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Dynamic microfunnel culture enhances mouse embryo development and pregnancy rates

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BACKGROUND: Despite advances in *in vitro* manipulation of preimplantation embryos, there is still a reduction in the quality of embryos produced leading to lower pregnancy rates compared with embryos produced *in vivo*. We hypothesized that a dynamic microfunnel embryo culture system would enhance outcomes by better mimicking the fluid-mechanical and biochemical stimulation embryos experience *in vivo* from ciliary currents and oviductal contractions.

METHODS AND RESULTS: Mouse embryos were cultured in microdrop-static control, microfunnel-static control or microfunnel-dynamic conditions with microfluidics. All groups tested had greater than 90% total blastocyst development from zygotes after 96 h culture. Blastocyst developmental stage was significantly enhanced ($P < 0.01$) under dynamic microfunnel culture conditions as evidenced by an increased percentage of hatching or hatched blastocysts (Microdrop-control 31%; Microfunnel-control 23%; Microfunnel-pulsatile 71%) and significantly higher ($P < 0.01$) average number of cells per blastocyst (Microdrop-control 67 $\pm$ 3; Microfunnel-control 60 $\pm$ 3; Microfunnel-pulsatile 109 $\pm$ 5). Blastocyst cell numbers in dynamic microfunnel cultures (109 $\pm$ 5) more closely matched numbers obtained from *in vivo* grown blastocysts (144 $\pm$ 9). Importantly, dynamic microfunnel culture significantly improved embryo implantation and ongoing pregnancy rates over static culture to levels approaching that of *in utero* derived preimplantation embryos.

CONCLUSIONS: The improved pregnancy outcomes along with the simple and user-friendly design of the microfluidic/microfunnel system has potential to alleviate many inefficiencies in embryo production for biomedical research, genetic gain in domestic species and assisted reproductive technologies in humans.

Key words: microfluidics / embryo culture / dynamic microfunnel culture / IVF

Introduction

Since the first *in vitro* development of fertilized mouse ova on a blood clot in the presence of oviduct tissue in 1941 (Kuhl, 1941), extensive research has been undertaken to improve developmental competence of mammalian embryos grown in culture. Embryonic developmental competence has been assessed and quantified as: (i) rate of preimplantation embryo development from the 1- or 2-cell stage to a total blastocyst (Naz *et al.*, 1986; Fleming *et al.*, 1987; McDowell *et al.*, 1988; van den Bergh *et al.*, 1996; Erbach *et al.*, 1994; Manser *et al.*, 2004); (ii) percentage of embryos developing to a specific stage of blastocyst development (early, full, expanded, hatching or hatched blastocyst) within a set time (Gardner and Lane, 1993; Biggers and McGinnis, 2001; Lane *et al.*, 2001; Biggers *et al.*, 2004b, 2005; Manser *et al.*, 2004); (iii) cellular constitution of blastocysts (counts of total cells, cells of the inner cellular mass and/or cells of the trophectoderm) within a set time (Gardner and Lane, 1993; Biggers and McGinnis, 2001; Lane *et al.*, 2001; Byrne *et al.*, 2002; Lane and Gardner, 2003; Biggers, 2005); (iv) percentage of embryos transferred into the oviduct/uterus that implant and/or percentage

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of embryos transferred that result in developmentally normal fetuses or offspring (Summers *et al*., 1995; Gardner and Lane, 1996; Lane and Gardner, 2003; Biggers *et al*., 2004a, 2005; Mitchell *et al*., 2009). Most studies have focused on modifications of soluble media components such as salts, energy or nitrogen sources and growth factor/hormone supplementation (Loutradis *et al*., 2000; Summers *et al*., 2005; Biggers and Summers, 2008; Perin *et al*., 2008). Although such studies have improved the developmental competence of mammalian embryos *in vitro*, current culture conditions do not consistently produce embryos with developmental competence equivalent to those derived from *in vivo* microenvironments of the oviduct and/or uterus (Lane and Gardner, 1996; Zander *et al*., 2006).

Contemporary mammalian *in vitro* fertilization (IVF) procedures, including that for humans, entail gamete isolation, fertilization and embryo culture in dishes, test tubes or microdrops using media volumes of 4–1000 μl under substantially static conditions (Trounson and Gardner, 2000; Brison *et al*., 2004; Thompson, 2007). Conversely, *in vivo* embryos develop from the zygote to blastocyst stage during a period of transit through the oviduct and into the uterus, where they reside spatially juxtapositioned between epithelial cells within luminal crypts representing a moist microenvironment (Leese *et al*., 2001). During most of this time, embryos experience dynamic mechanical (Fauci and Dillon, 2006) and biochemical conditions (Hardy and Spanos, 2002). Segmental muscular contractions in the oviduct are presumed to agitate tubal contents (Muglia and Motta, 2001) although oviductal epithelial cells contribute to ciliary currents (Gaddum-Rosse and Blandau, 1973; Talbot *et al*., 1999), which collectively exert fluid mixing and solid mechanical forces on embryos (Anand and Guha, 1978; Blake *et al*., 1983). Collectively, these *in vivo* mechanical agitations may significantly alter the biochemical microenvironment directly adjacent to the embryo periphery, thus facilitating the exchange of gases and biomolecules.

As early as the 1970s, the importance of microenvironment and embryo handling and culture was reported (Willadsen, 1979). Agar coating of embryos improved embryo development. Recently, microwells within the bottom of culture dishes (Vajta *et al*., 2000; Hoelker *et al*., 2009) have been shown to improve embryo development, presumably by impacting the culture microenvironment. Microfluidic technology may provide controllable microenvironments specialized for embryo development and provide an automated platform for performing multiple steps of IVF. For example, Choi *et al*. (2008) developed a microfluidic device capable of selecting normal oocytes with relatively high specificity. Similarly, intrinsic sperm mobility and microfluidic laminar flow were used to isolate motile sperm from non-motile sperm, debris and seminal plasma (Cho *et al*., 2003; Schuster *et al*., 2003). Zeringue *et al*. (2005) developed a microfluidic platform for control of embryo positioning, movement and zona pellucida removal for chimera and transgenic production. Although these devices provide convenient handling properties for sperm, oocytes and embryos, such studies did not address the potential of microfluidics to influence embryonic developmental competence.

Toward this goal, static microfluidic culture systems with submicroliter effective culture volumes (Raty *et al*., 2004) have been reported to enhance embryonic development rates presumably due to embryo self-conditioning of very small volumes of culture media. This microfluidic system, however, has not yet been shown to enhance pregnancy rates. A dynamic microfluidic culture system with continuous media perfusion to embryos within microchannels actually resulted in poor embryo development across a range of flow rates (Hickman *et al*., 2002). Whether this negative effect of microchannel perfusion on embryo development was due to shear stress, or continual removal of growth-promoting autocrine factors (Paria and Dey, 1990; Walker *et al*., 2004) is unknown. Interestingly, a non-microfluidic system using oil suspended microdrops with internal media recirculation has been used to expose embryos to shear stresses of 1.2 dynes/cm^2, which are orders of magnitude higher than physiological conditions, and this resulted in upregulation of stress signalling pathway constituents and embryo death (Xie *et al*., 2006). This would indicate that embryos can detect shear forces.

The objectives of the current studies were to utilize a microfluidic culture platform to provide embryos with media refresh in a physiologically based pulsatile manner and evaluate its impact on embryo developmental competence as assessed by kinetics of embryo development, cell composition, implantation rates and ongoing pregnancy rates. Finally, developmental and modelling experiments were performed to begin elucidating mechanisms influencing embryo development within this microfluidic culture system.

Materials and Methods

Device design, fabrication and fluid actuation

A microfluidic system was designed for testing the influence of continual pulsatile or peristaltic fluid flow on embryo development without placement of embryos into microchannels. This gave rise to design of a microfluidic system using microchannels as conduits for fluid flow through a microfunnel where embryos reside. Poly(dimethylsiloxane) (PDMS) was used for this microfluidics system because of its favourable mechanical properties, optical transparency, biocompatibility (Quake and Scherer, 2000; Johnson *et al*., 2001) and straightforward manufacturing by rapid prototyping (Duffy *et al*., 1998). A common challenge with PDMS-based microfluidic chips, however, is evaporation through PDMS which was alleviated by using PDMS-Parylene-PDMS membranes (Heo *et al*., 2007). This hybrid membrane minimized evaporation and osmolality shifts, yet possessed the thinness and flexibility necessary to interface with deformation-based microfluidic actuation systems; maintained clarity for optical microscopic cell assessment; and enabled successful development of single-cell mouse embryos into blastocysts on microfluidic systems. In addition, design of culture microfunnels rather than culture channels, was selected to minimize excessive fluid mechanical stress that may be associated with passage through narrow channels. Relief features of desired channel structures were composed of SU-8 (MicroChem, Newton, MA, USA) and fabricated on a thin glass wafer (200 μm thick) by using backside diffused-light photolithography (Futai *et al*., 2004). A thick (~8 mm) PDMS (Sylgard 184, Dow-Corning at a 1:10 curing agent-to-base ratio) slab with microfunnel and channel features (Duffy *et al*., 1998) were created by casting PDMS prepolymer against the SU-8 features and curing at 60°C for 120 min and at 120°C for 30 min. The resulting PDMS slab was attached to a thin PDMS-Parylene-PDMS hybrid membrane (Heo *et al*., 2007). The use of pin actuators that deformed PDMS channels to produce flow eliminated reliance on interconnects, tubings, and external pumps, which can make microfluidic systems complicated and inconvenient to users. In these studies, on-chip pulsatile or peristaltic pumping was performed using computer-controlled, piezoelectric, movable pins on a commercial Braille display (Braillex Tiny from F.H. Papenmeier GmbH & Co. KG, Germany) (Gu *et al*., 2004).

Embryo culture, transfer and development

All animal procedures were approved by the University of Michigan Animal Care and Use Committee. Six- to 8-week-old B6C3F1 females were administered 5 IU equine chorionic gonadotrophin (eCG; Sigma, St. Louis. MO, USA) followed by 5 IU human chorionic gonadotrophin (hCG; Sigma) 48 h later. Females were then placed with adult B6C3F1 males of known fertility overnight and examined the following morning for vaginal plugs indicating that mating had occurred. Presumptive zygotes were isolated from oviducts, and cumulus cells were removed (Hogan *et al.*, 1994) and pooled into Hepes-buffered Human Tubual Fluid media with 0.1% Serum Synthetic Substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) followed by random distribution of 13–15 zygotes into 10 μl of Potassium Simplex Optimized Medium (KSOM +1/2 AA with D-Glucose and phenol red Cat # MR 121-D, Specialty Media, Phillipsburg, NJ, USA) overlaid with mineral oil (Irvine Scientific) in (i) microdrop culture in culture dishes (Microdrop-control), (ii) microfunnel culture on PDSM chips without fluid flow (Microfunnel-static control) and (iii) microfunnel culture on PDMS chips with flow-through of fluid at a pin actuation rate of 0.1 Hz (Microfunnel-pulsatile). All cultures were performed in the same incubator with a humidified environment of 5% CO_2, 20% O_2 and balance N_2 at 37°C. In some experiments embryo development was assessed at 96 h of culture and categorized as blastocysts with subdivisions of early, full, expanded, hatching or hatched. All blastocysts were fixed, stained with Hoechst 33342 (Sigma) and visualized with fluorescent microscopy (Leica DMR, Leica Microsystems Inc., Bannockburn, IL; 400× magnification) by two individuals blinded to treatments. *In vivo* control embryos were collected from uteri corresponding to 72 or 96 h culture. Embryos used for transfer were cultured for 72 h and transferred to oviducts of (C57BL/6 X DBA/2) F1 mice (−0.5d asynchronous). Culture treatments were blinded and embryos were morphologically assessed for development. Embryos were transferred in groups of seven to each uterine horn according to the stage of embryo development starting from the seven furthest advanced to the seven least advanced-stage embryos. Two groups of embryos from different treatments were transferred to opposite uterine horns to control for uterine environment. This resulted in the seven most advanced embryos from dynamic culture being transferred to one recipient uterine horn and the seven most advanced embryos from static culture into the recipient's contralateral horn. *In vivo* derived embryos corresponding to 72 h culture were transferred and served as gold standard controls. After 15 days of gestation, hysterectomy was performed to evaluate and quantify embryo implantation sites and ongoing pregnancies. Fetal weight, stage of development and normality were also analysed. Parametric and non-parametric data were analysed with ANOVA/unpaired *t*-test and χ^2 statistics, respectively.

Design of 3D mesh geometries used in computer simulation

To model fluid flow, shear stresses and biomolecule retention and removal, a Fluent simulation system was employed. Briefly, to model microdrop culture in an organ well, the liquid drop was assumed to be a hemisphere with a radius of 1.68 mm calculated by the drop having a total volume of 10 μl of media covered by oil. The embryo is approximated to be a sphere with a 50 μm radius at the centre of the hemisphere. To model microchannel culture with a straight microfluidic channel and a barrier (Hickman *et al.*, 2002), the channel dimensions were 1.5 mm in length, 1 mm in width and 0.25 mm in height. The barrier had a cross-section of 0.1 mm in width and 0.11 mm in height (longitudinal cut of the channel) and was placed in the middle of the channel. A sphere, acting as an embryo, was placed in front of the barrier with a 10 μm gap. For microfunnel modelling, the funnel had a 0.25 mm bottom radius, 1.77 mm top radius and 2.63 mm height. Funnel dimensions were calculated based on the 10 μl volume of media and 60° angle of the funnel wall in respect to the bottom channel. The funnel and a 50 μm radius sphere were located at the middle of a straight channel with dimensions of 1.5 mm in length, 0.4 mm in width and 0.03 mm in height. Each sphere had a 10 μm gap from its respective bottom boundary. For microdrop and microfunnel modelling, the interfaces between media and covered oil were considered as walls for simulation purposes. The computational meshes consist of 59 856 elements, 181 202 elements and 55 765 elements for microdrop, microchannel and microfunnel, respectively, and the results were mesh-independent.

Embryo secretion and volumetric source of biomolecules

Assuming embryos secrete factors with a constant production rate over the culture period, and the transport of factors in media depends on mass diffusion in the absence of media flow, the secretion rate from an embryo can be modelled by Fluent computer simulation as the specified biomolecule mass production rate at the wall or surface of embryo. Here, a volumetric source term (kg/m^3 s) of a biomolecule in a fluid zone was defined only in the cells adjacent to the spherical wall (embryo) by using a user defined function (UDF). In this simulation, the properties of epidermal growth factor (EGF) were chosen as the representative biomolecule for beneficial factors. EGF has a molecular weight of 6045 Da and diffusion coefficient, D_{12}= 1.66E−10. In UDF, a constant mass production rate of EGF, 1.68E−15 (kg/s) was assigned and a volumetric source (kg/m^3 s) was obtained by dividing the production rate by volume of the cell. A waste product biomolecule, such as ammonia (molecular weight, 18 Da) showed similar retention ratios as EGF for the three conditions simulated. Retention rates in microfunnels were 49.3 versus 41.8% for ammonia and EGF, respectively. The slightly higher retention rate of ammonia can be explained by the faster diffusion of ammonia out of the main flow stream that exits towards the outlet microfluidic channels. Although 13–15 embryos were placed in each condition, simulations were done with a single embryo. Because normalized values of concentration were used for comparison of three different conditions, a single embryo model was adequate for comparisons of the retention rates. Though actual mechanical forces which each embryo experienced with the flow conditions would vary with the number and position of embryos loaded in the microfunnel, these single embryo models are still useful for semi-quantitatively evaluating differences that embryos may experience under dynamic conditions compared with static conditions.

Sampling volume

The 'sampling volume' was defined as the volume within which ligands are accessible for binding by receptors on an embryo. To determine a relevant sample volume, we considered the average distance, *l*, from within which an embryo can sample ligands. This distance can be approximated as $(D_L/k_r)^{1/2}$, where D_L is the translational diffusion coefficient of the ligand and k_r is the dissociation rate constant of the receptor for this ligand (Lauffenburger and Linderman, 1993). In the case of EGF in which characteristic parameters are used in simulation and which is known to be a relevant growth factor for embryo development, D_L is 1.66E−10 (m^2/s) (Thorne *et al.*, 2004) and k_r is 5.7E−3 (s^{-1}) (Lauffenburger and Linderman, 1993). Thus *l* is ∼171 μm. For other molecules such as insulin-like growth factor-I (IGF-I) with D_L = 1.59E−10 (m^2/s^1) (Nauman *et al.*, 2007) and k_r = 1.7E−3 (s^{-1}) (Jansson *et al.*, 1997), *l* is ∼306 μm. From these estimates, we used ∼250 μm as a characteristic distance of interest to determine a sampling volume for comparison.

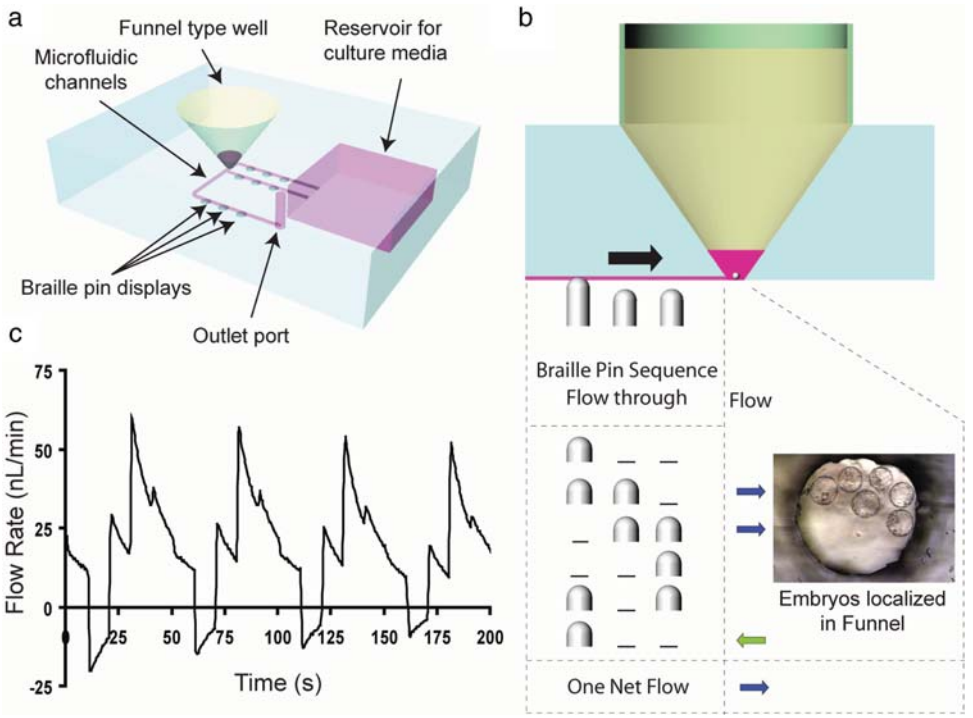

Figure 1 Dynamic microfunnel culture device and flow pattern.

(**a**) Schematic drawing of a microfunnel device. The device is placed on an array of piezoelectric pin actuators provided by Braille displays. (**b**) Embryos are loaded and cultured in the microfunnel under the flow-through condition created by the pin actuation sequence. (**c**) The flow rate generated over four cycles of the five-step Braille pin actuation sequence at 0.1 Hz was measured by a digital flow meter (SLC1430, http://www.sensirion.com). The average flow rate of 17.9 nl/min was used as the inlet velocity for FLUENT simulations in Fig. 6.

Results

The microfunnel culture system was constructed of PDMS and embryos were cultured on a ~500 μm diameter, flat, optically transparent floor (Fig. 1a and b). The microfunnel was connected to microchannels that provided periodic fluid pulses of media at physiological frequencies (0.135 Hz in rabbit oviduct; Bourdage and Halbert, 1980). These channels were thin (30 μm high) and wide (400 μm wide) to prevent embryos from entering and to enable peristaltic fluid pumping by deformation of the channels using computer-controlled piezoelectric pins. The microfunnel had four functions: (i) it localized multiple embryos to a small region (~500 μm diameter); (ii) it provided a fluid reservoir (up to tens of microliters) that buffered the biochemical exchange rate between the embryo and the fluid environment; (iii) it modulated the fluid mechanical stimulation to which embryos were exposed by fluid flow from the microchannels; and (iv) it provided a practical design whereby embryos were easily loaded and unloaded by simple pipetting.

The total number of blastocysts developed from zygotes over a 96 h culture period was not significantly different between culture conditions with all controls and treatments displaying greater than 90% blastocyst development. Embryo developmental kinetics were significantly enhanced ($P < 0.01$) under dynamic microfunnel culture conditions as evidenced by larger percentages of zygotes developing to hatching or hatched blastocysts (Microdrop-control 31%; Microfunnel-control 23%; Microfunnel-pulsatile 71%) and significantly higher ($P < 0.01$) average number of cells per blastocyst (Microdrop-control 67 ± 3; Microfunnel-control 60 ± 3; Microfunnel-pulsatile 109 ± 5) as shown in Figs 2 and 3. Blastocyst cell numbers in dynamic microfunnel cultures (109 ± 5) more closely resembled numbers obtained from *in vivo*-grown blastocysts (144 ± 9; Fig. 3). Importantly, this enhanced embryo development enabled by the dynamic microfunnel cultures led to correspondingly higher rates of implantation, lower rates of resorption and significantly higher ongoing pregnancy rates compared with embryos cultured in static microdrops ($P < 0.05$), as shown in Fig. 4. Fetuses originating from dynamic microfunnel culture demonstrated normal development as assessed by developmental age, fetal weight and placental weight.

To determine if enhanced embryo development in the dynamic microfunnel systems was time or stage dependent, we systematically varied the duration and developmental-stage at which embryos cultured in microfunnels were exposed to dynamic culture conditions (Fig. 5). Embryos in microfunnels were cultured under dynamic conditions for 48 h at the beginning, 48 h at the end or 96 h and compared with embryos under static condition over the entire 96 h. The developmental rate observed was proportional to the duration of dynamic culture regardless of the stage of embryo development. Thus, embryos cultured under dynamic conditions for 48 h had similar significantly enhanced development and cell numbers at 96 h compared with static controls ($P < 0.01$) regardless of whether the 48 h period was at the beginning or end of the 96 h.

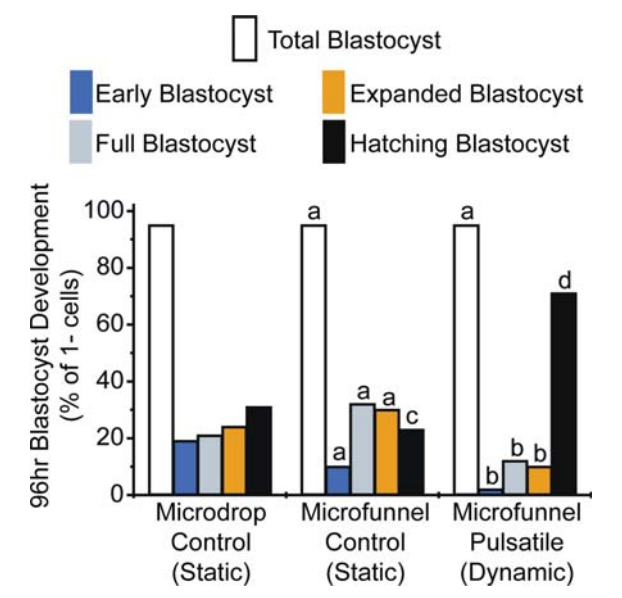

Figure 2 Dynamic microfunnel culture enhances blastocyst development.

Blastocysts obtained in each culture condition were morphologically categorized into four different stages: early, full, expanded and hatching blastocysts. Blastocyst development to each stage was not significantly different between static culture comparisons, thus subsequent statistical comparisons were performed between microfunnel-control (static) and microfunnel-pulsatile (dynamic). Blastocysts derived under microfunnel-pulsatile culture conditions developed faster than those from static conditions. Different letters between culture treatments within a stage of blastocyst development represent a significant difference between the microfunnel-control and microfunnel-pulsatile (a,b = $P < 0.05$; c,d = $P < 0.01$).

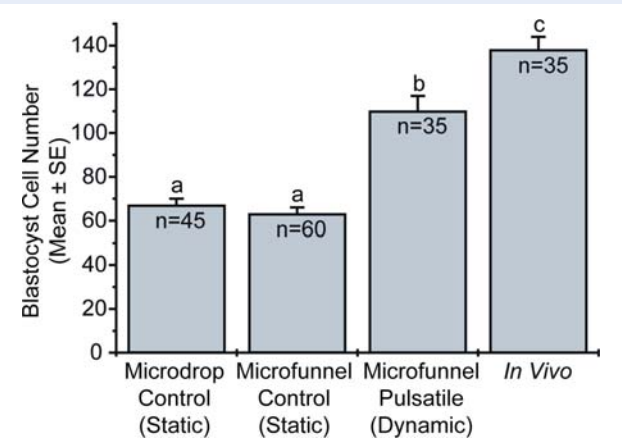

Figure 3 Dynamic chip condition (microfunnel-pulsatile) shows a greater number of cells per blastocyst compared with static culture, with results closer to *in vivo* conditions.

Different letters between culture treatments represent a significant difference (a,b,c = $P < 0.01$).

The differences in embryo culture microenvironments provided by the dynamic microfunnel culture system and previously described systems were highlighted by computational simulations (Fluent, www.fluent.com; Fig. 6). Three different 3D-meshes were modelled based on actual geometries: microdrop with 10 μl media, microchannel with barrier and a microfunnel. In each simulation, an embryo was modelled to secrete an autocrine factor for 48 h resulting in formation of a concentration gradient in its vicinity. Here it was useful to consider a sampling volume in the vicinity of the embryo (~250 μm radius region around an embryo) with which to compare the effective autocrine concentration in each device setting. For the microchannel and microfunnel, fluid flow was also simulated with average inlet flow velocities that corresponded to experimentally used values. Simulations

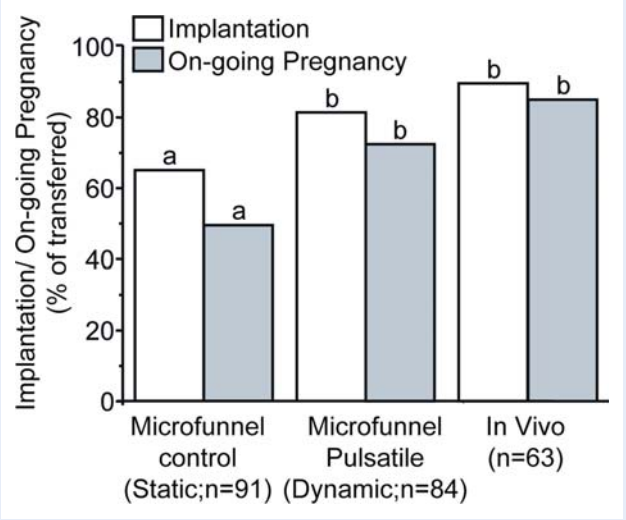

Figure 4 Dynamic microfunnel culture enhances implantation and on-going pregnancy rates.

Comparison of percentage of static, dynamic and *in vivo* grown and transferred embryos that implanted and maintained normal on-going pregnancies at 15 days post-transfer. Different letters indicate a statistical significance (a,b = $P < 0.05$).

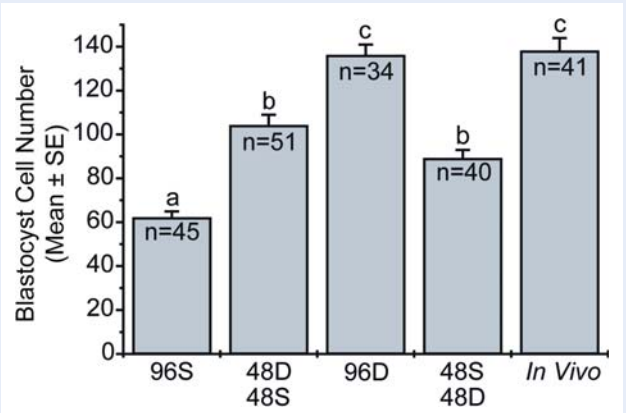

Figure 5 Effect of duration and timing of dynamic culture on embryo development.

Mean blastocyst cell counts from embryos cultured for 96 h under varying culture conditions of either dynamic (D) or static (S) media flow. Different letters indicate statistical significance ($P < 0.05$). Culture conditions of either dynamic or static media flow were interchanged at 48 h increments.

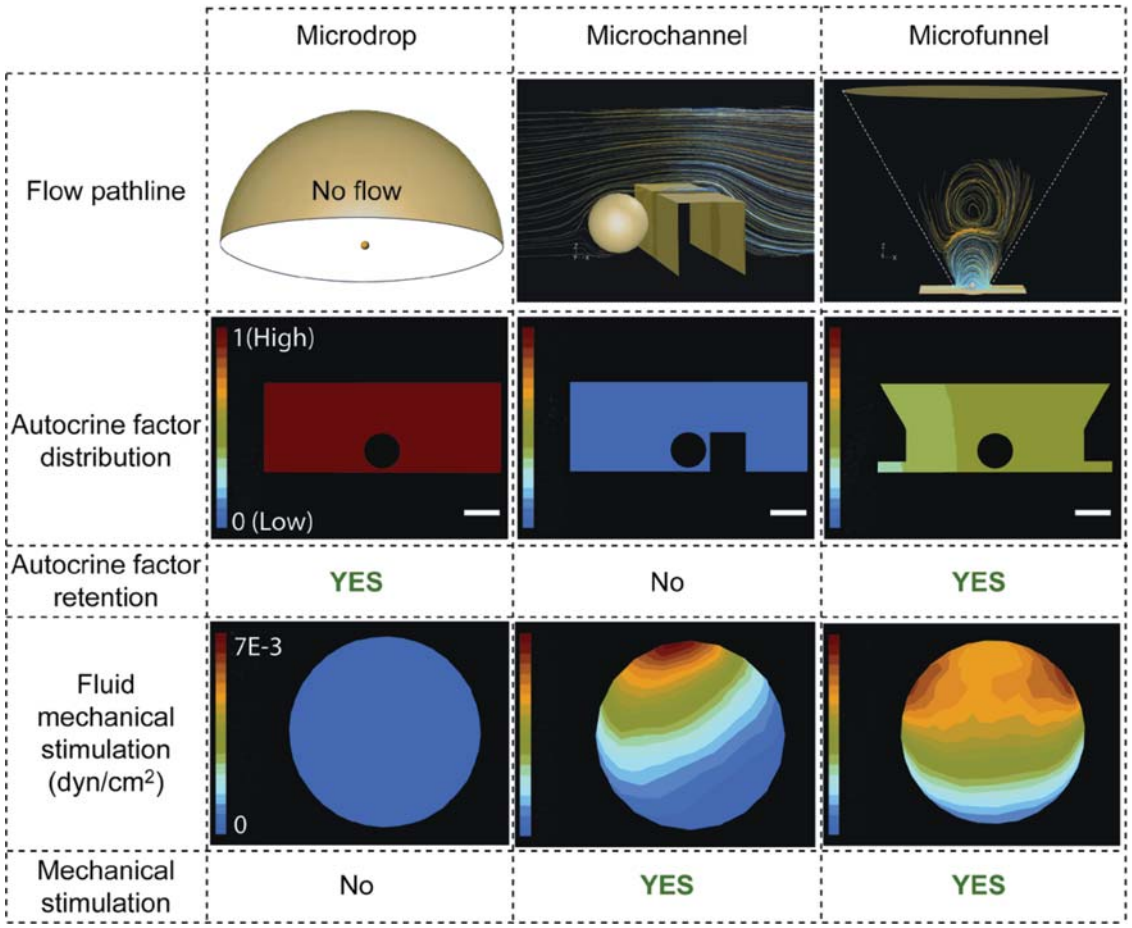

Figure 6 Dynamic microfunnel culture provides fluid mechanical stimulation with retention of autocrine factors.

Summary of flow pathlines, mechanical stimulation and autocrine factor distribution/retention provided by different embryo culture methods as simulated using Fluent. Microdrops retain autocrine factors but do not provide mechanical stimulation. Microchannels provide mechanical stimulation but do not retain autocrine factors. Dynamic microfunnel cultures provide both. Flow pathline: pathlines on the longitudinal plane for the channel and funnel. Pathlines represent the lines travelled by neutrally buoyant particles in equilibrium with the fluid motion. Within the straight microchannel, the pathlines predominantly pass over the embryo and barrier washing away embryo secreted factors. Pathlines within the microfunnel design show a vortex effect in the middle of the funnel which helps retain autocrine factors secreted from embryos. Autocrine factor distribution: concentration gradient formed within devices in the presence of an embryo secreting an autocrine factor at a constant physiological rate for 48 h. The normalized minimum value, zero is coloured with blue and the normalized maximum value, one, is coloured with red on the colour scale. The average concentration of autocrine factor in a sampling volume for the straight channel culture dramatically dropped to below 2% of the level found for the microdrop culture. Conversely, the funnel shaped culture retained 40% of the level found for the microdrop culture in the same sampling volume. Scale bar = 100 microns. Mechanical stimulation on embryo: shear stress experienced by the simulated embryo. Minimum shear stress, zero is coloured with blue and maximum value, 7E−3 (dyn/cm^2), is coloured with red on the colour scale. Here, the maximum instantaneous velocity (6.86E−3 cm/s) in Fig. 1c and the velocity (5.56E−4 cm/s) equivalent to a flow rate of 5 μl/h are applied at the inlet for the microfunnel and the microchannel, respectively. The average shear forces per area are 1.99E−3 dyn/cm^2 for the microchannel and 3.55E−3 dyn/cm^2 for the microfunnel.

showed that conventional microdrop cultures retain most of the autocrine factors produced by the embryo, but exert negligible fluid mechanical stimulation. The continuous perfusion microchannel culture provided fluid mechanical stimulation that may be beneficial to the embryo but the flow also removes most of the beneficial autocrine factors. Importantly, the dynamic microfunnel system can provide benefits of both fluid mechanical stimulation to the embryo and retention of significant amounts of autocrine factors simultaneously (Fig. 6). Although the sampling volume analysis was specifically performed for cell secreted factors, it also provided insights into how fluid flow enhances mass transport around the embryo for molecules embryos may consume.

Discussion

Development of *in vitro* based systems that mimic mechanical (Fauci and Dillon, 2006) and biochemical (Hardy and Spanos, 2002) environments that preimplantation embryos experience *in vivo* can lead to enhanced understanding of developmental mechanisms regulating normal embryo growth, and may improve embryonic developmental competence of those grown in the laboratory. In conventional assisted reproductive technologies, embryo culture occurs in Petri dishes with variations in media volume and/or distribution (Trounson and Gardner, 2000), yet *in vivo* environments are dynamic. Within the female reproductive tract, the preimplantation embryo is unique in

that it develops in the absence of direct cell contact with epithelial cells. It is free-floating, lacks a blood supply and is moved continuously through a changing fluid environment. The current research focused on assessing the impact of a dynamic microfluidic culture on mammalian embryo development, implantation and ongoing normal pregnancies.

Even when starting with the same media, protein source, gaseous environment and initial pooled zygote quality, significant enhancements in embryo development and fetus production can be achieved by dynamic microfunnel culture. Although the total blastocyst development rate at 96 h was not significantly different between culture systems tested, it was well in excess of 80% in all cases, demonstrating that all systems tested supported embryo development to a level typically required for quality control of human assisted reproductive technology contact materials and media (Clarke *et al.*, 1995; van den Bergh *et al.*, 1996; McCulloh, 2009). Improvement of embryo development in dynamic microfunnel culture was only delineated when the stage of blastocyst development and the total number of cells per blastocyst were assessed. In addition, these improved embryo development rates obtained in microfluidic dynamic culture experimentally translated into improved implantation and ongoing pregnancy rates. Most importantly these preimplantation embryo developmental kinetics, implantation and ongoing pregnancy rates more closely resembled those observed when embryos developed *in utero* in comparison to embryos grown under conventional static conditions. Collectively, results from these comparative controlled studies suggest that the microenvironment obtained by combining microfluidics, dynamic pulsatile fluid flow and microfunnel embryo detainment supports enhanced embryo development compared with conventional culture conditions. One could question whether this improved embryo development was a consequence of suboptimal development of control embryos. It is impossible to compare results from different laboratories, strains of mice, initial stage of embryo development, soluble culture components, gaseous environments, timing of end-point measures and specifics of end-point measures. However, our advanced blastocyst development rates, total blastocyst cell count, implantation rates and ongoing pregnancy rates for controls are representative of contemporary reports (Lane and Gardner, 2003; Manser *et al.*, 2004; Biggers, 2005; Summers *et al.*, 2005; Feil *et al.*, 2006; Mitchell *et al.*, 2009).

To begin elucidation of mechanisms imparting improved embryonic developmental competence in pulsatile dynamic microfluidic culture, we asked the question of whether fluid flow influenced embryo development in a temporal or developmental-stage specific manner. Forty-eight hours of pulsatile media flow enhanced blastocyst cell count in comparison to static culture, but was not as beneficial as 96 h of dynamic culture, indicating that the duration of pulsatile media flow is important. Secondly, embryo development was not differentially affected in relation to 48 h pulsatile media flow in the first or last half of a 96 h culture window. Thus developmental impact of pulsatile media flow is not developmental-stage specific. This is especially important when one considers that mouse zygotic genome activation occurs at the 1- to 2-cell transition (Schultz, 1993) during the first 48 h of culture, in conjunction with global chromatin remodelling, altered histone acetylation and release from a transcriptionally repressive state (Ma *et al.*, 2001). The lack of differential benefit of pulsatile media flow between the first and last 48 h of culture would also suggest that the developmental benefit is not specific toward latter preimplantation developmental events such as compaction at the 8-cell stage (Johnson and Ziomek, 1981), initiation of cellular polarity at the 8-cell to morula stage (Johnson *et al.*, 1986) or blastoceol formation at the transition form morula to blastocyst (Borland *et al.*, 1977). Collectively these results would suggest that benefits of microfluidic-generated pulsatile fluid flow on embryonic developmental competence may be due to subtle changes in the culture microenvironment that produce embryo development that is more like *in vivo* development and significantly advanced compared with that in static culture. This leads one to consider dynamic culture, embryo development and microenvironment in relation to retention of autocrine factors, removal of waste products and disruption of concentration gradients that may form at cell surfaces.

Embryos may develop better in small volumes or in the presence of multiple embryos, presumably due to beneficial autocrine effects (Diaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002). In the last decade, substantial work has been done to identify and characterize these embryo autocrine factors. Some identified autocrine factors include: leukemia inhibitory factor (Lavranos *et al.*, 1995), interleukin-1 (Zolti *et al.*, 1991), insulin-like growth factor (Rappolee *et al.*, 1992), platelet derived growth factor (Osterlund *et al.*, 1996), EGF (Brice *et al.*, 1993) and transforming growth factor (Babalola and Schultz, 1995). Utilization of embryo secreted autocrine factors is advantageous over exogeneous delivery of mitogens because: (i) embryos produce a complex mixture of biochemicals which is difficult to completely mimic with exogeneous biomolecules, and (ii) addition of exogenous growth factors has inherent limitations in spatial and temporal exposure, and can sometimes lead to developmental abnormalities such as large offspring syndrome (Young *et al.*, 1998; Young *et al.*, 2001). The Fluent modelling system (Fig. 6) demonstrated that the microfunnel culture system with pulsatile refresh retains greater amounts of biomolecules compared with microchannels with flow, which have had sub-optimal embryo development (Hickman *et al.*, 2002). However, retention of beneficial autocrine factors cannot fully explain the benefits of microfunnel pulsatile culture since this occurs to a greater extent in microdrops, which displayed reduced embryo developmental competence compared with the microfunnel dynamic system.

Culturing embryos in small droplets may allow accumulation of toxic substances such as ammonia (Gardner and Lane, 1993) and oxygen-derived radicals (Johnson and Nasresfahani, 1994), which may harm embryos (Lane and Gardner, 2003). Periodic media changes may prevent toxin accumulation, but needs to be balanced with elimination of positive acting auto- and paracrine factors (Fukui *et al.*, 1996). The microfunnel pulsatile embryo culture system employed in the current studies provided periodic media refresh, moderate retention of biomolecules, agitation of fluid surrounding the embryo and removal of media. This combination appears to be advantageous.

Importantly, fluid movement and mechanical agitation of embryos during culture can disrupt concentration gradients of substrates, secretory molecules, dissolved gases and waste products. Movement may ensure that unstirred layers do not form around the embryo, and may facilitate exchange of gases and/or metabolites. Although such point-of-contact concentrations of gases, substrates or metabolites are difficult to measure, one can appreciate that such disruptions of gradients could occur with media flow and embryo agitation.

However, in relation to dissolved gases, such as O_2, the importance of this agitation is questionable. In static microdrop or organ-well mouse embryo culture, the optimal oxygen concentration is still controversial. Increased mouse blastocyst development rates and cell numbers have been reported following culture under 7% (Gardner and Lane, 1996) or 5% oxygen (Orsi and Leese, 2001; Karagenc *et al.*, 2004), when compared with 20% oxygen. However, others have reported no difference in mouse blastocyst development in 2, 7 or 20% oxygen (Feil *et al.*, 2006). Maybe the most important consideration is that previous studies consider the influence of gaseous oxygen concentration which may not represent soluble O_2 concentrations at the embryo cell surface. A mathematical model demonstrated that mouse embryos in static culture are likely to satisfy their demand for oxygen by diffusion alone, but that human embryos may become marginally hypoxic, especially at lower oxygen levels (Byattsmith *et al.*, 1991). If we had used low gaseous oxygen concentrations in these studies, one could speculate that agitation with pulsatile fluid flow could prevent a hypoxic oxygen gradient at the embryo surface in comparison to static culture, and thus might explain observed embryo development benefits. However, because we used 20% gaseous O_2 in these experiments, it is difficult to propose that soluble O_2 levels fell below 5% or that hypoxic conditions existed in static culture, and that pulsatile fluid flow remedies this condition, and as a consequence enhances embryo development. Experiments focused on point-of-contact O_2 concentrations in static and dynamic embryo culture should be performed in the future.

In conclusion, a portable computerized microfluidic system has been designed for embryo culture. Its user-friendly architecture, flexibility in microchannel and chip design, and programmability of fluid actuation system allows convenient and practical manipulation of chemical and mechanical microenvironments for *in vitro* embryo production. Improved pregnancy outcomes may alleviate some current inefficiencies in embryo production for biomedical research, genetic gain in domestic species and fertility treatment in humans. Combining physiological mechanical stimulation with retention of beneficial autocrine factors may also benefit a broad range of cell culture technologies beyond the dynamic microfunnel embryo culture systems described here.

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