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Activin A inhibits activation of human primordial follicles in vitro

Chi Christina Ding · K. Joo Thong · Archie Krishna · Evelyn E. Telfer

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Abstract

Purpose To determine whether Activin A affects the activation and survival of human primordial follicles in vitro.

Methods Ovarian cortical biopsies from eight women undergoing elective caesarean sections or benign gynaecological procedures were taken and cut into small pieces (1–3 mm³), cultured in serum-free medium for 7 days with/without human recombinant Activin A at a concentration of either 50 or 100 ng/ml. Ovarian tissue were analysed by histology for follicle viability, development and density.

Result(s) Significant activation of primordial follicles within cultured cortical tissue was observed after 7 days in control medium. However, medium supplemented with Activin A at 50 ng/ml resulted in significant inhibition of follicular activation. Increasing the concentration of Activin A to 100 ng/ml reversed the inhibitory effect. The effect of Activin

A appeared to be specific to activation of non-growing (primordial) follicles into the growing population since no significant differences in follicle viability was observed between treatment groups.

Conclusion(s) Activin A at a concentration of 50 ng/ml can inhibit the spontaneous activation of human primordial follicles in vitro indicating that this may be a component of the signalling mechanisms that maintain follicular quiescence.

Keywords Primordial follicle · Activin A · In vitro culture · Ovarian cortical tissue

Capsule This study observed a dose dependent inhibitory action of Activin A on the activation of human primordial follicles in vitro.

C. C. Ding
Reproductive Medicine Unit, Liverpool Women's Hospital,
Liverpool, UK

C. C. Ding · K. J. Thong
Assisted Conception Unit,
Edinburgh Fertility and Reproductive Endocrine Centre,
Royal Infirmary of Edinburgh,
Edinburgh, UK

A. Krishna
St. Georges Hospital,
London, UK

E. E. Telfer (✉)
Institute of Cell Biology, The Darwin Building,
University of Edinburgh,
The King's Buildings, Mayfield Road,
Edinburgh, UK
e-mail: Evelyn.Telfer@ed.ac.uk

Introduction

It is estimated that one in 715 of the adult population has been treated for cancer in childhood [1, 2]. Advances in treatments now highlight the adverse affects of such medicines on female fertility. Chemotherapy regimens vary in their gonadotoxicity [3] but for many young women premature ovarian failure (POF) is a likely outcome. To preserve their fertility options many women are now able to store ovarian cortical tissue prior to treatment. However, the option to use this tissue is currently limited to transplantation that may not be suitable for all women [4]. There is therefore a pressing demand for improvement in the area of female fertility preservation and this underpins the drive to develop techniques to support oocyte growth from the abundant population of primordial follicles to maturity in vitro [5]. Such culture systems have been developed for mice and offspring have been produced from oocytes developed in vitro from primordial follicles [6, 7]. Similar culture systems have been difficult to develop in humans but the feasibility of this approach has been demonstrated by the development of human antral follicles from

primordial / primary stages using a two-step culture system [8]. To optimise these systems a greater understanding of factors regulating early follicle development is required.

Maintenance of the “resting” primordial pool of follicles appears mainly to be regulated by inhibitory factors [9] and the removal of ovarian tissue from its *in vivo* environment induces spontaneous activation and growth of primordial follicles [8, 10–13]. This spontaneous activation may lead to increased degeneration therefore it is important to optimise culture conditions to support these early stages. Previous work has shown that Activin A promotes growth and improves health of human preantral follicles grown *in vitro* and the aim of this study was to determine whether Activin had a similar effect at primordial stages.

Activins, which belong to the superfamily of TGF β , are homodimers composed of two inhibin β subunits termed β A and β B. Three isoforms of Activin exist, differing in their β subunits (β A β A as Activin A, β B β B as Activin B and β A β B as Activin AB). Activin A is the most abundant isoform of activins and the most extensively studied member of the TGF β family. Activin is produced by granulosa cells of the ovary and it has been localised in granulosa cells from primary to tertiary follicles [14]. Similar to other TGF β family members, Activin A influences many facets of cell activities such as proliferation, differentiation, motility and apoptosis through the transmembrane receptor mediated Smad pathway. It has complex regulatory mechanisms modulated by extracellular binding proteins (follistatin, β glycan), intracellular antagonistic I-Smads and post transcription modulators [15, 16]. Activin A has been demonstrated to promote granulosa cell proliferation in a dose dependant manner [17, 18].

Studies into the role of Activin A in early folliculogenesis have produced conflicting results that appear to be stage, age and dose dependent. While, Activin A is generally believed to have a stimulatory role on the preantral stage of follicular development [7, 8, 19–22], there are studies that show its inhibitory role in follicular growth and development at this stage [23, 24]. The aim of this study was to determine whether Activin affected the activation of primordial follicles *in vitro* and the subsequent health of growing follicles.

Materials and methods

Patients

Ovarian cortical biopsies of approximately 5–10 mm² were collected after informed consent from 8 women aged 26–39 years (mean \pm SD=33 \pm 5 years) undergoing elective caesarean sections ($n=5$) or benign gynaecological procedures ($n=3$) at the Edinburgh Royal Infirmary. Written

consent was obtained from each patient, and the study was approved by the local ethics committee.

Ovarian tissue culture

Tissue collected during the surgical procedure was transported immediately to the laboratory on a gauze soaked in normal saline ($n=8$). Under sterile conditions, the biopsy tissue was cut into smaller pieces of 2 mm² (range of 1–3 mm²). A piece from each fresh biopsy sample was taken and fixed immediately for histological analysis, whilst the remainder were cultured in pre-warmed 24 well culture dishes (Nunclon, Nunc, Denmark) with inserts (0.4 μ m Millicell Culture Plate Inserts, Millipore, France) for 7 days. Three pieces of tissue from each specimen were added to each well containing 200 μ l of either control medium [McCoy's 5A modified medium supplemented with Human Serum Albumin (5 mg/ml), Penicillin G/Streptomycin Sulphate (0.1 mg/ml), cGMP (1.1 mg/ml), Fresh Ascorbic Acid (50 μ g/ml), L-Glutamine (3 mM), Transferrin (2.5 μ g/ml), Selenium (4 ng/ml), Insulin (10 μ g/ml) and FSH (0.5 IU/ml), all Sigma Chemicals Poole] with or without either 50 or 100 ng of Activin A (Invitrogen) inside and outside of the insert in a humidified incubator at 37°C with 5% CO₂ in air. Every second day 200 μ l of culture medium within the inserts was removed and replaced with fresh medium.

Fixation and histological analysis

Cultured tissue was removed after 7 days and fixed in Bouin's solution for 12 h then dehydrated in increasing concentrations of ethanol (70%, 90% and 100%). Following dehydration, tissue was immersed in cedar wood oil (BDH Laboratory Supplies, Poole UK) for 24 h then placed in toluene (Fisher Scientific UK Ltd, Loughborough UK) for 30 min to remove all traces of the oil. Tissue was then embedded in paraffin wax at 60°C for 4 h, the wax being renewed every hour to ensure complete clearance of all the toluene. 6 μ m thick sections were cut, mounted on gelatine coated slides and left to dry overnight prior to staining with haematoxylin and eosin.

A complete morphological analysis and follicle count was carried out on every 10th section. Follicles were only counted and photographed when the oocyte nucleolus was present to avoid double counting. Developmental stages of viable follicles were classified based on granulosa cell morphology surrounding the follicle: (1) Primordial stage: oocyte surrounded by a layer of flattened granulosa cells; (2) Primary stage: oocyte surrounded by a complete layer of cuboidal granulosa cells; (3) secondary stage: oocyte surrounded by two or more layers of cuboidal granulosa cells. The proportion of follicles at different developmental stage is defined as percentage of viable follicle over the total count.

Table 1 Display data on age and the number of follicles studied for each individual patient

| Patient | A | B | C | D | E | F | G | H | Mean | SEM | Total |
|--------------|-----|-----|-----|-----|-----|----|-----|----|------|------|-------|
| Age | 34 | 36 | 29 | 38 | 30 | 34 | 39 | 26 | 33 | 0.57 | |
| Follicle No. | 258 | 188 | 116 | 180 | 170 | 52 | 111 | 62 | 142 | 8.7 | 1137 |

Follicles were classified for viability using a previously described method [8]: type 1: intact oocyte (regular shape and even cytoplasm) in contact with a complete layer of granulosa cells and less than 10% of pyknotic granulosa cells present; type 2: misshapen oocyte still surrounded by granulosa cells with more than 10% pyknotic granulosa cells; type 3: oocyte dissociated from granulosa cells with more than 10% pyknotic granulosa cells; type 4: fragmented oocyte with more than 10% pyknotic granulosa cells. For analytical purposes, results were then grouped into viable follicle (including type 1 and 2 follicles) and nonviable follicle (including type 3 and 4 follicles). Follicle viability or proportion of viable follicles is defined as percentage of the number of viable follicles out of the total scored.

The integrated measuring tool in the ImageJ software (<http://rsbweb.nih.gov/ij/>) was used to measure tissue area after calibration with a stage micrometer. The volume of a tissue slide is computed by multiplying the tissue area with the known thickness (6 μ m) of each section. The sum of the volume of all slides for a tissue sample is therefore the volume of the tissue. Follicle density of a tissue is defined as the number of follicles divided by the tissue volume.

Statistical analysis

Statistical comparisons between groups for all data (viability, development and density) were carried out using *t*-test. Before analysis, the percentage data were normalized using arcsine transformation. Bonferroni’s correction was used to correct for multiple testing. Significance is reported at the

0.017 level (0.05 before Bonferroni’s correction). Data are presented as mean \pm SEM.

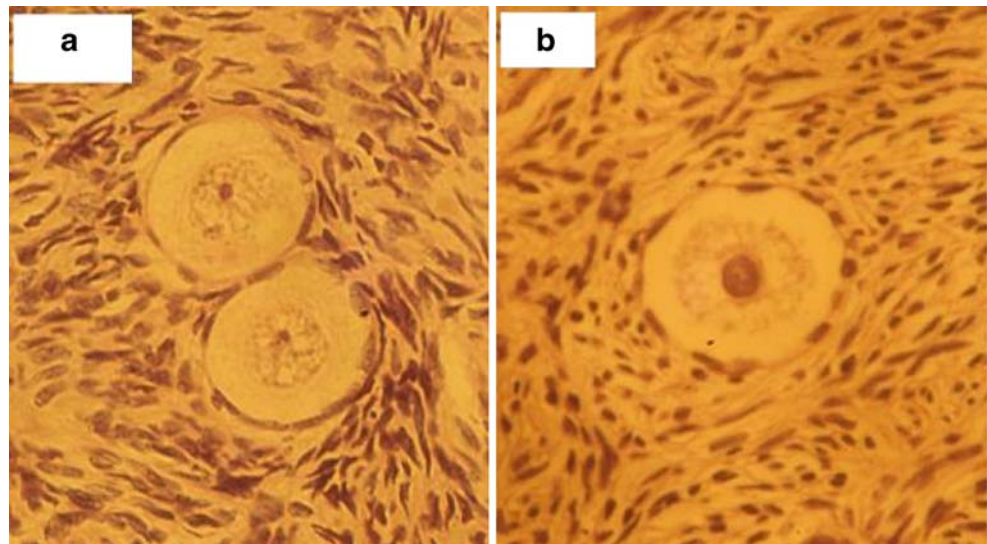
Results

A total of 1137 follicles from eight patients were examined under the light microscope. The numbers of follicles obtained from each patient together with their age are shown in Table 1.

71% of follicles in freshly isolated uncultured tissue (Fig. 1) were identified as primordial. After 7 days of culture significant activation of primordial follicles was observed (73.7% growing) in the control medium, however tissue cultured in medium supplemented with Activin A at 50 ng/ml demonstrated significant inhibition of activation of the primordial population compared with the control medium [53.6% of follicles at primordial stage in presence of 50 ng/ml Activin A compared with 26.3% in control medium ($p < 0.003$)] (Fig. 2). Tissue cultured at the higher dose of Activin A (100 ng/ml) did not show this inhibitory effect after 7 days in culture and the proportion of primordial follicles was not significantly different from that in control media (31.6%).

The proportion of viable follicles after 7 days of culture was significantly reduced by culturing but not affected by treatment with Activin A (42.5% in control group, 41.1% in 50 ng/ml and 51.3% in 100 ng/ml Activin A) compared with the fresh uncultured tissue at day 0 (92.9%) ($P = 0.001$ between the uncultured tissue and all cultured groups, $P > 0.313$ between all cultured groups). (Fig. 3)

Fig. 1 Follicles photographed under 400 \times magnification using a light microscope (a) Uncultured fresh ovarian tissue with primordial follicles (b) Primordial follicle after 7 days culture supplemented with 50 ng/ml Activin A



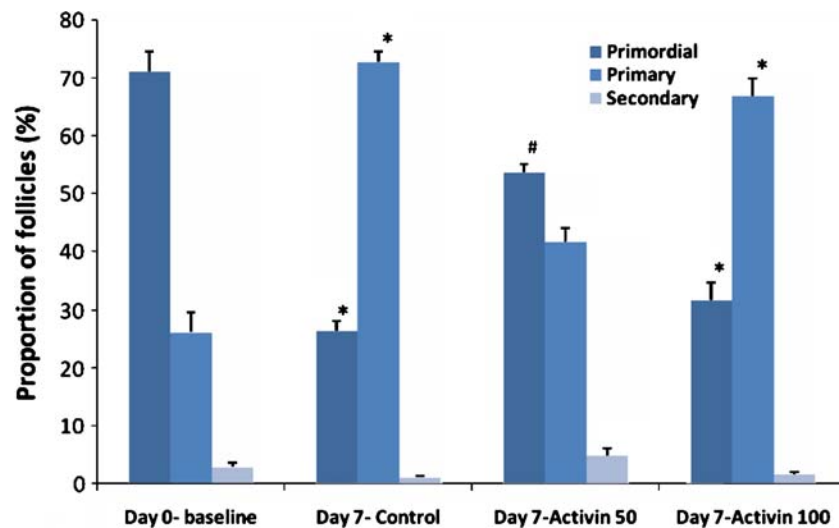


Fig. 2 Histogram showing the proportion of follicles at the primordial (dark blue bars), primary (medium blue bars) and secondary (light blue bars) stages of development in histological samples taken from human ovarian cortical biopsies at the time of collection (Day 0-baseline) and after 7 days of culture in mediums (control medium, medium supplemented with 50 ng/ml or 100 ng/ml of Activin A) of different

treatment groups ($n=8$ for all group). Compared to uncultured Day 0 – baseline, all cultured tissues, with the exception of medium supplemented with 50 ng/ml of Activin A, had significantly less primordial follicles and more primary follicles. * $P<0.01$ versus Day 0 –baseline group for the same follicle developmental stage, # $P<0.01$ versus Day 7- control group

Whilst variation in follicle density was observed between patients, no significant differences were observed between any of the treatment groups either before or after culture (Fig. 4).

Qualitative assessment of the surrounding cellular compartments of follicles indicates there may be a correlation between the inhibition of follicles and increased stromal cell density (Fig. 5). The most dense stroma was found in fresh uncultured tissue followed by tissue cultured with supplemented Activin A (50 ng/ml similar to 100 ng/ml) and the least dense stroma was found in the tissue cultured with control medium.

Discussion

Central to our understanding of female fertility and developing in vitro growth systems is to know how the “resting” population of primordial follicles is regulated. Components of this regulation involve suppressive and activating factors [25, 26]. This is the first study to examine the role of Activin A in the activation of human primordial follicles and we have found that Activin A has a dose dependent inhibitory effect on this process. The proportion of primordial follicles activated to grow was significantly

Fig. 3 Histogram demonstrating the proportion of viable follicles in Day 0 baseline – uncultured fresh tissue and tissue cultured in control medium or medium supplemented with 50 ng/ml or 100 ng/ml Activin A for 7 days ($n=8$ for all groups). * $P<0.001$ versus Day 0 baseline uncultured group

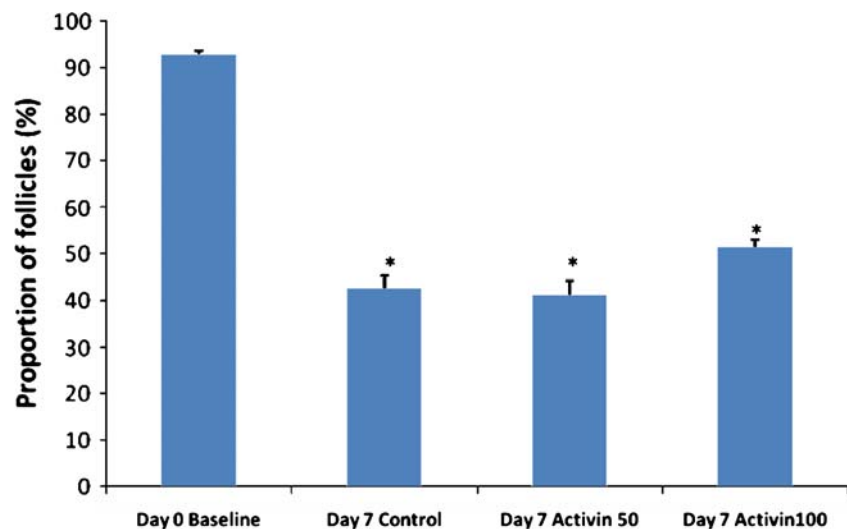
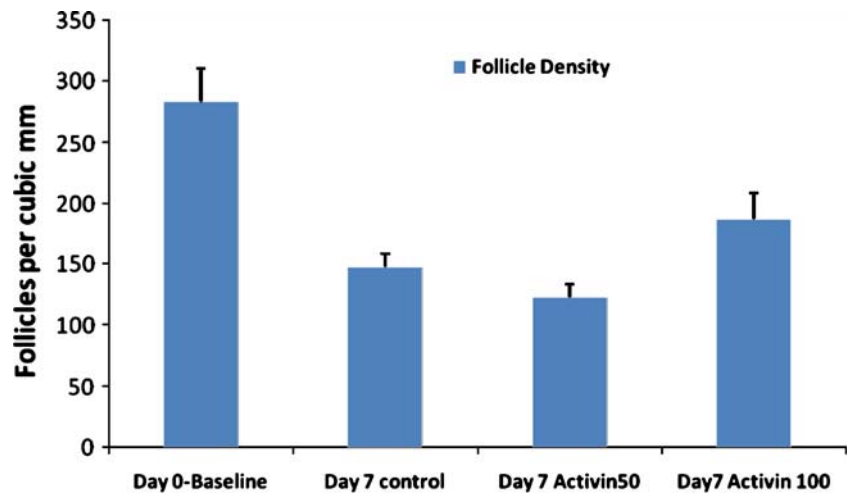


Fig. 4 Histogram displaying the follicle density (mean ± SEM) measured as the total number of follicles per cubic millimetre observed in all treatment groups ($n=8$)



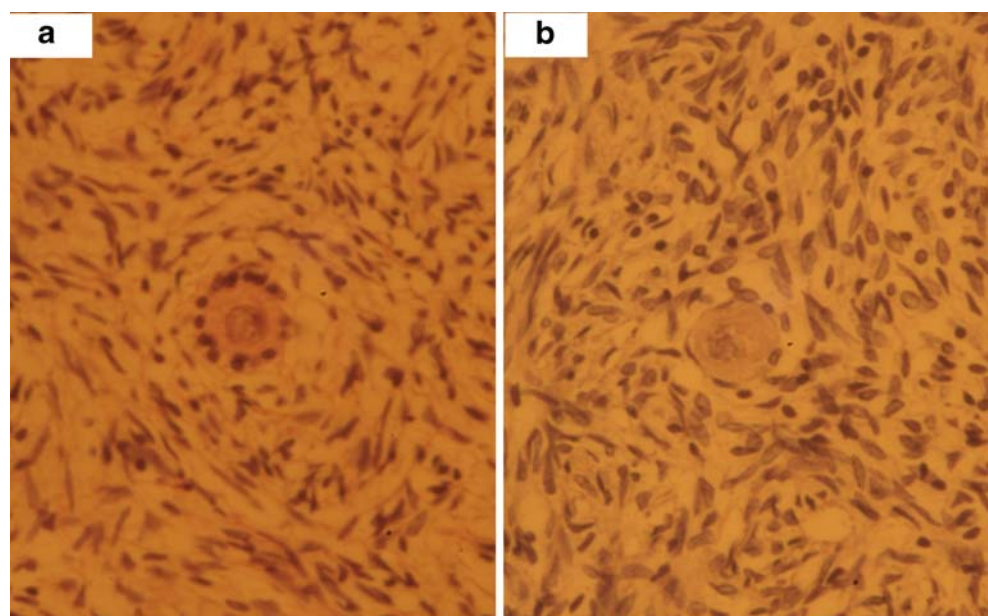
lower in the presence of 50 ng/ml of Activin A compared with control or medium containing 100 ng/ml Activin A.

Activin A has been associated with a stimulatory role during the growing stages of follicle development in a range of species and we have previously shown that its presence enhances in vitro growth of human preantral follicles [8]. However, inhibitory actions of Activin A on small secondary murine follicles have been reported [23]. Indeed, inhibitory roles of activin A have been documented in various cells and tissues such as CHO-K1 (Chinese hamster ovary) cell line [27], and more recently in the luteinisation of human granulosa cells [28]. The exact mechanism of Activin A's inhibitory action is not known but there is some evidence in mice that changes in intraovarian concentration of Activin affects follicle development in a stage dependent manner [23] and the data presented here agrees with that.

Activin ligands act as homodimers of β A subunits (activin A) or β B subunits (activin B) binding transmembrane receptors, which signal through either Smad or extracellular signal-regulated protein kinase (Erk) pathways to affect cell function [29]. The subunits along with the activin receptors type I, IIA and IIB are expressed in ovarian cells during follicular development in human ovaries [30]. Activin or activin receptors have not been localised in human primordial or primary follicles but activin type II receptor has been localised in human secondary follicles [31]. Therefore the effect on primordial follicle activation observed in this study may be the result of an indirect effect of Activin acting via another cell type, possibly stromal cells.

Little is known about the underlying signalling pathways that affect primordial follicle activation but a growing

Fig. 5 Photographed cultured human ovarian cortical tissue demonstrating the differences in stromal density found in tissue cultured in control medium (a) and Activin A (50 ng/ml) (b)



body of evidence is now emerging to show that the phosphatidylinositol-3'-kinase (PI3K-AKT) pathway is a major regulator of early follicle/oocyte development and that components of this pathway are involved in controlling the rate of activation from the non-growing population of follicles [32]. A recent study showed that the phosphatase PTEN acts within the oocyte as a negative regulator of PI3K-AKT and suppresses activation of follicle development in mice [33]. Whether Activin signalling in this culture model affects the PI3K pathway either directly or indirectly should be investigated further.

The absence of an inhibitory effect on follicle activation observed at the higher concentration of Activin A (100 ng/ml) is in accordance with findings from other published animal studies. Mouse ovaries cultured in the presence of 100 ng/ml of Activin A showed no significant difference on the activation of primordial follicles when compared to the control group [34]. This higher dose of Activin-A may be stimulating an increased production of follistatin therefore reducing the biological activity of Activin or it could be altering the differentiation of somatic cells and therefore altering the downstream effect of activin signalling. Further studies using our culture models will be required to elucidate these actions.

It has been suggested that Activin A may inhibit follicular development through atretogenic action. Intra-bursal injection of Activin A has been reported to increase the number of atretic follicles and decrease granulosa cell proliferation [35]. The results presented here indicate that the increased proportion of non-growing follicles observed in 50 ng/ml Activin (54%) compared to control group (26%) was not due to an increase in atresia since no differences in total numbers or viability were detected between groups suggesting that the reduction of the growing follicle in Activin A (50 ng/ml) group is a result of growth inhibition. Our result is in accordance with other reported studies [23, 27, 34] demonstrating a neutral effect of Activin A on follicle survival.

Conclusions

In summary, based on our results, we suggest Activin A may play a role in inhibiting and controlling the recruitment of primordial follicles and their early growth in humans. The exact mechanism for the inhibitory action of Activin still needs to be determined. This may involve studies into the possible differential effects on the neighbouring stromal cell compartment. Further studies to elucidate the reversibility of this dose dependent effect may shed light on the complex interplay/mechanism responsible for the fine regulation of selection and the activation of growth of primordial follicles from the dormant pool.

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Investigations on Human Subjects This study was approved by the local ethics committee.

Conflict of Interest Statement No conflict of interest for any of the submitting authors in reference to the submitted material.

References

1. Scottish Intercollegiate Guidelines Network (SIGN) Long-term follow-up of survivors of childhood cancer. SIGN Publication No. 76. Edinburgh: SIGN 2004.
2. Edgar AB, Morris EM, Kelnar CJ, Wallace HB. Long-term follow-up of survivors of childhood cancer. *Endocr Dev.* 2009; 15:59–80.
3. Meirou D, Schiff E. Appraisal of chemotherapy effects on reproductive outcome according to animal studies and clinical data. *J Natl Cancer Inst Monogr.* 2005;34:21–5.
4. Meirou D. Fertility preservation in cancer patients using stored ovarian tissue: clinical aspects. *Curr Opin Endocrinol Diabetes Obes.* 2008;15(6):536–47.
5. Telfer EE, McLaughlin M. Natural history of the mammalian oocyte. *Reprod Biomed Online.* 2007;15(3):288–95.
6. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod.* 1996;54:197–207.
7. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod.* 2003;68:1682–6.
8. Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod.* 2008;23:1151–8.
9. Fortune JE, Cushman RA, Wahl CM, Kito S. The primordial to primary follicle transition. *Mol Cell Endocrinol.* 2000;63(1–2):53–60.
10. Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. Initiation in vitro of growth of bovine primordial follicles. *Biol Reprod.* 1996;55(5):942–8.
11. Hovatta O, Silye R, Abir R, Krausz T, Winston RM. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Hum Reprod.* 1997;12:1032–6.
12. Hovatta O, Wright C, Krausz T, Hardy K, Winston RM. Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. *Hum Reprod.* 1999;14:2519–24.
13. Fortune JE, Kito S, Wandji SA, Srsen V. Activation of bovine and baboon primordial follicles in vitro. *Theriogenology.* 1998;49(2): 441–9.
14. Rabinovici J, Spencer SJ, Doldi N, Goldsmith PC, Schwall R, Jaffe RB. Activin-A as an intraovarian modulator: actions, localization, and regulation of the intact dimer in human ovarian cells. *J Clin Invest.* 1992;89(5):1528–36.
15. Xu L. Regulation of Smad activities. *Biochim Biophys Acta.* 2006;1759(11–12):503–13.
16. Massagué J, Chen YG. Controlling TGF-beta signaling. *Genes Dev.* 2000;14(6):627–44.
17. Li S, Maruo T, Ladines-Llave CA, Samoto T, Kondo H, Mochizuki M. Expression of transforming growth factor-alpha in

- the human ovary during follicular growth, regression and atresia. *Endocr J*. 1994;41(6):693–701.
18. Zhao J, Taverne MA, van der Weijden GC, Bevers MM, van den Hurk R. Effect of activin A on in vitro development of rat preantral follicles and localization of activin A and activin receptor II. *Biol Reprod*. 2001;65:967–77.
 19. Zhou Y, Sun H, Danila DC, Johnson SR, Sigai DP, Zhang X, et al. Truncated activin type I receptor Alk4 isoforms are dominant negative receptors inhibiting activin signaling. *Mol Endocrinol*. 2000;14(12):2066–75.
 20. Ethier JF, Findlay JK. Roles of activin and its signal transduction mechanisms in reproductive tissues. *Reproduction*. 2001;121:667–75.
 21. Findlay JK, Drummond AE, Dyson ML, Baillie DM, Robertson DM, Ethier JF. Recruitment and development of the follicle; the roles of the transforming growth factor- β superfamily. *Mol Cell Endocrinol*. 2002;191:35–43.
 22. Thomas FH, Walters KA, Telfer EE. How to make a good oocyte: an update on in vitro models to study follicle regulation. *Hum Reprod Update*. 2003;9:1–15.
 23. Mizunuma H, Liu X, Andoh K, Abe Y, Kobayashi J, Yamada K, et al. Activin from secondary follicles causes small preantral follicles to remain dormant at the resting stage. *Endocrinology*. 1999;140:37–42.
 24. Yokota H, Yamada K, Liu X, Kobayashi J, Abe Y, Mizunuma H, et al. Paradoxical action of activin A on folliculogenesis in immature and adult mice. *Endocrinology*. 1997;138:4572–6.
 25. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, et al. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology*. 2002;143(3):1076–84.
 26. Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen APN, Hovatta O. Anti-Müllerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. *Human Reprod*. 2006;21:2223–7.
 27. Gonzalez-Manchon C, Vale W. Activin-A, inhibin and transforming growth factor- β modulate growth of two gonadal cell lines. *Endocrinology*. 1989;125(3):1666–72.
 28. Myers M, van den Driesche S, McNeilly AS, Duncan WC. Activin A reduces luteinisation of human luteinised granulosa cells and has opposing effects to human chorionic gonadotropin in vitro. *J Endocrinol*. 2008;199(2):201–12.
 29. Pangas SA, Woodruff TK. Activin signal transduction pathways. *Trends Endocrinol Metab*. 2000;11(8):309–14.
 30. Roberts VJ, Barth S, el-Roeiy A, Yen SS. Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle. *Clin Endocrinol Metab*. 1993;77(5):1402–10.
 31. Pangas SA, Rademaker AW, Fishman DA, Woodruff TK. Localization of the activin signal transduction components in normal human ovarian follicles: implications for autocrine and paracrine signaling in the ovary. *J Clin Endocrinol Metab*. 2002;87:2644–57.
 32. Jagarlamudi K, Liu L, Adhikari D, Reddy P, Idahl A, Ottander U, et al. Oocyte-specific deletion of Pten in mice reveals a stage-specific function of PTEN/PI3K signaling in oocytes in controlling follicular activation. *PLoS One*. 2009; 9;4(7):e6186.
 33. Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, et al. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science* 2008; 1;319(5863):611–3.
 34. Oktay K, Karlikaya G, Akman O, Ojakian GK, Oktay M. Interaction of extracellular matrix and activin-A in the initiation of follicle growth in the mouse ovary. *Biol Reprod*. 2000;63(2):457–61.
 35. Woodruff TK, Lyon RJ, Hansen SE, Rice GC, Mather JP. Inhibin and activin locally regulate rat ovarian folliculogenesis. *Endocrinology*. 1990;127(6):3196–205.