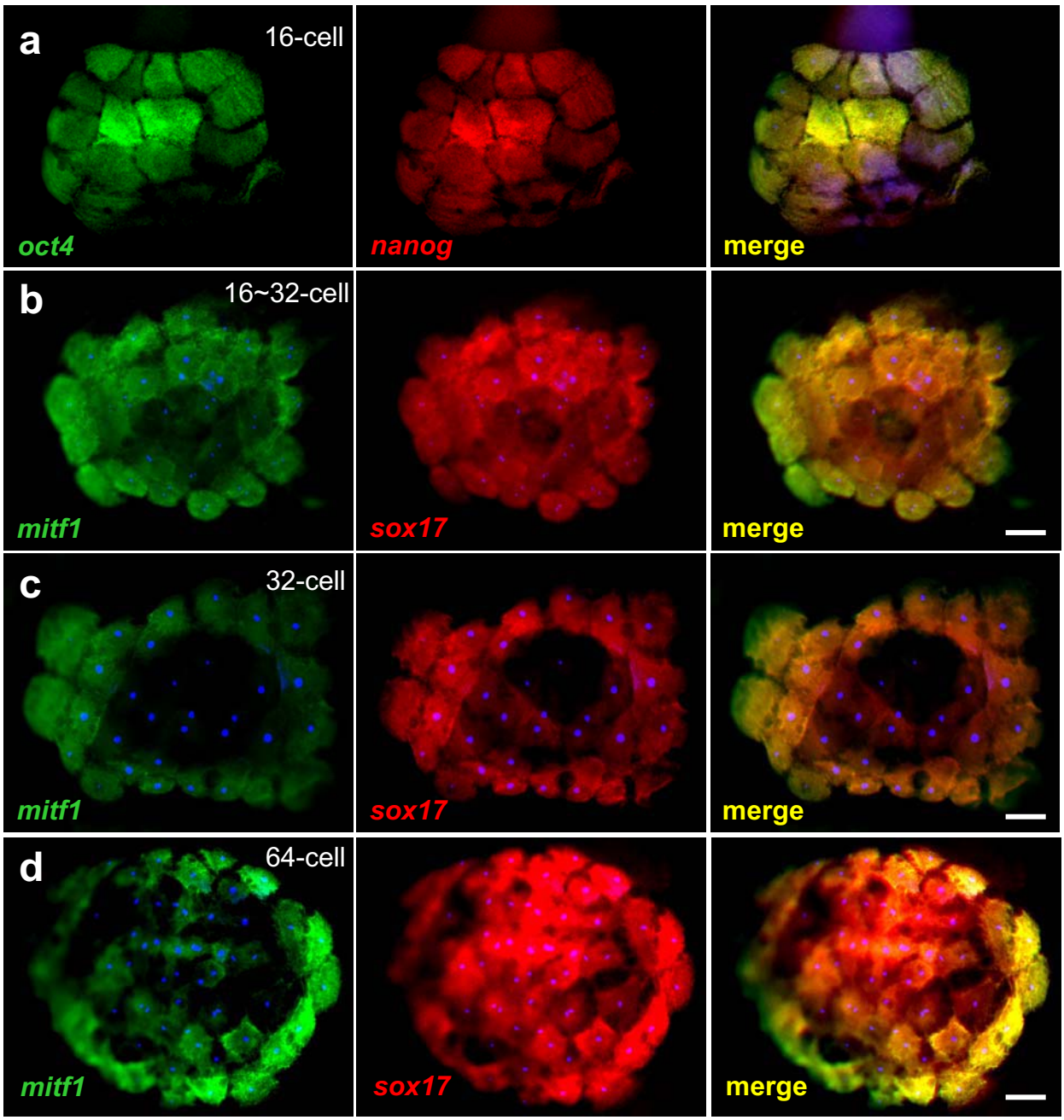

Figure 3 | RNA Expression of lineage markers.