Expression of anti-Mullerian hormone in hens selected for different ovulation rates

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Abstract

In hens, the granulosa layer is the primary source of anti-Mullerian hormone (AMH), as it is in mammals. Small follicles express the greatest amount of *Amh* mRNA with less in the larger follicles. Laying hens have a distinct ovarian hierarchy of follicles while broiler breeder hens often have excessive follicle growth with a disrupted hierarchy. The objective of Experiment 1 was to examine *Amh* expression in two strains of hens differing in ovulatory efficiency. *Amh* expression was greater ($P < 0.01$) in broiler breeder hens ($n = 6$) as compared with laying hens ($n = 6$). Experiment 2 was designed to examine whether alterations in follicular development due to diet, within the broiler breeder hens, were correlated with changes in the expression of *Amh*. Restricted feeding (RF) in broiler breeder hens promotes optimal follicular development. Egg production in broiler breeder hens on full feed (FF; $n = 8$) was 78% that of hens on RF ($n = 9$). The number of large follicles ($P < 0.05$), total ovarian weight ($P < 0.01$), and *Amh* mRNA expression were greater in FF hens as compared with RF hens ($P < 0.01$). There was no difference in FSH receptor expression between the two groups. A direct nutritional effect was not supported because culture of granulosa cells with varying concentrations of glucose and insulin showed no effect on granulosa *Amh* expression. Finally, testis-conditioned medium resulted in a dose-related increase in granulosa cell proliferation, which could be inhibited by preincubation with AMH antibody. AMH may enhance granulosa cell proliferation through an autocrine or paracrine mechanism although excessive AMH may inhibit optimal follicle selection.

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Introduction

In mammals, anti-Mullerian hormone (AMH or Mullerian inhibiting substance) has the well-described role of regressing the Mullerian duct system in males and no apparent function, prenatally, in females. Immuno-reactive AMH is detected only postnatally in female mammals (Ueno *et al.* 1989a, 1989b). In most female birds, only the left ovary and oviduct develop. AMH has been shown to have a role in regression of the Mullerian ducts in male birds and of the right Mullerian duct in female birds (Tran & Josso 1977, Teng 1987, Teng *et al.* 1987). In birds, both embryonic ovaries express AMH (Teng 1987, Carre-Eusebe *et al.* 1996) although ovarian estrogen (from the left ovary) protects the left Mullerian duct of the chick from AMH action (Teng 2000, 2001) and therefore permits development of the left oviduct.

In postnatal mammals, AMH is localized to the granulosa cells with the strongest expression in the preantral class of follicles. No immunohistochemical staining is observed in the primordial follicles (Ueno *et al.* 1989a, 1989b). AMH is expressed most strongly in granulosa cells adjacent to the oocyte (cumulus), and less expression is present in mural granulosa cells (Ueno

et al. 1989a, Hirobe *et al.* 1992). Although a variety of functions have been proposed for AMH (McGee *et al.* 2001), data from the AMH knock out mouse provide additional insight into possible ovarian function. Female mice null for AMH are fertile (Behringer *et al.* 1994) but detailed analysis of the ovaries indicated that follicular growth is accelerated in the absence of AMH (Durlinger *et al.* 1999). As a consequence, young, cycling mice of the AMH null genotype have a greater number of small, growing follicles that ultimately results in a significant depletion of primordial follicles in older mice.

The cDNA for chicken AMH has been cloned and sequenced (Carre-Eusebe *et al.* 1996, Neeper *et al.* 1996) and the expression pattern of mRNA in embryonic chick gonads determined (Carre-Eusebe *et al.* 1996). The predicted amino acid sequence for chick AMH is ~52% identical to mammalian forms of this hormone in the biologically active C-terminal region (Carre-Eusebe *et al.* 1996). In oviparous species, like the hen, yolk accumulation by the rapidly growing oocyte is the most obvious aspect associated with follicle development. In contrast to mammals, no follicular fluid accumulates and all granulosa cells are in contact with the oocyte (Perry *et al.* 1978). We have recently demonstrated that the

granulosa cells of the hen follicle express *Amh* in a highly regulated pattern (Johnson *et al.* 2008) with the greatest amount in the small follicles and decreasing expression with increased follicle size. In addition, *Amh* expression does not appear to be regulated by estradiol (E_2) or progesterone but is inhibited by a heat-labile factor (not GDF9) associated with the oocyte (Johnson *et al.* 2008).

Laying breeds of chickens typically have very organized follicular development and high egg production in contrast to broiler breeds of hens with excessive, unorganized follicle growth and a lower rate of egg production. The poorly organized hierarchies of broiler breeder hens frequently result in simultaneous, multiple ovulations. Eggs are laid at inappropriate times and eggshell quality is often poor (Yu *et al.* 1992). This ovarian overgrowth can be somewhat controlled by restricted feeding (RF) which slows the rate of follicle growth and therefore, improves egg quality and production (Renema *et al.* 1999, 2001). The average production rate of RF broiler breeder hens is ~180 eggs per production cycle as compared with 250 eggs per production cycle for laying hens.

The association between *Amh* and rate of follicle recruitment in mammals (Durlinger *et al.* 1999) suggests that *Amh* could be involved in the rate of follicle growth in hens. In the present study, we examined the expression of *Amh* in a strain of hens selected for optimal ovulation rate (laying hens) and compared it to expression in a strain of hens selected primarily for optimal growth (broiler breeder hens, the parental line for broiler chicks). Because of the excessive (and inefficient) level of follicle growth in broiler breeder strains of hens, we hypothesized that *Amh* would be decreased in this strain, in line with enhanced follicle development in *Amh* knock-out mice (Durlinger *et al.* 1999). In addition, we investigated whether the commercial practice of RF, which is associated with decreased follicle growth and increased ovulatory efficiency, was associated with alterations in *Amh* expression. We also examined *in vitro* whether likely nutritional signals (glucose or insulin) had a direct effect on *Amh* mRNA expression. Finally, we treated granulosa cells with bioactive AMH (obtained from testis-conditioned medium (TCM)) and examined the effect on cell proliferation.

Results

Results from Experiment 1 are presented in Fig. 1. The comparison in this experiment was made to examine *Amh* expression in two strains of hens differing in ovulatory efficiency during the first year of lay. A significant difference ($P < 0.01$) was found in the overall expression of *Amh* mRNA between broiler breeder and layer hens. In addition, *Amh* mRNA expression was greater in the granulosa layer of 6–12 mm follicles and total ovary (TO; $P < 0.05$, for each) from broiler breeder hens as compared with those tissues from layer hens. It is important to note

Figure 1 Quantitative analysis of expression of *Amh* mRNA in liver, granulosa layer from F1 and 6–12 mm follicles and in TO of laying hens and broiler breeder hens (which were restricted-fed). There was an overall greater expression of *Amh* mRNA in broiler breeder hens ($P < 0.01$) as compared with laying hens. Asterisks indicate differences ($P < 0.05$) in expression between types of hens for a particular tissue ($n = 6$ replicates). Error bars represent mean $\pm$ S.E.M.

that the broiler breeder hens in this experiment had been subjected to RF (87% of *ad libitum*) as is the commercial practice. Experiment 2 was designed to examine whether alterations in follicular development due to diet, within the broiler breeder strain, were correlated with changes in expression of *Amh*. Feed restriction of the broiler breeder hen is associated with decreased follicle growth and a more ordered follicular hierarchy, approaching that observed in laying breeds of hens. By contrast, allowing the broiler breeder hens to feed *ad libitum* (full feed (FF)) results in excessive follicle growth without a defined hierarchy. Figure 2 shows representative ovaries from broiler breeder hens after 1 month of RF (Panel A) or FF (*ad libitum*; Panel B). Body weight, TO weight, and the number of yellow follicles (LYF) larger than 15 mm in the FF hens were significantly greater ($P < 0.05$, $P < 0.01$ and $P < 0.05$ respectively) compared with the RF hens (Table 1). Egg production in the FF hens (calculated over ~1 month) was 78% of that in the RF hens.

Analysis of *Amh* mRNA expression in FF versus RF broiler breeder hens showed that overall, FF broiler breeder hens had an increased level of *Amh* expression (Fig. 3A; $P < 0.01$). In addition, *Amh* expression in the FF hens was significantly higher ($P < 0.05$) in granulosa cells

Figure 2 Representative ovary from a broiler breeder hen after 1 month of restricted feed (A; 87% of *ad libitum*) and from a broiler breeder hen after 1 month of full feed (B; fed *ad libitum*).

Table 1 Comparison of restricted feed (RF) and full feed (FF) broiler breeder hens.

Type of broiler breeder hen	Body weight (kg)	Ovary weight (g)	Number of LYF > 15 mm
RF (n=9)	4.3	75.66	5.0
FF (n=8)	4.9 [†]	99.05*	7.5 [†]

*Indicates significance level of $P < 0.01$. [†]Indicates significance level of $P < 0.05$. LYF, large yellow follicles.

from 3 to 5 mm follicles and in TO. There was no difference in FSH receptor (*Fshr*) expression between the two groups of hens in the same tissues (Fig. 3B). Culture of granulosa cells from 3 to 5 mm follicles (from laying hens) in medium with varying concentrations (relatively high doses) of glucose or insulin had no effect on expression of *Amh* mRNA (data not shown).

Figure 4A shows western blot results of TCM and crude protein lysate generated from very small hen follicles (~1 mm) as a positive control. A band of protein was detected at the expected size of ~70 kDa in all four lanes indicating the presence of AMH in the TCM as well as in small follicles. Figure 4B (3–5 mm follicles) and C (6–8 mm follicles) shows a significant dose-related increase of cell proliferation in response to TCM. Preincubation of the TCM with AMH antiserum reduced the stimulatory effect of TCM on granulosa cell proliferation (3–5 and 6–8 mm follicles). AMH antiserum alone also decreased granulosa cell proliferation.

Discussion

We have shown that ovarian *Amh* mRNA expression is significantly greater in broiler breeder hens as compared with laying hens. The first comparison was made between laying hens and broiler breeder (RF) hens. Both laying and broiler breeder hens were in the first year of production and the data were analyzed with the same standard curve. Greater expression of *Amh* was found in the broiler ovary as compared with the layer ovary although a similar pattern of expression was found for both types of chickens. Most reproductive studies have used laying breeds of hens. These strains are very prolific and a favored model of avian reproductive biologists because of their highly organized ovarian structure and predictable ovulation and oviposition pattern. Reproductive efficiency has been optimized in these breeds and productivity leaves little room for improvement. In contrast to these breeds of chickens, however, are the broiler breeder hens which have good reproduction but also the genetics of fast gain. When allowed to eat *ad libitum*, these chickens become very fat and have excessive ovarian follicular development that often results in erratic ovulations (Yu *et al.* 1992). This ovarian overgrowth can be somewhat controlled by RF (Yu *et al.* 1992, Renema *et al.* 1999, 2001) which improves egg production by enhancing dominant follicle

selection but follicle development is still not as precise as that in laying hens. Feeding level in broiler breeder hens is not associated with any difference in basal or surge amplitude of plasma LH or progesterone or mean level of plasma E_2 (Liu *et al.* 2004).

We had originally hypothesized that broiler breeder hens with increased follicle growth would have lower expression of *Amh* compared with laying hens. We based this on data from the *Amh*-null mouse, where disruption of the *Amh* gene results in enhanced follicle development and premature depletion of oocytes (Durlinger *et al.* 1999). The mouse study indicated that AMH has a role at very early stages in recruitment from the primordial follicle pool. Ovulation and fertility in the *Amh*-null mouse was apparently normal and only upon close examination was the enhanced rate of follicle development observed (Behringer *et al.* 1994, Durlinger *et al.* 1999). Our hypothesis was not correct in that AMH

Figure 3 (A) Quantitative analysis of expression of *Amh* mRNA granulosa layer from 6 to 12 and 3 to 5 mm follicles and in TO. There was greater expression of *Amh* mRNA in tissue from full fed broiler breeder hens ($P < 0.01$) as compared with restricted fed hens. Asterisks indicate differences ($P < 0.05$) in expression for a particular tissue on different feeding regimens ($n = 3$ –5 replicates). (B) Quantitative analysis of expression of *Fshr* mRNA expression in granulosa layer from 6 to 12 and 3 to 5 mm follicles and in TO. There was no difference in *Fshr* mRNA between tissue from hens on different feeding regimens ($n = 5$ replicates). Error bars represent mean $\pm$ s.e.m.

Figure 4 (A) Western blot of AMH protein in testis-conditioned medium (TCM). Protein from TCM (lanes 2, 3, and 4) and from ~1 mm follicles (5 µg; lane 5) was run and probed with AMH antibody. Approximate size of 70 kDa is indicated. Molecular weight standard (std) is in lane 1. (B) Granulosa cell (3–5 mm follicles) proliferation in response to TCM, TCM+AMH antibody or antibody alone. Bars with different letters are significantly different ($P < 0.05$). (C) Granulosa cell (6–8 mm follicles) proliferation in response to TCM, TCM+AMH antibody or antibody alone. Bars with different letters are significantly different ($P < 0.05$).

expression was greater in the FF broiler breeder hens. The condition in the broiler breeder hens is different from the *Amh*-null mouse in that excessive growth of follicles is associated with limited follicle selection from the growing pool of large follicles, resulting in a lack of a defined hierarchy. This resembles polycystic ovary syndrome (PCOS) in women, which is associated with increased serum AMH (Cook *et al.* 2002) which has been used as a diagnostic tool for the disease (Pigny *et al.* 2006). Ovaries in women with PCOS differ from normal ovaries in that dominant follicle selection does not take

place (Visser *et al.* 2006). As a result, many large antral follicles develop, increased over the usual number, although ovulation in these women is irregular at the best.

In the second experiment, we determined that increased follicle growth, resulting from FF, was associated with increased expression of *Amh*. A major function for AMH is that it decreases FSH sensitivity in the growing early antral/small antral follicles (Durlinger *et al.* 2001). It is proposed that this function permits the orderly (and species specific) selection of follicles. We found that allowing the broiler breeder hens to feed *ad libitum* (FF) further increases *Amh* expression relative to RF and is associated with excessive and disrupted follicle development. It is important to point out that *Amh* mRNA concentration is increased (per µg of RNA) and the total number of follicles is also increased in the FF hens. This would likely result in a dramatically increased amount of paracrine and/or endocrine AMH.

Our recent data characterizing *Amh* mRNA expression in the laying hen (Johnson *et al.* 2008) indicate that the highest expression of *Amh* (in laying hens with defined hierarchies) occurs earlier in follicle development (<1–5 mm follicles) than *Fshr*, previously found to be maximal in the granulosa layer of 6–8 mm follicles (You *et al.* 1996). It has been reported that in groups of 6–8 mm follicles isolated from laying hens, one follicle of the group (presumably the selected follicle) shows dramatically increased *Fshr* mRNA in the granulosa layer, compared with the others (Woods & Johnson 2005). It is possible that AMH (through an autocrine/paracrine mechanism) may promote the appropriate and timely development of FSHR.

In vitro and *in vivo* experiments in mice suggest that AMH decreases FSH sensitivity (Durlinger *et al.* 2001). In addition, differential *Amh* expression among morphologically indistinguishable preantral and early antral follicles (Baarends *et al.* 1995) suggests that *Fshr* could be inversely expressed relative to *Amh* (Visser & Themmen 2005). A decrease in FSH sensitivity (through receptors or other mechanisms) associated with enhanced AMH, may permit increased numbers of growing follicles to be maintained (Durlinger *et al.* 2001, Visser *et al.* 2006) with an impairment of follicle selection. Although the overall amount of *Amh* mRNA was higher in FF hens, the expression was found to decrease with increasing follicle size in the FF broiler breeder hens as in RF hens and laying hens. Timely inhibition of *Amh* expression during follicular development may be critical in maintenance of the follicular hierarchy. Although we found an increase in *Amh* mRNA expression, no change in *Fshr* was detected between the FF and RF groups. Our data represent pooled tissue from several small follicles and may not reveal a difference in a single follicle as observed by Woods & Johnson (2005). Also, it may be that the effect of AMH is not direct through the number of FSHR but through secondary mechanisms modulating FSH sensitivity.

We hypothesized that a paracrine or endocrine factor associated with over-nutrition in the FF broiler breeder hen may have increased the expression of *Amh*. It is interesting to note that PCOS with elevated AMH is often associated with obesity and insulin resistance (Eisenhardt *et al.* 2006), suggesting that a factor related to metabolic state may be involved in AMH regulation. Our data (not shown) suggest that glucose and insulin are not responsible for this effect. We tested this in granulosa cells from laying hens which have lower levels of *Amh* mRNA compared with broiler breeder hens and therefore, may more readily show an increase in response to treatment. We have previously shown that neither E₂ nor progesterone, at a variety of doses, regulate *Amh* expression in granulosa cells from laying hens. A heat-labile factor associated with the oocyte (but not GDF9) caused a dose-related inhibition of *Amh* expression (Johnson *et al.* 2008). These results suggested that an oocyte factor could be involved in regulation of AMH expression in the hen as was previously suggested by Salmon *et al.* (2004), for the mouse ovary. It remains to be determined how nutritional factors may modulate signals between the oocyte and the surrounding granulosa cells.

It has been established that mammalian AMH is not biologically active in avian systems (di Clemente *et al.* 1992) and avian AMH is not commercially available. Interestingly, mammalian antibodies recognize avian AMH (di Clemente *et al.* 1992, Johnson *et al.* 2008) so we utilized this to identify AMH in TCM. Bioactive AMH was effective in increasing granulosa cell proliferation and this effect could be blocked by preincubation with AMH antiserum. Antiserum alone reduced granulosa cell proliferation, perhaps by inhibiting endogenous AMH activity, with a greater amount likely produced by 3–5 mm granulosa cells. Although it is at present uncertain how feed intake modulates AMH expression, a possible mechanism for the increase in follicle numbers is the stimulatory action of AMH on granulosa cell proliferation. In spite of the presence of many large follicles, they lack functional maturity. Data from women with PCOS (Cook *et al.* 2002, Pigny *et al.* 2003, Pellatt *et al.* 2007) suggest that excessive expression of AMH during follicle development may be very detrimental to follicle selection. The present data suggest that the timely inhibition of *Amh* in the granulosa cells of hens may similarly be critical for follicle selection and optimal reproductive efficiency.

Materials and Methods

Animals

Laying hens were Single-comb White Leghorn hens of the Babcock B300 strain. The birds were individually caged, with egg records maintained at 2 h intervals during daylight. All hens had free access to water and food, and were maintained on a lighting schedule of 15 h light:9 h darkness (lights on at 0600 h). Hens between 22 and 74 weeks of age and

laying regular sequences were selected and killed at 1.5–2 h after oviposition for collection of tissues. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Ovaries were obtained from broiler breeder hens in the first year of lay which were maintained in pens at a commercial facility (Hubbard Farms, Walpole, NH, USA). Broiler breeder hens used in Experiment 1 ($n=6$) had been feed-restricted according to commercial practices (87% of FF) and egg records were maintained daily. Ovaries from hens used in Experiment 2 had been raised using conventional commercial practice (RF) but one pen of hens was maintained on a FF diet ($n=8$) and one pen on a RF; 87% of FF diet ($n=9$) for one month prior to euthanasia. Total egg production was recorded daily.

TCM was made by harvesting testes removed from day 11 to day 18 chicken embryos (di Clemente *et al.* 1992). For culture, two testes were placed in a tube with 0.5 ml Medium 199 plus BSA (0.1%) for 3 days at 37 °C. The TCM was subsequently filter-sterilized and frozen until pooled with other preparations and used for western blot or in cell culture. Aliquots of medium were removed for western blot analysis which utilized the anti-AMH antiserum previously described (Johnson *et al.* 2008) at a dilution of 1:1000. The medium was run on a 10% polyacrylamide gel and transferred to nitrocellulose. Detection of signal was with LumiGLO chemiluminescent substrate (KPL, Gaithersburg, MD, USA).

RNA extraction

For Experiment 1, tissue was removed from laying ($n=6$) and broiler breeder hens ($n=6$) after euthanasia and immediately placed into ice-cold Krebs-Ringer bicarbonate buffer. The liver, pieces of the TO excluding the LYF, the granulosa layer from the largest (F1) follicle, and the granulosa layer from small yellow follicles (SYF; 6–12 mm) were collected in Buffer RLT (supplied in Qiagen RNeasy Mini Kit). Tissue was homogenized and extracted using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. In Experiment 2, ovaries from FF hens ($n=8$) and RF hens ($n=9$) were removed from birds after euthanasia, weighed (complete ovary weight including large follicles), and LYF > 15 mm were counted. Tissue from the TO, the granulosa layers from SYF (6–12 mm) and from large white follicles (LWF; 3–5 mm) were collected in Buffer RLT (supplied in Qiagen RNeasy Mini Kit). Tissue was homogenized ($n=5$ of each tissue type for FF and RF) and extracted using the Qiagen RNeasy Mini Kit as above.

Real-time PCR

Probes and primers for quantitative PCR were designed using Primer Express Software v2.0 (Applied Biosystems, Foster City, CA, USA). Primers for chicken *Amh* (GenBank accession number U61754) defined a cDNA of 71 bp (forward: 5'-CCCCTCTGTCCCTCATGGA-3'; reverse: 5'-CGTCATCCTGGT-GAAACACTTC-3') and the probe was: 6FAMAGCTCCTC TTTGGCTCA MGBNFQ. For the *Fshr* (GeneBank accession number U51097), the primers defined a product of 70 bp (forward: 5'-GCACCTTCCAAGCCTCAGATAT-3'; reverse: CCCTATGGACGACGGGTAAA-3'), and the probe was:

6FAMTGTTAATATCAAACACAGGCCTMGBNFQ. Control reagents were TaqMan rRNA (18S) primers and probe and a control reaction was run for each sample. Serial dilutions of cDNA (made from RNA from pooled granulosa layers from 3 to 8 mm follicles) were made to establish seven points on a standard curve which was run in duplicate. Samples of RNA (as described above) from the various tissues and treatments were also subjected to RT to cDNA. The quantitative PCR reactions were performed (using an ABI 7000 Sequence Detection System) in a 25 µl volume of reaction buffer containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and 900 nM of the *Amh* and *Fshr* primer pairs or 50 nM of the 18S primer pair and each respective (*Amh* and *Fshr* at 250 nM and 18S at 200 nM) TaqMan probe. Control reactions containing no template and reactions lacking RT were also used. All PCR amplifications were carried out in duplicate for each sample and the mean value was calculated relative to 18S reactions (also run in duplicate). The relative amount of mRNA in a particular sample was determined by the comparative threshold cycle (C_t) method using Sequence Detection System Software (Applied Biosystems).

Granulosa cell culture

The granulosa layers from 3 to 5 and 6 to 8 mm follicles were isolated, pooled, and dispersed as previously described (Davis *et al.* 1999). The number of cells and viability (trypan blue exclusion) were estimated using a hemocytometer. Cell viability was 95% or greater at the start of an experiment. For the glucose/insulin experiment ($n=8$ replications), cells (from 3 to 5 mm follicles) were plated in 6-well plates (in M199 plus 5% FBS) at a density of 3×10^6 cells/well and incubated for 24 h as per conditions that have been previously described (Johnson *et al.* 2005), except that they were cultured in 1.5 ml volume. After 24 h, the medium (which contained serum) was removed and replaced with control medium M199 (1 mg/ml glucose + 0.5 µg/ml insulin) with 0.1% BSA, medium with 2 mg/ml glucose, 5 mg/ml glucose, or 1.0 µg/ml insulin. The plates were then returned to the incubator and cultured for an additional 24 h. RNA was extracted from the granulosa cells at the termination of culture and quantitative PCR was performed.

For the cell proliferation assay ($n=6$ replications each), granulosa cells from 3 to 5 as well as 6 to 8 mm follicles were plated in 96-well dishes (at a density of 1.2×10^5 cells/well) and cultured for 24 h as described above. After 24 h, the medium (which contained serum) was removed and replaced with M199 with 0.1% BSA and various treatments. TCM was added in amounts of 0, 25 or 50 µl out of 100 µl total volume. To determine specificity of any effect, the TCM was pre-incubated with AMH antiserum (1:100 dilution of antiserum diluted in M199 + 0.1% BSA; the antiserum was incubated 1:1 with TCM for 30 min at room temperature) to neutralize the effect of AMH in the TCM before adding to cultures. The plates were then returned to the incubator and cultured for an additional 24 h. Granulosa cell proliferation was quantified using the Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions (Promega) and visual inspection of plate confluence confirmed proliferation.

Statistical analysis

Data were analyzed with SAS using Proc GLM with protected least-significant difference. The significance level was set at $P < 0.05$.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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