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***In Vitro* Embryo Culture in Defined, Sub-microliter Volumes**

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Abstract

The high attrition rate of *in vitro* human embryo culture presents a major obstacle in the treatment of clinical infertility by *in vitro* fertilization (IVF). Physical and genetic requirements are not well understood for human or mouse pre-implantation embryo development. Group culture is an established requirement for optimal embryo development in the mouse model. However, conventional microdrop culture limitations hinder investigations of the effects of physical parameters on *in vitro* embryo development. We report a microfluidics platform that enables embryo culture in precisely defined, sub-microliter volumes (5 – 500nL) which cannot be investigated using conventional methods. Groups of two embryos per microfluidic well successfully developed to the blastocyst stage, at a rate of over 80%, which is comparable to those cultured in 20µL microdrops. This system can be used to dissect physical requirements of *in vitro* single or group embryo culture, and be made highly parallel to increase experimental throughput.

INTRODUCTION

Human embryos exhibit high rates of developmental arrest in *in vitro* fertilization (IVF) for the treatment of infertility. The highly differentiated egg and sperm fuse to form the zygote (also known as 1-cell or 2 pronuclei stage embryo), which then undergoes dramatic reprogramming during the 2-cell, 4-cell, multicell and morula stages to reach the blastocyst stage (1–3). While blastomeres may be totipotent prior to the morula stage, the morula stage is characterized by compaction of cells and is the earliest stage at which the first two lineages of differentiation can be distinguished by molecular markers (4). The blastocyst stage is a critical developmental milestone because it comprises the trophectoderm, which is the precursor of the placenta, and the inner cell mass (ICM), from which the developing fetus and embryonic stem cell (ESC) lines are derived. However, only up to half of human embryos conceived by IVF develop to the blastocyst stage in *in vitro* culture, and ~18% arrest at or prior to the 4-cell stage (5,6). High attrition rates may be due to underlying genetic or biological defects that are intrinsic to embryos of infertility patients, suboptimal culture media composition or physical culture parameters, or a combination of all these factors. Understanding how genetic and environmental factors affect and determine *in vitro* embryo development and reprogramming is essential for improving infertility treatment, understanding embryo origins of human diseases, and advancing stem cell and regenerative medicine (7,8).

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Paria and Dey previously established that *in vitro* development requires group culture of 10 embryos in 20 μ L culture drops (9). Since addition of secreted factors such as epidermal growth factor (EGF), to the culture media improved the development of singly cultured embryos, secreted factors, and along the same lines, metabolic wastes were thought to contribute to the group culture or an optimal embryo-per-volume requirement (10–12). Consistent with those findings, our IVF outcomes analysis on 665 IVF cycles and developmental data of more than 4000 embryos, revealed that the total number of embryos (called group size hereafter) is one of four non-redundant predictors of positive pregnancy test after IVF treatment (6). Although the number of embryos is dependent on biological factors such as the number of oocytes, sperm and oocyte quality, and the age of the woman, we do not know whether the prognostic value of the total number of embryos is due to an intrinsic, biological difference in embryos belonging patients who have few or many embryos, or if it is merely a biological consequence of the differential culture group size.

The latter is possible because the number of embryos cultured together is dependent on the number of fertilized eggs that are obtained for each patient. There is currently no method to adjust for the varying number of embryos amongst cases or patients. Hence, clinical *in vitro* embryo culture protocols are not patient- or IVF case-specific, nor do we know whether customizing culture protocols would improve treatment outcomes. Finally, we do not understand how each physical parameter, such as culture volume, culture volume per embryo, group size, and surface area, independently or coordinately affect *in vitro* development.

We sought to dissect how specific physical parameters, such as culture volume, determine pre-implantation embryo development, and whether optimal parameters change according to specific characteristics of the embryos, such as group size or other genetic traits or deficiencies. Specifically, our ultimate goal was to identify optimal parameters for single-embryo culture. We reasoned that if threshold concentrations of certain secreted factors underlay cooperativity amongst embryos and the requirement for group culture, as suggested by Paria and Dey (9), then a decrease in the group size may still develop optimally if the culture volume is decreased to maintain critical concentrations of secreted factors.

The conventional microdrop culture method has numerous physical constraints, including evaporation and poor maintenance of smaller culture volumes, which have limited the ways in which physical parameters could be investigated in embryo culture (11). Further, relatively low surface area-to-volume (SA/V) ratios of conventional microdrops are very different from the physiological oviductal or tubal environment of *in vivo* mouse and human embryo development. Therefore, the first step in pursuing this line of investigation was to establish a culture system that allowed embryos to be cultured in precisely defined, sub-microliter volumes that could be well protected from evaporation, and whose SA/V ratios were in the physiological range. Microfluidics technology satisfied these criteria. Although microfluidics has been successfully applied to cell culture (13), single cell genetic analysis (14,15), protein crystallography (16), human sperm manipulation (17), and IVF in animal husbandry (18,19), precisely defined sub-microliter culture chambers have not been applied to mammalian germ cell or embryo culture.

Here, we describe the design and fabrication of our microfluidics embryo culture platform and report successful blastocyst development of mouse embryos in 100 nL culture chambers. This culture system can be applied to dissect the physical requirements of *in vitro* embryo culture, determine the optimal parameters for single-embryo culture, and study the effects of secreted factors on embryo development. Finally, this platform design can be made highly parallel to increase experimental throughput and accelerate the pace of discovery.

RESULTS

Group Culture Requirement

Since the landmark studies on group culture requirement were performed more than a decade ago, superior culture media containing serum supplements and lacking ammonium have become available (20). Therefore, we first tested whether group size affected development using current culture media. We used the F1 hybrid C57BL6/DBA2 mouse strain for all our experiments because in our hands, embryos of this background have 90–100% *in vitro* blastocyst developmental and live pup rates when cultured in groups of 10 embryos per 20 μ L starting at the 1-cell stage followed by transfer to uterine horns of surrogate mothers. Further, these mouse embryos are thought to be genetically identical, so that effects due to genetic variation were considered minimal.

Single-embryo culture and group culture comprising 10 embryos per drop were performed in parallel, from the 1-cell to blastocyst stages while the culture volume was held constant. For embryos cultured singly in 5 and 20 μ L, the blastocyst developmental rates were $50.0 \pm 8.9\%$ and $86.6 \pm 3.3\%$, respectively, which were lower compared to those of embryos cultured in groups of 10 at the same volumes (p -value = 0.01 and 0.08 for the 5 and 20 μ L drops, respectively, in paired t -test; mean $\pm$ standard error of the mean, SEM; Fig. 1a). Although a decrease in culture volume would presumably increase the concentration of secreted factors, single-embryo culture at 5 μ L did not improve or “rescue” development, but resulted in development that was further compromised (p -value = 0.018 in paired t -tests). Therefore, despite the use of improved culture media and consistent with the literature, development was compromised in embryos cultured singly compared to those cultured in groups. Further, we confirmed that reducing the culture volume to 5 μ L did not “rescue” development, possibly due to significant evaporation and suboptimal gas exchange, which had been described by Lane and Gardner (11).

We reasoned that if critical levels of secreted factors were required, then development would be further compromised if single-embryos were removed from the culture drop containing their secreted factors at the 2-cell stage. Indeed, single-embryos that were transferred to new culture drops of the same volume, showed decreased blastocyst development rates at $0 \pm 0.0\%$ and $70.5 \pm 5.2\%$, for 5 μ L and 20 μ L drops, compared to rates of $50.0 \pm 8.9\%$ and $87.1 \pm 3.3\%$ for single-embryos that remained in the same 5 μ L and 20 μ L drops ($p=0.005$ for 5 μ L and $p=0.003$ for 20 μ L in unpaired t -tests; Fig. 1b). In contrast, embryos cultured in groups of 10 did not show differential developmental rates between transferred and non-transferred embryos. Therefore, single-embryo culture and removal of single-embryos from culture drops containing their secreted factors compromised development and supported that critical amounts of secreted factors were necessary for optimal development.

Design and Fabrication of Microfluidic Embryo Culture Chip

Conventional culture volumes at and below 5 μ L were suboptimal. Since it was not possible to differentiate between truly suboptimal parameters such as SA/V ratio from technical issues of evaporation, the conventional culture method could not be used to investigate the impact of various physical parameters on development. Thus, we aimed to create microfluidics embryo culture chips that could support a wide range of precisely defined, sub-microliter culture volumes without technical issues such as significant evaporation, that were inherent to the conventional culture methods. We designed a vertically-mounted chip that features an insertion funnel, a cylindrical culture chamber that can be closed off by two membrane valves, and an outlet port (Fig. 2a, b). This design allows embryos to enter the culture chamber by gravity after being placed into the funnel by micropipette, thereby reducing any mechanical stresses that may compromise viability (Fig. 2a, side view) Hollow

steel pins (0.017 μm internal diameter, 0.025 μm internal diameter) were inserted into the inlet and outlet ports to allow easy attachment of tubing to the outlet and to provide a stable guide for the micropipette when inserting embryos into the funnel (Fig. 2b).

Different culture volumes, 20 nL, 40 nL, 100 nL, and 500 nL, were accommodated by altering the diameter of the cylindrical chambers while keeping the chamber height constant. Four replicates of the same chamber size were placed in parallel on each chip. Two perpendicular control lines, one at the inlet and another at the outlet, can be activated to simultaneously open or close the membrane valves of all four chambers. Previously, the design and fabrication of this “push-up” type of valves were reported for the culture and manipulation of somatic cells, but those valves only accommodated flow channels with $\sim 45\mu\text{m}$ height. Here, we have significantly increased the flow channel height to 140 μm , which was required for embryos to flow freely through the channels. In addition, two separate layers of flow channel features a round profiled valve “patches” and a rectangular profiled flow channels were created to equalize surface tension and to allow valves to close completely (see Supplementary Fig. 1 online). These valves were critical for maintaining defined culture volumes throughout the duration of embryo culture to produce a tightly controlled, closed environment, while allowing embryo development to be examined daily by light microscopy.

Briefly, the design was drawn using computer-aided design (CAD) software and transparency masks depicting the design features were printed (Fineline Imaging, Colorado Springs, USA) to facilitate the separate production of molds containing control layer and flow layer features. Multilayer soft lithography was used to fabricate the embryo culture device (21)(Fig. 3). Polydimethylsiloxane (PDMS) was poured or spun onto each mold, cured, peeled from the mold and bonded together. This dual layer PDMS device was then bonded onto a glass substrate (see Supplementary Fig. 1 online for details). PDMS has previously been shown to support cell and mouse embryo culture with no adverse effects, and had also been used to seal culture chambers in some clinical IVF laboratories (22,23).

Preparation of Microfluidic Embryo Culture Chips

Microfluidic chips were baked continuously at 80°C for 36 hours, after which the control lines were filled with oil to test the functionality of the valves. Microfluidic channels in the flow layer were filled with 0.2% Pluronic 127 (Sigma-Aldrich), a detergent, in phosphate buffered saline (PBS) for one hour, and replaced with a continuous flow of PBS solution at a flow rate of 50 mL/minute for 5 minutes per chamber, followed by a continuous flow of double distilled H₂O (ddH₂O) at the same rate. The flow channels were filled with ddH₂O and ready to use for the next 24 hours. Prior to insertion of embryos by micropipette, the microfluidic chamber and channels were completely filled with media and all air bubbles were purged from the chamber. After the insertion and exit channels were both plugged with solid steel pins to ensure a closed system, the entire microfluidics chip was equilibrated in a desiccator filled with 5% CO₂, 5% O₂, and 90% N₂ (5-5-90; Praxair), at 37°C.

Embryo Placement and Culture

The solid steel pins at the insertion channels were temporarily removed for embryo placement and the microfluidic chip was mounted on a mobile X-Y-Z stand facing a camera that connected to a video recorder for real time observation. To introduce the embryos into a microfluidic chamber, a glass capillary pipette attached to a mouth piece was used for gentle transfer of the embryos. The glass pipette was inserted into the funnel region of the microfluidic chamber through a guiding hollow steel pin placed into the inlet and the embryos were released into the funnel. The guidance steel pin was then removed from the inlet and the solid steel pin was reinserted. This process was repeated until all the funnels on

the chip were occupied. The valve at the base of the funnel was opened and the embryos fell freely into the chamber through gravity. After all the embryos had flowed into the chamber region, the valve was closed again at a positive pressure of 9 pounds per square inch (PSI). Subsequently, the embryo-loaded microfluidic chip was dismounted from the X-Z stand and the control lines were disconnected from the pump and reattached to a pressure accumulator (Fluidigm) that was maintained at 9 PSI. The chip is then placed in a Pyrex glass dish, covered with embryo-grade mineral oil (Sigma), and examined under the microscope to confirm that embryos are in the enclosed chamber. Embryos in the microfluidics chip and conventional culture controls are cultured in a dessicator containing mixed gas and 37°C. Embryos are observed daily at 20× magnification, on an inverted microscope (Olympus IX90) equipped with heated stage and a Nikon camera.

***In Vitro* Blastocyst Development in Microfluidic Chips**

We tested whether this microfluidic embryo culture chip could support *in vitro* development to the blastocyst stage. Briefly, 1-cell stage embryos were harvested and placed in conventional group culture of 10 embryos per 20 μ L. At the mid-2-cell stage (~ 44 hours after human chorionic gonadotropin injection), embryos were placed into the 100 nL chambers of the microfluidic embryo culture chip in groups of 2 embryos per chamber. We chose to test this minimal group size of 2 embryos because it approached single-embryo culture while still allowing for cooperativity between the two embryos. Of a total of twenty-two 2-cell embryos that were placed into eleven 100 nL chambers, $81.8 \pm 10.1\%$ developed to blastocyst stage by Day 4. This blastocyst developmental rate was comparable to the $83.3 \pm 8.8\%$ observed for embryos placed into 20 μ L conventional drops in groups of 2 embryos per drop. In contrast, $3.3 \pm 3.3\%$ of embryos that were placed into 5 μ L conventional drops in groups of 2 embryos per drop, developed to blastocysts (p-value <0.01 for comparison with the 100 nL or 20 μ L drop in unpaired t-test). Thus, we showed that blastocyst development is possible in precisely defined, 100 nL culture volumes, and comparable to the results achieved in standard 20 μ L conventional culture drops. Most importantly, although this culture volume was 50 folds smaller than the 5 μ L drop and 200 folds smaller than the 20 μ L drop, it supported blastocyst development significantly better than the 5 μ L microdrop, for which problems with evaporation had been reported (11).

DISCUSSION

We provided proof-of-concept that mammalian embryos can develop to the blastocyst stage in *in vitro*, sub-microliter volumes. We further showed that sub-microliter culture volumes could be defined precisely in a microfluidic platform. This culture platform can be modified to further define the group culture requirement or critical group size. Further, it can potentially be applied to customize *in vitro* culture of human embryos in IVF, after in-depth testing of a wide range of sub-microliter culture volumes to identify optimal parameters according to clinically relevant endpoints such as implantation, live birth, neonatal survival and subsequent measures of development, health and reproduction. Since hundreds of embryos may be required to detect a statistical significance in blastocyst developmental rates, especially since our group culture control embryos consistently develop to blastocyst stage at 90–100%, the use of *in vivo* endpoints may facilitate identification of optimal parameters. Finally, the culture chip is designed with a view to integrate functionalities, such as sampling of culture media, testing of a wide range of sub-microliter volumes, and automated microscopy.

The work presented here is novel and opens up an entire research area that was not previously amenable to scientific investigation. We have provided the tools and methods for investigating the effects of physical parameters on embryo development associated with sub-microliter culture volumes that more closely simulate the *in vivo* environment. Most

interestingly, these methods can be applied to study basic mechanisms and requirements of preimplantation embryo culture, and they can be further adapted and explored for clinical application to improve human embryo development in the clinical IVF laboratory.

EXPERIMENTAL PROCEDURES

Mouse embryo harvest, conventional culture, and observation

Hybrid F1 (C57BL6 x DBA/2) mouse embryos at the 2 pronuclei (2PN) stage were harvested from 3–5 week old female mice (Charles River) that had been primed by pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG), mated with stud males, and sacrificed by cervical dislocation at 17–22 hour after HCG injection. Cumulus cells were removed by hyaluronidase (Sigma, H4272) treatment and mechanical pipetting, recovered in M2 media (Chemicon International), washed in ten drops of culture media comprised of Human Tubal Fluid (HTF) with 10% serum supplement (Cooper Surgical), and cultured in microdrops under mineral oil in mixed gas (90% nitrogen, 5% oxygen, 5% carbon dioxide; Praxair) at 37°C, at 10 embryos per 20 µL drop. At ~ 24 hours of *in vitro* culture, or the mid-2-cell stage, embryos were placed in the experimental control microdrop and culture conditions being tested. Embryos in the environmental control drop were not further manipulated.

Embryo culture in microfluidic chips

The mouse embryos were harvested on Day 0 at the 1-cell stage. On Day 0, the surface of the microfluidic chamber was flushed with 0.02% Pluronic solution in PBS and then rinsed and maintained with distilled water. On Day 1, the microfluidic chambers were filled with embryo culture media, HTF supplemented with 10% serum replacement. Mouse embryos at the 2-cell stage were then placed into control microdrops at 10 embryos per drop, or inserted into the microfluidic chamber at 2 embryos per drop, in four sessions over two independent embryo harvests. After the embryos are inserted into the culture chamber and the valves are closed, an accumulator is attached to the chip to maintain pressure in the control channels and to prevent the valves from opening during the incubation period. The microchip is placed in a glass dish and covered with mineral oil. The glass dish is then placed into a desiccator along with the accumulator and the microdrops and flushed with bio-mix gas (5% O₂, 5% CO₂, 90% N₂). The flushed desiccator is placed into the 37° C incubator for incubation. Similarly, control experiments were performed in the same way in three independent experiments, except that groups of two embryos per drop were placed into 5 or 20 µL conventional culture drops instead of the microfluidic chambers.

Statistical methods

Data were analyzed by paired or unpaired Student's t-test, as noted for each experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Group culture is required for *in vitro* mouse embryo development. **(a)** At 5 µL culture volume, the blastocyst development rate was $50.0 \pm 8.9\%$ for embryos cultured singly, which was lower than the rate of $90.0 \pm 0.0\%$ observed for embryos cultured in groups of 10 (p-value = 0.01). At 20 µL culture volume, the blastocyst development rate was $86.6 \pm 3.3\%$ for embryos cultured singly, which was lower than the rate of $97.0 \pm 2.1\%$ observed for embryos cultured in groups of 10 (p-value = 0.08). Paired t-tests were performed. Mean $\pm$ standard error of the mean (SEM). **(b)** Singly-cultured embryos that were transferred to a new culture drop resulted in decreased blastocyst developmental rates at $0.0 \pm 0.0\%$ for 5 µL and $70.5 \pm 5.2\%$ for 20 µL, compared to $50.0 \pm 8.9\%$ and $87.1 \pm 3.3\%$ observed for non-transferred embryos (i.e. embryos that remained in the same drops) for 5 and 20 µL, respectively. (p-value = 0.005 for 5 µL, and 0.03 for 20 µL in unpaired t-tests.) Transferred and non-transferred embryos developed to blastocysts at comparable rates of $94.0 \pm 4.0\%$ and $97.0 \pm 2.1\%$, respectively, for embryos cultured in groups of ten.

Fig. 2. Microfluidic embryo culture chip design. (a) Front view. (b) Side view. (c, d) Top view (top panels) and cross-sectional view (bottom panels) of a flow channel crossing over a control channel respectively, show how a membrane valve is created. If there is no pressure difference across the PDMS membrane, the membrane valve remains open (c). When the pressure in the control channel exceeds the flow channel pressure (by approx 10 psi) the membrane will flex and close off the flow channel (d). (e) Photograph of the microfluidic chip. The flow channels are filled with dark color dye and the control channels are filled with a lighter color dye. Chip shown contains two 100 nL and two 500 nL chambers.

Fig. 3.

Embryos flowing into the microfluidic culture chamber by gravity. Two 2-cell stage mouse embryos have fallen to the bottom part of the funnel (**a**), entered the microfluidic channel (**b**), to cross the open valves (**c**), and enter the circular, 100 nL culture chamber (**d**). (**d**) The valve was closed immediately after embryos passed it.

Fig. 4.

Experimental protocol and blastocyst development in microfluidic chip. **(a)** 1-cell stage embryos were harvested on Day 0, cultured in groups of 10 embryos per 20 μ L drop, and transferred to 100 nL microfluidic chambers for culture in groups of 2 embryos per chamber, while a transfer control comprising 10 embryos per group was performed in parallel. The same protocol is performed to test groups of 2 embryos in conventional 5 μ L and 20 μ L microdrops, in parallel with transfer and non-transfer controls. **(b)** Blastocyst development for groups of 2 embryos was comparable between 100 nL and 20 μ L, but it was significantly compromised in the 5 μ L drop. Blastocyst developmental rates were above 90% for groups of 10 embryos cultured in the transfer control and non-transfer control drops, which indicated that *in vitro* environment was well maintained in all the experiments.