Published in final edited form as:

Biotechnol Bioeng. 2013 December ; 110(12): . doi:10.1002/bit.24986.

Supplemented α MEM/F12-based medium enables the survival and growth of primary ovarian follicles encapsulated in alginate hydrogels

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Abstract

Hydrogel-encapsulating culture systems for ovarian follicles support the *in vitro* growth of secondary follicles from various species including mouse, non-primate human, and human; however, the growth of early stage follicles (primary and primordial) has been limited. While encapsulation maintains the structure of early stage follicles, feeder cell populations, such as mouse embryonic fibroblasts (MEFs), are required to stimulate growth and development. Hence, in this report, we investigated feeder-free culture environments for early stage follicle development. Mouse ovarian follicles were encapsulated within alginate hydrogels and cultured in various growth medium formulations. Initial studies employed embryonic stem cell medium formulations as a tool to identify factors that influence the survival, growth, and meiotic competence of early stage follicles. The medium formulation that maximized survival and growth was identified as MEM/F12 supplemented with fetuin, insulin, transferrin, selenium, and follicle stimulating hormone (FSH). This medium stimulated the growth of late primary (average initial diameter of 80 μ m) and early secondary (average initial diameter of 90 μ m) follicles, which developed antral cavities and increased to terminal diameters exceeding 300 μ m in 14 days.

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Author Disclosure Statement

No competing financial interests exist.

Survival ranged from 18% for 80 μm follicles to 36% for 90 μm follicles. Furthermore, 80% of the oocytes from surviving follicles with an initial diameter of 90–100 μm underwent germinal vesicle breakdown (GVBD), and the percentage of metaphase II (MII) eggs was 50%. Follicle/oocyte growth and GVBD/MII rates were not significantly different from MEF co-culture. Survival was reduced relative to MEF co-culture, yet substantially increased relative to the control medium that had been previously used for secondary follicles. Continued development of culture medium could enable mechanistic studies of early stage folliculogenesis and emerging strategies for fertility preservation.

Introduction

In vitro ovarian follicle culture systems are enabling tools for the advancement of fertility preservation technologies for cancer patients (Jeruss and Woodruff 2009; Woodruff 2010). The fertility of girls and women is threatened by cancer therapies, such as chemotherapy and radiation. These lifesaving treatments diminish the follicle pool and trigger premature ovarian failure. Cancer patients have the opportunity to cryopreserve their ovarian tissue before receiving treatment; however, the autotransplantation of cryopreserved ovarian tissue post-treatment risks the reintroduction of cancer cells (Meirow et al. 2008). To avoid this risk, *in vitro* follicle culture and fertilization techniques could be used to produce mature eggs, and subsequently embryos, from cryopreserved ovarian tissue. Remarkable progress towards accomplishing this goal has been achieved via various *in vitro* culture systems (Eppig 1977; Eppig and O'Brien 1996; Lenie et al. 2004; Nayudu and Osborn 1992; O'Brien et al. 2003; Pangas et al. 2003; Torrance et al. 1989).

Biomaterials, such as alginate hydrogels, have been employed to maintain the three-dimensional structure and cell-cell interactions of developing follicles (Pangas et al. 2003; Shikanov et al. 2011). Alginate is an algae-derived polysaccharide polymer, which has been utilized in many tissue growth applications (Augst et al. 2006). Alginate-based culture systems have supported the *in vitro* growth of preantral mouse, non-human primate, and human follicles (Pangas et al. 2003; Shikanov et al. 2009; West et al. 2007; Xu et al. 2013; Xu et al. 2011; Xu et al. 2009a; Xu et al. 2006b; Xu et al. 2009b), with mouse follicles producing oocytes capable of fertilization and live births (Xu et al. 2006a). Additionally, these systems have facilitated mechanistic studies of folliculogenesis, including mechanics, extracellular matrix and gene expression (Kreeger et al. 2006; Kreeger et al. 2005; Parrish et al. 2011; Skory et al. 2013; West et al. 2007; Xu et al. 2006b). While effective for secondary and multilayer follicles, alginate-based culture systems do not support the consistent growth of early stage follicles (primordial and primary). Various culture approaches have been developed for early stage follicles; however, success has been limited (Abir et al. 2006; Itoh and Hoshi 2000; Lenie et al. 2004; Muruvi et al. 2005; Muruvi et al. 2009; Saha et al. 2000). These systems have not focused on maintaining the follicle structure as a component of the culture system. Success with the culture of early stage follicles has been achieved via *in situ* organ culture or two-step culture systems (Eppig and O'Brien 1996; Jin et al. 2010), in which ovarian tissue containing early stage follicles is cultured *in vitro*. The tissue and stromal cells provide physical support as well as signals that stimulate follicle growth. Recently, fibrin has also been shown to promote the growth of primate primary follicles (Xu et al. 2013).

Hydrogels function to maintain the three-dimensional structure of follicles (Hornick et al. 2012). The culture of early stage follicles on traditional culture substrates can lead to a disruption of cell-cell connections and normal follicular structure, which often results in the separation of the oocyte from the somatic cells. Physical manipulations have been used to prevent cell attachment and migration of somatic cells in primary follicle culture systems

that utilize flat surfaces (Lenie et al. 2004). In contrast, hydrogels encapsulate follicles and preserve cell-cell connections, which promotes survival of early stage follicles. However, the physical support provided by the hydrogel must be combined with signals to promote follicle growth. The culture medium formulations that support secondary follicle growth have not been effective in stimulating primordial and primary follicle growth. Furthermore, the supplementation of various growth factors, including fibroblast growth factor 2 (FGF2 or bFGF), leukemia inhibitory factor (LIF), epidermal growth factor, (EGF) and nerve growth factor (NGF) (Berkholtz 2006; Tagler et al. 2012), has been unsuccessful at promoting the growth of isolated early stage follicles.

The growth of primary follicles within alginate hydrogels has been successful via co-culture with ovarian stromal cells (theca-interstitial cells, macrophages, and other supporting cell types that surround follicles in the ovary) (Tingen et al. 2011), mouse embryonic fibroblasts (MEFs) (Tagler et al. 2012), and multiple follicles (Hornick et al. 2013). These feeder cell populations secrete paracrine factors that enable the survival, growth, and development of early stage follicles, which can produce mature eggs. While effective, these co-culture methods promote growth by undefined mechanisms, have batch-to-batch variability, and clinical translation would risk of transmission of pathogens. Hence, the development of feeder-free culture medium for early stage follicles would benefit both basic science and clinical applications.

In this report, we investigated the design of culture medium for the feeder-free culture of early stage mouse ovarian follicles within alginate hydrogels. This work utilized alginate hydrogels to support follicle structure/survival and medium to stimulate follicle growth/development. Human embryonic stem cell (ESC) culture medium formulations (Chen et al. 2011; Ludwig et al. 2006a; Ludwig et al. 2006b) that replaced co-culture with feeder cells, such as MEFs, served as the foundation for these studies. Numerous factors that significantly influence the survival, growth, and meiotic competence of ovarian follicles were identified from ESC medium formulations. These results led to the systematic development of feeder-free culture medium for early stage follicles that can produce metaphase II (MII) eggs.

Materials and Methods

Animals and Materials

Follicles were isolated from the ovaries of 8–12 day old CD1 female mice. Mice were maintained in accordance with the policies of the National Institutes of Health and Northwestern University's Animal Care and Use Committee. A temperature, humidity, and light (12hL/12hD) controlled barrier facility within Northwestern University's Center of Comparative Medicine (Chicago, IL) was used to house and breed the mice. Mice were provided with food and water *ad libitum*. The mice were fed Teklad Global (Madison, WI) irradiated chow (2919 or 2916) that does not contain soybean or alfalfa meal but does contain minimal phytoestrogens. Unless otherwise specified, all chemical were purchased from Sigma-Aldrich (St Louis, MO) and medium formulations were purchased from Life Technologies (Calsbad, CA). Sodium alginate (55–65% guluronic acid) was provided by FMC BioPolymers (Philadelphia, PA).

Follicle Isolation and Alginate Encapsulation

Previously established procedures (Tagler et al. 2012; Xu et al. 2006b) for follicle isolation and alginate encapsulation were followed with slight modifications. Briefly, individual follicles (diameter: 70–90 μ m) were mechanically isolated using insulin gauge needles in dissection medium (Leibovitz's L-15 medium supplemented with 1% fetal bovine serum and

0.5% penicillin-streptomycin). Follicles were separated into size classes based on average initial diameter (70, 80 and 90 μm). After isolation, the follicles were washed and stored in maintenance medium (Minimum Essential Medium Eagle Alpha Modification [MEM] supplemented with 1% fetal bovine serum and 0.5% penicillin-streptomycin). Follicles were then transferred into 5–6 μL solutions of 0.25% alginate and immersed into a 50 mM CaCl_2 and 140 mM NaCl solution for 1–2 minutes to crosslink. Beads were then placed into separate wells of a 96-well flat-bottom tissue culture plate (Corning Costar #3596) containing 100 μL growth medium. Follicle culture was conducted at 37°C in 5% CO_2 for 6–14 days. Every 2 days, half the medium (50 μL) was replaced with fresh growth medium.

Growth Medium Formulations and Components

The base medium of each growth medium formulation was either Minimum Essential Medium Eagle Alpha Modification (MEM) (Life Technologies #32561037), Dulbecco's Modified Eagle Medium Nutrient Mixture (DMEM) (Life Technologies #10567014), Ham's F12 Nutrient Mixture (F12) (Life Technologies #31765035), Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) (Life Technologies #11330032), mTeSR1 human embryonic stem cell (hESC) medium (STEMCELL Technologies, Vancouver, Canada) (Ludwig et al. 2006a), STEMPRO® (Life Technologies), or E8 human induced pluripotent stem cell (iPS) medium (Chen et al. 2011). Growth medium formulations were supplemented with the following: 3–13 mg/mL bovine serum albumin (MP Biomedicals, Solon, OH), 1 mg/mL bovine fetuin, 5–25 $\mu\text{g/mL}$ insulin, 5–25 $\mu\text{g/mL}$ transferrin, 5–25 ng/mL selenium, 10 mIU/mL recombinant human follicle-stimulating hormone (FSH) (A.F. Parlow, NHPP, NIDDK), 10–100 ng/mL recombinant human fibroblast growth factor 2 (FGF2 or bFGF) (Life Technologies), 0.5–2 ng/mL transforming growth factor beta 1 (TGF-1) (R&D Systems, Minneapolis, MN), 64 $\mu\text{g/mL}$ ascorbic acid 2-phosphate magnesium, 50 $\mu\text{g/mL}$ l-ascorbic acid, 6 $\mu\text{g/mL}$ thiamine, 2 $\mu\text{g/mL}$ glutathione, 0.1 $\mu\text{g/mL}$ pipercolic acid (MP Biomedicals), 41 $\mu\text{g/mL}$ lithium chloride, 100 $\mu\text{g/mL}$ gamma-aminobutyric acid, 1% non-essential amino acid solution (NEAA) (Life Technologies), 0.2% chemically defined lipid concentrate solution (Life Technologies), 0.2%/0.1% trace element solutions B/C (Corning CellGro Mediatech, Manassas, VA), 8 $\mu\text{g/mL}$ 2-mercaptoethanol (Life Technologies), and 540 $\mu\text{g/mL}$ sodium bicarbonate. All chemicals were prepared, stored, and utilized according to the manufacturer's protocols. The control growth medium, which supports secondary follicle culture (Xu et al. 2006b), was MEM supplemented with 3 mg/mL bovine serum albumin, 1 mg/mL bovine fetuin, 5 $\mu\text{g/mL}$ insulin, 5 $\mu\text{g/mL}$ transferrin, 5 ng/mL selenium, 10 mIU/mL and recombinant human follicle-stimulating hormone (FSH).

Follicle Survival and Growth

Images of follicles were collected every other day using an inverted Leica DM light microscope (Leica, Wetzlar, Germany). Follicle diameters were measured using ImageJ software (National Institutes of Health, Bethesda, MD). An average of two perpendicular diameter measurements was used for each follicle at each time point. Survival was determined via follicle/oocyte morphology. Non-growing follicles or follicles with fragmented, shrunken, degenerated, or detached oocytes were considered dead.

Oocyte Meiotic Competence

Follicles were removed from the alginate beads by incubating with 50 μL of MEM medium containing 10 IU/mL alginate lyase for 5–10 minutes. Next, follicles were incubated in MEM medium supplemented with 10% fetal bovine serum, 5 ng/mL epidermal growth factor (EGF), and 1.5 IU/mL human chorionic gonadotropin (hCG) for 14–16 hours (Smitz et al. 1998). Oocytes were then removed from the follicles using 0.3% hyaluronidase. Images were captured via light microscope (inverted Leica DM) to assess oocyte meiotic competence. Oocytes were classified as follows: germinal vesicle (GV) if the

germinal vesicle was present, germinal vesicle breakdown (GVBD) if the germinal vesicle was not present, metaphase II (MII) if there was germinal vesicle breakdown and a polar body in the perivitelline space, or degenerated (DG) if the oocyte was fragmented or shrunken.

Statistical Analysis

Survival and growth data were collected via thirty-five independent cultures of approximately 80–120 follicles each. For each medium condition, oocyte meiotic competence data were collected from at least four independent *in vitro* maturation experiments. Statistical analysis was performed using Microsoft Excel and GraphPad Prism. Follicle and oocyte growth data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey for single time point comparisons. Survival data were analyzed using the Kaplan-Meier log-rank test. Oocyte meiotic competence data were evaluated by χ^2 (chi-square test or Fisher's exact test) analysis. A p-value less than 0.05 was considered statistically significant.

Results

Embryonic Stem Cell (ESC) Medium Formulations

Two commercially available ESC medium formulations, mTeSR1 and STEMPRO® were initially investigated as replacements for ovarian follicle co-culture with feeder cells (i.e. MEFs). The survival and growth of early secondary (average initial diameter of 90 μm) and late primary follicles (average initial diameter of 70–80 μm) in mTeSR1 was significantly enhanced compared to the control medium (Table 1 and Figure 1). The control growth medium, which supports secondary follicle culture (Xu et al. 2006b), consisted of MEM supplemented bovine serum albumin (BSA), fetuin, insulin, transferrin, selenium (ITS), and follicle-stimulating hormone (FSH). None of the 90 μm follicles in control medium survived past day 10, and none of the control 70–80 μm follicles survived past day 6. The survival and growth of follicles cultured in mTeSR1 were not significantly different from co-culture with mouse embryonic fibroblasts (MEFs) (Tagler et al. 2012). After 14 days in mTeSR1, the 70, 80 and 90 μm follicles reached 275 ± 15 , 309 ± 49 , and 365 ± 37 μm (mean $\pm$ standard deviation). Survival was 21%, 53% and 67% respectively. STEMPRO® (DMEM/F12 base medium supplemented with mercaptoethanol, non-essential amino acids, albumin, lipids, activin A, fibroblast growth factor 2, and insulin-like growth factor 1) did not significantly improve follicle survival or growth relative to the control medium (**data not shown**).

Numerous medium formulations based on mTeSR1 were tested in order to determine the factors influencing follicle survival and growth (Supplemental Table 1 and Supplemental Figure 1). The mTeSR1 components were separated into groups based on growth factors, antioxidants, vitamins, lipids, and trace elements. Numerous candidate factors that significantly influenced follicle development were identified including DMEM/F12 medium, ascorbic acid, fibroblast growth factor 2 (FGF2 or bFGF), and transforming growth factor beta 1 (TGF- β 1). While these formulations were effective at promoting follicle growth similar to mTeSR1, none of the formulations were able to attain the survival rate for follicles cultured in mTeSR1.

A refined/simplified mTeSR1-based medium, E8, (Table 1 and Figure 2) (representative images of cultured follicles: Figure 3) was investigated to further identify candidate factors and create a simplified culture medium. E8 is serum-free and contains 7 components (insulin, selenium, transferrin (ITS), ascorbic acid, FGF2, TGF- β 1, and sodium bicarbonate) added to DMEM/F12 base medium, while mTeSR1 contains 10 additional components (albumin, non-essential amino acids, trace elements, glutathione, mercaptoethanol, thiamine,

lipids, lithium chloride, aminobutyric acid, and pipecolic acid). Both of these medium formulations were equally effective at ESC culture (Chen et al. 2011). Similarly, the survival and growth of follicles in E8 was not significantly different from mTeSR1. After 12 days in E8, the 80 and 90 μm follicles reached diameters of 260 ± 32 and 281 ± 48 μm respectively. Survival for the 80 and 90 μm follicles was 67% and 70% respectively.

Studies subsequently investigated the components of E8 in order to isolate the critical factors influencing follicle development. The E8 growth factors (FGF2, TGF- β 1 and ITS) were tested separately (E8-A, E8-B, E8-C, and E8-D medium formulations) (Table 1 and Figure 2). Without FGF2 and TGF- β 1 (E8-D), 80 and 90 μm follicles reached 282 ± 31 and 310 ± 50 μm , and survival was 63% and 67% respectively after 12 days, which was not significantly different than the full E8 medium. FGF2 (E8-B) significantly increased follicle growth compared to E8-D, with 80 and 90 μm follicles reaching diameters of 365 ± 42 and 388 ± 57 μm , and survival was 67% and 65% respectively. No significant differences were observed between high and low ITS concentrations (E8-B and E8-C). TGF- β 1 (E8-A) significantly decreased follicle survival (33% and 42% for 80 and 90 follicles respectively) compared to all other tested medium formulations. In addition, the survival and growth of secondary and multilayer follicles was enhanced by E8-D (Supplementary Figure 2), which suggests that the novelty of these medium formulations is not limited to early stage follicles.

The maximal growth condition (E8-C) and minimal medium formulation (E8-D) were further investigated for oocyte meiotic competence. Follicles cultured in E8-C and E8-D were able to produce metaphase II (MII) oocytes. MII rates were significantly lower for E8-C (24%) and E8-D (8%) (Table 2) relative to MEF co-culture (62%) (Tagler et al. 2012). The rate of degenerated (DG) oocytes was significantly greater in E8-C and E8-D (24–25%) compared to MEF co-culture (13%). Moreover, E8-C and E8-D resulted in significantly smaller terminal oocyte diameters (62–65 μm) than MEF co-culture (68 ± 2 μm) (Table 3) (Tagler et al. 2012).

Sequential Medium

A sequential medium culture system was tested to investigate the stage-specific medium requirements for early stage follicles (Figure 4). Follicles were cultured in E8-C medium for 4 days (time point when follicles reached approximately 120 μm in diameter) and then cultured in control growth medium for an additional 8 days. Previous reports have demonstrated the growth of isolated two-layer secondary follicles (120 μm) in the control medium without co-culture (Xu et al. 2006b). The survival and growth of 80–90 μm follicles in sequential medium was not significantly different from E8-C for 14 days. Also, the meiotic competence of oocytes from follicles cultured in sequential medium was not significantly different from E8-C (**data not shown**).

Base Medium

Multiple base medium formulations, MEM, DMEM, F12, DMEM/F12 (1:1 ratio), and MEM/F12 (1:1 ratio), were investigated for their ability to promote early stage follicle development (Table 4 and Figure 5). Each medium contained equal concentrations of glucose, pyruvate, ascorbic acid, ITS, and FSH. Follicles cultured in the individual base medium formulations of MEM, DMEM, and F12 had minimal growth (<150 μm terminal diameter) and did not survive past day 10. Follicle growth and survival was not significantly different among these medium formulations. Interestingly, supplementing MEM or DMEM with F12 significantly enhanced survival and growth compared to MEM, DMEM, or F12 individually, identifying F12 as a supplement that supports early stage follicle development. After 12 days in DMEM/F12 and MEM/F12, the 90 μm follicles increased in diameter to 289 ± 67 and 306 ± 55 μm , and survival was 56% and 63% respectively. Survival

and growth in DMEM/F12 and MEM/F12 were not significantly different from E8-D. Also, meiotic competence and oocyte size in MEM/F12 was not significantly different from E8-C or E8-D (Tables 2–3).

Serum Proteins

Bovine serum albumin (BSA) and fetuin, which are present in conventional growth medium for secondary follicles (Xu et al. 2006b), were added to the MEM/F12 medium formulation to investigate their influence on early stage follicle development (Table 4 and Figure 6). In particular, serum proteins were investigated as a means to improve meiotic competence. Each medium also contained equal concentrations of ascorbic acid, ITS, and FSH. For 90 μm follicles, the combination of fetuin and BSA had survival and growth (40%, $314 \pm 44 \mu\text{m}$) that was not significantly different from fetuin alone (36%, $331 \pm 42 \mu\text{m}$) or without serum proteins (58%, 347 ± 38) at day 14. BSA alone significantly inhibited follicle survival and growth (0%, $146 \pm 6 \mu\text{m}$). For 80 μm follicles, survival and growth was significantly decreased by the combination of fetuin and BSA (0%, $106 \pm 8 \mu\text{m}$) compared to fetuin alone (18%, $302 \pm 27 \mu\text{m}$).

Supplementing the MEM/F12 medium formulation with BSA and fetuin significantly improved oocyte meiotic competence (50–63% MII rate) and terminal oocyte diameter ($68 \pm 3 \mu\text{m}$) compared to E8-C, E8-D, and MEM/F12 medium without serum proteins (Tables 2–3). In addition, BSA and fetuin significantly decreased the number of degenerated oocytes (5–11%) compared to E8-C and E8-D (25–26%). The improved oocyte meiotic competence and terminal oocyte size results were statistically consistent with MEF co-culture (Tagler et al. 2012). The addition of serum proteins to E8-based medium significantly impaired follicle survival and growth, and *in vitro* maturation was unable to be performed (**data not shown**).

Taken together, these studies have led to the systematic development of a medium formulation for the culture of late primary and early secondary follicles capable of producing MII eggs. The maximal medium condition was identified as MEM/F12 (1:1) supplemented with fetuin, ITS, and FSH (Table 4). Terminal follicle growth (331 ± 42 and $302 \pm 27 \mu\text{m}$ for 90 and 80 μm follicles respectively) was not significantly different from MEF co-culture (344 ± 39 and $314 \pm 52 \mu\text{m}$). Survival (36% and 18%) was significantly less than MEF co-culture (62% and 48%), but significantly greater than the control medium (0% and 0%). Terminal oocyte size, GVBD rate, and MII rate ($68 \pm 2 \mu\text{m}$, 80%, and 50%) were not significantly different from MEF co-culture ($68 \pm 2 \mu\text{m}$, 78%, and 62%).

Discussion

This study investigated culture medium formulations for late primary and early secondary mouse ovarian follicles within alginate hydrogels. Biomaterials and culture medium were combined to maintain follicle structure/survival and stimulate follicle growth/development. These results build upon our previous co-culture study that utilized mouse embryonic fibroblasts (MEFs) (Tagler et al. 2012). In this report, we sought to develop a feeder-free culture medium that supports the survival, growth, and meiotic competence of early stage follicles at levels similar to MEFs. Previous reports have identified various factors, including base medium, growth factors, or serum proteins, in early stage follicle development (Knight and Glister 2006; Picton et al. 2008); however, this study uniquely investigated the effect of these factors in the context of biomaterials and three-dimensional follicle culture. The influence of geometry and mechanics on various cellular processes, such as proliferation, migration, gene expression, and differentiation, has been well established (Abir et al. 1999; Engler et al. 2006; Petersen et al. 1992; Tanaka et al. 2004).

This study further demonstrated that the combination of signals from both the matrix and medium environment is critical to achieving early stage follicle survival and growth.

Our previous studies with MEFs motivated the use of embryonic stem cell (ESC) culture medium formulations, such as mTeSR1 and E8 (Chen et al. 2011; Ludwig et al. 2006a). Initially, various factors associated with MEFs and follicle development, including leukemia inhibitory factor (LIF) and fibroblasts growth factor 2 (FGF2 or bFGF), were tested unsuccessfully (Tagler et al. 2012). Furthermore, bioinformatics tools were used to identify additional candidate factors, including as insulin-like growth factor 1 (IGF-1), bone monogenetic proteins 4/7 (BMP-4/7), and transforming growth factor beta 1 (TGF- 1), by comparing MEF proteomic data (Buhr et al. 2007; Chin et al. 2007) with *in vitro* and *in vivo* follicle development microarray data. Nevertheless, these factors were unsuccessful at improving growth as well (**data not shown**). Hence, we were led to investigate alternatives, such as complex medium formulations that replaced MEFs in ESC culture. As reported, mTeSR1 and E8 enhanced the survival and growth of early stage ovarian follicles equal to MEF co-culture.

Factors in mTeSR1 and E8, such as FGF2, TGF- 1, and ascorbic acid, are also associated with ovarian follicle development. FGF2 is known to promote the primordial-primary transition (Chaves et al. 2012; Nilsson and Skinner 2004), and TGF- 1 has been reported to have both stimulating and inhibitory effects on early stage follicles (Knight and Glister 2006). Ascorbic acid has been associated with increased follicle survival, tissue remodeling, and steroid synthesis (Murray et al. 2001; Rossetto et al. 2009; Thomas et al. 2001). The influence of FGF2 and TGF- 1 was further explored in this study. In the E8-based medium, TGF- 1 resulted in significantly lower follicle survival, and FGF2 promoted significantly larger follicle growth. However, larger follicle growth did not significantly improve oocyte meiotic competence, which suggests uncoordinated growth/development between the somatic cells (granulosa and theca) and oocyte. Surprisingly, these factors did not influence growth when added to the control growth medium (MEM supplemented with BSA, fetuin, ITS, and FSH), which could be explained by different base medium, serum proteins, and/or interactions among factors.

Base medium had a significant influence on follicle survival and growth, which supports the notion that the metabolic environment plays a critical role in early follicle development. Previous reports have investigated various base medium formulations, such as MEM, DMEM/F12, Waymouth, and McCoy's 5A, on follicle and oocyte development (Ding et al. 2012; Eppig et al. 1990; Hirao 2011; Nonowaki 2010; Picton et al. 2008; van de Sandt et al. 1990; Yoshida et al. 1992). However, results vary due to differences in species, medium supplements, and culture system. In this study, F12 was identified as a critical factor for early stage mouse follicle survival and growth, but had no influence on meiotic competence. While similar to MEM and DMEM, F12 contains various inorganic salts (cupric sulfate and zinc sulfate) and other components (hypoxanthine, linoleic acid, putrescine, and thymidine) not present in MEM or DMEM. Zinc has been shown to influence oocyte maturation (Gao et al. 2007; Kim et al. 2011; Kim et al. 2010; Riggio et al. 2003). Hypoxanthine, linoleic acid, and putrescine have also been linked to oocyte development and meiotic arrest (Eppig et al. 1985; Homa and Brown 1992; Marei et al. 2010; Sunkara et al. 1981; Zeron et al. 2001). Moreover, the concentrations of various amino acids, vitamins, sugars, and other components vary between MEM, DMEM, and F12. While the specific critical factor(s) and mechanism have yet to be elucidated, this study supports the hypothesis that the metabolic environment has a significant influence in early stage follicle development. In addition, this study parallels the past development of *in vitro* fertilization and embryo culture medium formulations (Biggers and Summers 2008; Biggers et al. 1967; Summers and Biggers 2003).

Furthermore, this study confirmed previous reports about the significance of serum proteins on follicle growth. Fetuin significantly improved meiotic competence and oocyte growth. Without fetuin, the somatic cells survived and proliferated, but oocyte growth and maturation was significantly decreased. Thus, supplementing fetuin enabled the coordinated growth of both the somatic cells (granulosa and theca) and oocyte. Previous studies have also demonstrated that serum proteins improve meiotic competence (Eppig et al. 1992), and fetuin inhibits zona pellucida hardening (Schroeder et al. 1990). Serum proteins are also hypothesized to regulate osmolality, remove toxins, and transport lipids or growth factors. In contrast to the positive effects of fetuin, albumin had toxic effects on late primary follicles and inhibited growth, which could be due to impurities.

In conclusion, we have developed a culture system that supports early secondary (initial diameter of 90 μm) and late primary (initial diameter of 80 μm) mouse ovarian follicles. This system combined biomaterials (alginate hydrogels) and medium (soluble factors) to support follicle structure/survival and initiate growth. The medium replicated the effects of feeder-cell populations, such as stromal cells and MEFs, on early stage follicles. Follicles with a diameter in the range of 80–90 μm were cultured to the antral stage. Furthermore, follicles with a diameter ranging from 90–100 μm were matured to produce metaphase II eggs. The maximal medium condition was identified as MEM/F12 supplemented with fetuin, ITS, and FSH. Base medium, growth factors, and serum proteins were major contributors to follicle survival, growth, and meiotic competence. This culture medium motivates further investigations into follicle/oocyte metabolism and may lead to novel strategies for fertility preservation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Mr. Raymond Lee, Mr. Brendan Tran, Dr. Jessica Hornick, and Dr. Francesca Duncan for technical assistance, guidance, and discussion. This research was supported by grants from the National Institutes of Health (NIH) (PL1EB008542), Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research (SCCPIR) (U54HD41857 and U54HD076188), National Health and Medical Research Council (NHMRC) (Australia) (C.J. Martin Fellowship to Y.M., GNT1016460), and Northwestern University (NIH Biotechnology Training Program and McCormick School of Engineering Fellowships to D.T.). The content of this research is solely the responsibility of the authors and does not necessarily represent the official views of the NIH, NICHD, NHMRC, or Northwestern University.

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Figure 1. Embryonic Stem Cell (ESC) Medium Enabled Early Secondary and Primary Ovarian Follicle Culture

The mTeSR1 medium significantly enhanced the survival and growth (diameter mean $\pm$ standard deviation) of early secondary (90 μm) (A–B), late primary (80 μm) (C–D), and primary (70 μm) (E–F) follicles compared to the control medium. The control medium, which supports secondary follicle culture, was MEM supplemented with BSA, fetuin, ITS, and FSH. In the control medium, no follicles survived (n.s.) on days 10 and 14. The survival and growth of follicles in mTeSR1 was not significantly different than MEF co-culture (Tagler et al., 2012). Survival trajectories with different lower case letters (a–b) are

significantly different ($p < 0.001$). Growth bars on each day labeled with different lower case letters (a–b) are significantly different ($p < 0.001$).

Figure 2. Growth Medium Formulations based on E8 Medium Enhanced Early Stage Follicle Development

The survival and growth (diameter mean $\pm$ standard deviation) of early secondary (90 μ m) (A–B) and late primary (80 μ m) (C–D) follicles was significantly enhanced by E8 and E8-based medium formulations. Survival trajectories with different lower case letters (a–b) are significantly different ($p < 0.05$). Growth bars labeled with different lower case letters (a–b) are significantly different ($p < 0.01$) on day 12.

Figure 3. Follicle and Oocyte Morphology

Representative images of encapsulated follicles in alginate hydrogels on (A) day 0, (B) day 2, (C) day 6, (D) day 10, and (E) day 12 of culture. Images of (F) follicle with antral cavity (*), (G) metaphase II (MII) egg with polar body (arrow), (H) detailed view of multilayer follicle morphology on day 4, and (I) degenerated follicle with extruded oocyte from control medium. Scale bars are (A–F) 100 μ m, (G) 25 μ m, and (H–I) 50 μ m.

Figure 4. Sequential Medium

The survival and growth (diameter mean $\pm$ standard deviation) of early secondary (90 μm) (A–B) and late primary (80 μm) (C–D) was not significantly different between E8 medium (12 days) and sequential medium (E8 medium for 4 days and then control medium for 8 days). Both E8 medium and sequential medium had significantly greater survival and growth than the control medium. In the control medium, no follicles survived (n.s.) on days 10–12. Survival trajectories with different lower case letters (a–b) are significantly different ($p < 0.001$). Growth bars on each day labeled with different lower case letters (a–b) are significantly different ($p < 0.01$).

Figure 5. Base Medium Formulations Influenced Follicle Development

The (A) survival and (B) growth (diameter mean $\pm$ standard deviation) of early secondary (90 μ m) was significantly enhanced by supplementing MEM or DMEM base medium with F12. Each medium also contained equal concentrations of glucose, pyruvate, ascorbic acid, ITS, and FSH. In the MEM, DMEM, and F12 medium formulations, no follicles survived (n.s.) on day 12. Survival trajectories with different lower case letters (a–b) are significantly different ($p < 0.001$). Growth bars labeled with different lower case letters (a–b) are significantly different ($p < 0.05$) on day 10.

Figure 6. Serum Proteins Influenced Follicle Development

The survival and growth (diameter mean $\pm$ standard deviation) of early secondary (90 μm) (A–B) and late primary (80 μm) (C–D) was significantly influenced by serum proteins (BSA and fetuin). Each medium also contained equal concentrations of ascorbic acid, ITS, and FSH. In the MEM/F12/BSA and MEM/F12/BSA/Fetuin medium formulations, no follicles survived (n.s.) on days 10–14. Survival trajectories with different lower case letters (a–b) are significantly different ($p < 0.05$). Growth bars labeled with different lower case letters (a–b) are significantly different ($p < 0.01$) on day 12.

Growth Medium Formulations

Table 1

Medium Formulation	Control	mTeSR1 ^a	ESC Medium	E8 ^a	ESC Medium	TGF- β 1	E8-B	FGF2 High ITS	FGF2 Low ITS	E8-D
Description	Follicle Medium	ESC Medium	ESC Medium	ESC Medium	ESC Medium	TGF- β 1	FGF2 High ITS	FGF2 Low ITS	Minimal Condition	Minimal Condition
Base Medium	MEM	MEM/F12	MEM/F12	MEM/F12	MEM/F12	MEM/F12	HEPES	HEPES	HEPES	HEPES
	GlutaMAX	HEPES	HEPES	HEPES	HEPES	HEPES	HEPES	HEPES	HEPES	HEPES
Bovine Serum Albumin (BSA)	3 mg/ml	13 mg/ml								
Fetuin	1 mg/ml	1 mg/ml								
Follicle Stimulating Hormone (FSH)	10 mIU/ml	10 mIU/ml								10 mIU/ml
Insulin	5 μ g/ml	19 μ g/ml								5 μ g/ml
Transferrin	5 μ g/ml	11 μ g/ml								5 μ g/ml
Selenium	5 ng/ml	14 ng/ml								5 ng/ml
Sodium Bicarbonate (NaHCO ₃)		540 μ g/ml								540 μ g/ml
Non-Essential Amino Acids (NEAA)		1%								
Trace Elements B/C		0.2% 0.1%								
Ascorbic Acid 2-Phosphate		64 μ g/ml								64 μ g/ml
Glutathione		2 μ g/ml								
Mercaptoethanol		8 μ g/ml								
Thiamine		6 μ g/ml								
Lipids		0.2%								
Lithium Chloride (LiCl)		41 μ g/ml								
Fibroblast Growth Factor 2 (FGF2/bFGF)		100 ng/ml								100 ng/ml
Transforming Growth Factor (TGF- β 1)		580 pg/ml								2 ng/ml
Aminobutyric Acid (GABA)		100 μ g/ml								
Pipecolic Acid		130 ng/ml								

^a mTeSR1 commercial medium (Ludwig et al., 2006) supplemented with FSH and fetuin

Table 2Oocyte Meiotic Competence (90–100 μ m Follicles)

Medium Condition	Oocytes	MII (%) [*]	GVBD (%)	GV (%)	DG (%)
E8-C: FGF2, Low ITS	54	9 (24%) ^a	38 (70%) ^a	2 (4%) ^a	14 (26%) ^a
E8-D: Minimal Condition	56	3 (8%) ^a	38 (68%) ^a	4 (7%) ^a	14 (25%) ^a
MEM/F12	22	2 (14%) ^a	14 (64%) ^a	3 (14%) ^a	5 (23%) ^{ab}
MEM/F12 BSA Fetus	106	53 (63%) ^b	84 (80%) ^a	10 (9%) ^a	12 (11%) ^b
MEM/F12 Fetus	20	8 (50%) ^b	16 (80%) ^a	3 (15%) ^a	1 (5%) ^b
MEF Co-Culture (Tagler et al., 2012)	103	50 (62%) ^b	81 (78%) ^a	9 (9%) ^a	13 (13%) ^b

^{*} MII rate is relative to follicles that achieved GVBD^{a–b} Different lower case letters (a–b) are significantly different (p<0.05)

MII: Metaphase II Egg

GVBD: Germinal Vesicle Breakdown

GV: Germinal Vesicle

DG: Degenerated

Table 3Terminal Oocyte Size (90–100 μm Follicles) (diameter mean $\pm$ standard deviation)

Medium Condition	MII (μm)	GVBD (μm)
E8-C: FGF2, Low ITS	62 $\pm$ 4 ^a	65 $\pm$ 3 ^a
E8-D: Minimal Condition	63 $\pm$ 2 ^a	65 $\pm$ 3 ^a
MEM/F12	62 $\pm$ 2 ^a	63 $\pm$ 2 ^a
MEM/F12 BSA Fetuin	68 $\pm$ 3 ^b	68 $\pm$ 3 ^b
MEM/F12 Fetuin	68 $\pm$ 2 ^b	68 $\pm$ 2 ^b
MEF Co-Culture (Tagler et al., 2012)	68 $\pm$ 2 ^b	68 $\pm$ 2 ^b

^{a-b} Different lower case letters (a–b) are significantly different ($p < 0.05$)

Table 4

Base Medium and Serum Protein Medium Formulations

Medium Formulation	MEM	DMEM	F12	DMEM/F12	MEM/F12	MEM/F12 BSA/Fetuin	MEM/F12 Fetuin	MEM/F12 BSA
Description	MEM	DMEM	F12	DMEM/F12	MEM/F12	MEM/F12	MEM/F12	MEM/F12
	Serum-Free	Serum-Free	Serum-Free	Serum-Free	Serum-Free	BSA and Fetuin	Fetuin	BSA
Base Medium	MEM	DMEM	F12	DMEM	MEM	MEM	MEM	MEM
				F12 (1:1)	F12 (1:1)	F12 (1:1)	F12 (1:1)	F12 (1:1)
	GlutaMAX	GlutaMAX	GlutaMAX	GlutaMAX	GlutaMAX	GlutaMAX	GlutaMAX	GlutaMAX
	Low Glucose	Low Glucose	Low Glucose	Low Glucose	Low Glucose	Low Glucose	Low Glucose	Low Glucose
	Pyruvate	Pyruvate	Pyruvate	Pyruvate	Pyruvate	Pyruvate	Pyruvate	Pyruvate
BSA						3 mg/ml		3 mg/ml
Fetuin						1 mg/ml	1 mg/ml	
FSH	10 mIU/ml	10 mIU/ml	10 mIU/ml	10 mIU/ml	10 mIU/ml	10 mIU/ml	10 mIU/ml	10 mIU/ml
Insulin	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml
Transferrin	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml
Selenium	5 ng/ml	5 ng/ml	5 ng/ml	5 ng/ml	5 ng/ml	5 ng/ml	5 ng/ml	5 ng/ml
Ascorbic Acid	50 µg/ml	50 µg/ml	50 µg/ml	50 µg/ml	50 µg/ml	50 µg/ml	50 µg/ml	50 µg/ml