**Title: Effects of extracellular matrices and the lectin DBA on cell adhesion and self-renewal of bovine gonocytes cultured *in vitro*.**

**Short title: Effects of ECM and a lectin on cultured bovine gonocytes**

Authors: Sung-Min Kim[1], Mayako Fujihara[1,2], Mahesh Sahare[1], Naojiro Minami[1], Masayasu Yamada[1] and Hiroshi Imai[1]

Address: [1]Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

[2]Smithsonian Conservation Biology Institute, Center for Species Survival, National Zoological Park, 1500 Remount Road, Front Royal, VA, USA

Correspondence and reprint requests: Dr. Hiroshi Imai, Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan. Tel: 81-75-753-6058 Fax: 81-75-753-6329 e-mail: imai@kais.kyoto-u.ac.jp

**Abstract**

Surface molecules of primitive male germ cells, gonocytes, are essential components for regulating cell adhesion and maintaining self-renewal in mammalian species. In domestic animals, a stage-specific glycan epitope, *α-N-acetylgalactosamine* (GalNAc), is recognized by the lectin *Dolichos biflorus agglutinin* (DBA) and is found on the surface of gonocytes and spermatogonia. Gonocytes from bovine testis formed mouse embryonic stem (ES)-like cell colonies on plates, which were previously coated with DBA or extracellular matrix (ECM) components such as gelatin (GN), laminin (LN) and poly-L-Lysine (PLL). The number of colonies on the DBA plate was significantly higher than the numbers of colonies on the GN, LN

and PLL plates. Pretreating gonocytes with DBA to neutralize the terminal GalNAc residues strongly suppressed colony formation. Furthermore, the expressions of a germ cell-specific gene and pluripotency-related transcription factors were increased considerably on the DBA plates. These results suggest that the GalNAc residues on gonocytes can recognize pre-coated DBA on plates and the resulting GalNAc-DBA complexes support germ cell and stem cell potentials of gonocytes *in vitro*. These glycan complexes through the GalNAc epitope may provide a suitable microenvironment for the adhesion and cell proliferation of gonocytes in culture.

**Introduction**

A population of germ cells has the unique ability to transmit genetic information to the next generation. Gonocytes are primitive germ cells that are present in the early stage of the neonatal testis and that give rise to spermatogonia. Spermatogonia have the potential for self-renewal and differentiation to spermatozoa, thereby initiating spermatogenesis. In rodents, gonocytes growing in culture acquire the characteristics of spermatogonia, exhibit stem-cell potential as indicated by their self-renewal (Kanatsu-Shinohara *et al.* 2003; 2005), and can contribute to spermatogenesis after transplantation into immune-deficient nude mouse testes (Orwig *et al.* 2002a; 2002b). However, in domestic animals, little is known about whether gonocytes have stem-cell activity during germ cell development. Culture conditions for maintaining germ cells have been established for various species including mouse (Nagano *et al.* 1998; 2003; Kubota *et al.* 2004; Kanatsu-Shinohara *et al.* 2005), rat (Hamra *et al.* 2005), hamster (Kanatsu-Shinohara *et al.* 2008a) and rabbit (Kubota *et al.* 2011). In domestic animal species, however, culture systems have not been available and cell lines such as embryonic germ (EG) cells in mouse have not been established.

In the testis, the dynamic events during spermatogenesis occur through the basement membrane of the seminiferous tubule and the interaction with Sertoli cells. In fact, the basement membrane of the seminiferous tubule is composed of extracellular matrix (ECM), whose major components are collagen and laminin (Siu and Cheng 2004). Recent studies have revealed that adhesion molecules on the surface of SSCs specifically recognize ECM components, which have been used to identify and

purify the population of germ cells in mixed testicular cells (Shinohara *et al.* 1999; Orwig *et al.* 2002c; Hamra *et al.* 2005). Furthermore, adhesion molecules, such as β1- and α6-integrin are known to be receptors of laminin. These molecules, which are present on the surface of mouse SSCs, support the long-term proliferation of SSCs in culture (Shinohara *et al.* 1999; Kanatsu-Shinohara *et al.* 2005) and play critical roles in the reconstruction of the stem cell niche after transplantation into immunodeficient mouse testis (Kanatsu-Shinohara *et al.* 2008b). Therefore, the adhesion of cells to ECM molecules seems to be associated with their survival and proliferation, both *in vitro* and *in vivo*. However, in the case of cattle, little is known about the mechanism by which germ cells adhere to ECM matrices.

One approach to distinguishing and characterizing germ cells in a mixed testicular cell population is to identify a stage-specific glycosylation event. A lectin, *Dolichos biflorus agglutinin* (DBA), which recognizes a terminal *N-acetylgalactosamine* (GalNAc) residue (Piller *et al.* 1990), is a specific marker for germ cells such as gonocytes and type A spermatogonia in both pig (Goel *et al.* 2007) and cattle (Ertl and Wrobel 1992; Izadyar *et al.* 2002). In addition, DBA can be used to enrich germ cells by using magnetic-activated cell sorting (MACS) (Herrid *et al.* 2009). Therefore, germ cells isolated by DBA can be a useful model for understanding the roles of cell surface glycans in adhesion and proliferation of germ cells both *in vivo* and *in vitro*.

In domestic animals, a procedure for a long term culture of germ cells has not been established. To achieve this, the expressions of vital pluripotency-related genes such as *NANOG* and *POU5F1* are essential, but their expressions gradually decrease as the passage number increases (Goel *et al.* 2009). The pluripotent state in cultured germ cells can be supported by using ECM components that interact with adhesion molecules on the cell surface (Chai and Leong 2007), which suggest that some cell surface molecules can regulate the expression of genes associated with a pluripotent state in cultured germ cells. However, the effects of biomaterials, such as ECM molecules and DBA, on the adhesion, proliferation and stem cell potential of germ cells remain unknown in domestic animals.

In the present study, we tested the hypothesis that adhesion molecules including

carbohydrate chains on the surface of germ cells affect cell survival and proliferation in culture. Our results suggest that the terminal glycan residues of cell surface carbohydrates are involved in the proliferation and the stem cell potential of bovine gonocytes in culture.

## Materials & Methods

### *Collection of the testes and the isolation of gonocytes*

Testes were collected from Holstein bulls (*Bos taurus*) aged 3 months old from a local farms and were immediately placed in DMEM/F12 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 100 IU/ml$^{-1}$ penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 mg/ml$^{-1}$ streptomycin (Sigma-Aldrich), 40 mg/ml$^{-1}$ gentamycin sulfate (Sigma-Aldrich) and 15 mM HEPES (Wako, Osaka, Japan). The collected testes were transported to the laboratory at 4°C within 24 hr. The part of the testis was fixed with Bouin's fixative or 4% (w/v) paraformaldehyde (PFA) solution for immunohistochemical analysis.

To collect testicular cells, the testes were treated with three-step enzymatic digestions and isolated cells were subjected to the discontinuous density gradient Percoll centrifugation as described previously with some modifications (Fujihara *et al.* 2011). Briefly, to obtain a testicular cell suspension, the decapsulated testicular tissue was minced into small pieces and treated with a first enzymatic solution that was supplemented with 2mg/ml collagenase (type IV; Sigma-Aldrich) and 1 mg/ml deoxyribonuclease I (DNase I; Sigma-Aldrich) in DMEM/F12 for 30 min. at 37°C. Testicular cells were washed 3 times in DMEM/F12 and sequentially digested with a second enzymatic solution containing 2mg/ml collagenase (type IV; Sigma-Aldrich), 2 mg/ml hyaluronidase (Sigma-Aldrich) and 1 mg/ml deoxyribonuclease I for 30 min at 37°C and washed with DMEM/F12. The collected cells were incubated with third enzymatic solution (0.25% trypsin and 0.53 mM EDTA in PBS) containing 5 mg/ml deoxyribonuclease I for 10 min. at 37°C, washed with DMEM/F12, filtered with 50 ㎛ nylon meshes (Kyoshin Rikoh, Tokyo, Japan), and the isolated cells were subjected to the discontinuous density gradient Percoll centrifugation. Gonocytes were fractionated between 40 to 50% and identified by DBA-staining and morphological definition with large diameter in cell size.

The viability of purified cells was ≥95%, as determined by trypan blue exclusion assay.

***In vitro culture of gonocytes***

Freshly collected gonocytes were seeded at a density of $2 \times 10^5$ cells/$cm^{-2}$ onto culture dishes (Iwaki, Tokyo, Japan). The culture medium used was DMEM/F12 supplemented with 10 $\mu g/mL^{-1}$ insulin (Sigma-Aldrich), 10 $\mu g/mL^{-1}$ apotransferrin (Sigma-Aldrich), 100 IU/$mL^{-1}$ penicillin (Sigma-Aldrich), 50 $\mu g/mL^{-1}$ streptomycin (Sigma-Aldrich), 40 $\mu g/mL^{-1}$, gentamycin sulfate (Sigma-Aldrich), single strength non-essential amino acid solution (Gibco, Invitrogen), 1mM pyruvate (Sigma-Aldrich), 1.5 $\mu\ell$/ml 60% (w/v) sodium lactate (Sigma-Aldrich), 0.01mM β-mercaptoethanol (Wako), 20 ng/$mL^{-1}$ basic fibroblast growth factor (bFGF; Upstate, Temecula, CA, USA), 20 ng/$mL^{-1}$ glial-derived neurotrophic factor (GDNF; R&D System, Minneapolis, MN, USA), 50 ng/$mL^{-1}$ epidermal growth factor (EGF), 1% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) and 15% (v/v) knockout serum replacement (KSR). The culture medium was changed every other day and passaged at every 7 to 10 days interval using 0.25% (w/v) trypsin and 0.53 mM EDTA solution or mechanical dissociation methods using a fire-polished Pasteur pipette. Cells were cultured in a $CO_2$ incubator at 37ºC in a water-saturated atmosphere with 95% air and 5% $CO_2$.

***Preparation of ECM matrix plates and assessment of binding affinity of germ cells***

Culture dishes were pre-coated with ECM molecules (0.2% (w/v) gelatin (GN) (Sigma-Aldrich), 20 $\mu g/mL^{-1}$ laminin (LN) (Sigma-Aldrich) and 10 $\mu g/mL^{-1}$ poly-L-lysine (PLL) (Sigma-Aldrich) and 30 $\mu g/mL^{-1}$ DBA (Vector Laboratories, Burlingame, CA, USA) for overnight at 37ºC, then washed with PBS and were blocked with 5% BSA in PBS for 1 hr at 37ºC to prevent non-specific binding.

To analyze the binding affinity of gonocytes to culture dishes, freshly collected gonocytes were plated in 4-well or 24-well culture dishes (Iwaki) pre-coated with different ECM molecules.

Cells were incubated for 4 hr at 37 ºC in an adherent medium: which was DMEM/F12 supplemented 10% FBS without KSR and growth factors to enhance the attachment of germ cells on ECM matrices. After 4 hr of culture, floating cells were discarded, and adhered cells were gently washed and collected in the culture medium. Adhered cells were characterized by immune-cytochemical staining to distinguish germ-cell and somatic-cell populations. Antibodies were used for germ-cell markers (UCHL1 and DBA) and Sertoli-cell marker (VIMENTIN). The average numbers of positive cells for specific markers were counted in the microscopic field (magnification: 200x) that were randomly selected six fields per sample (n= 4-5) and were subjected for the statistical analysis.

***Assessment of colony formation on the different ECM matrices***

Freshly isolated gonocytes were seeded at a density of $2 \times 10^5$ cells/$cm^{-2}$ onto 4-well, 24-well or 35 mm dishes. Gonocytes were incubated in the adherent medium on the pre-coated dishes, which were pre-coated with different ECM matrices and DBA for 12 hr at 37ºC. Gonocytes were then pre-incubated with DBA (30$\mu$g/$mL^{-1}$) for 30 min at 37 ºC to neutralize GalNAc residues on the surface of gonocytes. After pre-incubation, gonocytes were seeded at a density of $2 \times 10^5$ cells/$cm^{-2}$ onto 4-well, 24-well or 35 mm dishes. DBA pre-treatment cells were incubated on the GN plates (D30_GN) and DBA plates (D30_DBA) for 12 hr at 37ºC. After 12 hr of culture, floating cells were decanted and the adhered cells were washed with culture medium, and then cultured with the adherent medium for another 4 to 7 days on different ECM matrices or DBA. To examine the glycan epitopes on colony formation, gonocytes were then pre-incubated with DBA (30$\mu$g/$mL^{-1}$) for 30 min at 37 ºC to neutralize GalNAc residues on the surface of gonocytes. After pre-incubation, gonocytes were seeded at a density of $2 \times 10^5$ cells/$cm^{-2}$ onto 4-well, 24-well or 35 mm dishes and were incubated on the GN plates (D30_GN) or DBA plates (D30_DBA) for 12 hr at 37ºC. The culture medium was changed every 2 days. At 5 days, the total numbers of colonies were counted on each well of a 4-well or 24-well plate to obtain the average number of colonies. The above procedure was replicated four times for the each group.

***Immunochemistry of testicular tissues and cultured gonocytes***

Gonocytes were identified in the testicular tissues and cultured testicular cells using DBA-FITC (1:50; Vector Laboratories, Burlingame, CA, USA) and anti-UCHL1 (PGP9.5; 1:100; Biomol, Exeter, UK). The presence of Sertoli cells in cultured testicular cells were confirmed by using anti-VIMENTIN (clon v9, 1:100; Sigma-Aldrich). The expression of pluripotency specific-markers on gonocytes in bovine testis and cultured testicular cells was examined using anti-NANOG (1: 200; Chemicon International, USA) and anti-POU5F1 (1:50; C-10, Santa Cruz Biotechnology, CA, USA), as described previously (Goel *et al.* 2008; Fujihara *et al.* 2011). Briefly, testis sections were fixed with Bouin's fixative or 4% PFA, washed several times with 0.2% (v/v) Tween 20 in TBS (TBS-T), incubated in 5% (w/v) BSA in TBS for 90 min to block non-specific binding, incubated with the DBA-FITC and primary antibodies overnight at 4˚C, washed with TBS-T three times,~~.~~ incubated with the corresponding secondary antibody as an anti-rabbit IgG antibody conjugated with Alexa 546 (1:500; Molecular Probes, Eugene, Oregon, USA) and anti-mouse IgG antibody conjugated with Alexa 546 (1:500; Molecular Probes, Eugene, Oregon, USA) for 1 hr at 37℃, rinsed three times with TBS-T, stained with Hoechst 33342 (Sigma-Aldrich) for 10 min, mounted with 50 % glycerol in PBS and observed under an immune-fluorescence microscope (Olympus BX 50, Tokyo, Japan).

Cultured cells were examined for the presence of gonocytes by germ-cell-specific markers (DBA, and anti-DDX4) and for stem-cell potential by pluripotent-specific markers (anti-NANOG and anti-POU5F1). Samples were fixed with Bouin's fixative or 4% PFA, washed several times with TBS-T, incubated 0.3% (v/v) $H_2O_2$ in PBS for 15 min to block endogenous peroxidase activity, washed with PBS several times, incubated in 5% (w/v) BSA in PBS for 30 min to block non-specific binding and incubated with DBA and primary antibodies overnight at 4℃. The primary antibodies were anti-NANOG (1:200 dilution),

anti-POU5F1 (1:50 dilution), and anti-DDX4 (1:300, Chemicon, USA). After incubation with primary antibodies, samples were washed with TBS-T three times, incubated with substrate-chromogen mix for DBA or the corresponding HRP-conjugated secondary antibodies, i.e., sheep anti-rabbit IgG (1:100; GE Healthcare, Buckinghamshire, UK), sheep anti-mouse IgG (1: 100; Amersham Biosciences, UK) for 1 h at room temperature, rinsed several times with TBS-T, mixed with substrate-chromogen for 3-5 min to colorimetrically measure peroxidase activity, washed with TBS several times, counterstained with hematoxylin, mounted on slides, and observed under the microscope (Olympus BX 50, Tokyo, Japan).

To examine a stem-cell-potential of gonocytes in cattle, purified gonocytes were double stained with DBA-FITC, anti-UCHL1, anti-NANOG and anti-POU5F1 antibodies using immune-fluorescence labeling as described above.

***RT-PCR analysis***

Testicular cells were cultured for 4 days on the different ECM matrices. Total RNAs were prepared from these cells using a ToTally RNA kit (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. RNAs were also isolated from 3-month-old testes as a positive control (T). Oligo (dT) primers and RNase OUT (both from Invitrogen) were added to the RNA solution, incubated for 5 min at 65°C and set on ice. For reverse transcription, ReverTra Ace (MMLV reverse transcriptase RNaseH-; Toyobo) was added to the RNA solution and incubated for 10 min at 30°C, for 60 min at 42°C, and for 5 min at 99°C (RT+). At the same time, the reaction without the addition of ReverTra Ace was done to check genomic DNA contamination (RT-). The PCR amplification was carried out on 2 μl of cDNA per 20 μl of PCR reaction mixture containing, 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 × PCR buffer, 10 pmol of each primers and 1U of Taq DNA polymerase (ExTaq, TaKaRa, Ohtsu, Japan). The primer sequences used for the amplification of specific genes are shown in Table 1. PCR products were separated and visualized on 2 % (w/v) agarose gels containing 0.5 $\mu g/ml^{-1}$ ethidium bromide. All PCR products were sequenced to confirm their identity.

### *Statistical analysis*

All data are presented as the mean ± SEM (n= 4-5) in each group. To determine the differences among experimental groups, one-way or two-way ANOVA was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego CA, USA). All data were subjected to Tukey's multiple-comparison test to determine the significance of differences between groups. Differences were considered to be significant at $P < 0.05$.

## Results

### *Characterization of stem cell potential of developing germ cells*

To examine the DBA binding affinity and the expression pattern of pluripotent-specific markers in bovine testis, 3-month-old testes sections were immunohistochemically stained. The binding of DBA and expression of UCHL1 were observed in populations of gonocytes. These cells were easily distinguished from other somatic cell populations by two morphological features with a large nucleus and a basal location in the seminiferous tubules (Fig. 1*a-d*). DBA was found on cell surface or cytoplasmic part of gonocytes (Fig. 1*a-b*), while UCHL1 expression was observed in the germ cells (Fig. 1*c-d*). Double-immunostaining for UCHL1 (a germ cell-specific marker) and DBA show that UCHL1 is expressed in most of the DBA-positive cells (Fig. 1*e-g*), while a small number of UCHL1-positive cells (one is indicated by *white arrow* in Fig. 1*e*) were negative for DBA (Fig. 1*f-g*). To examine the stem-cell potential of DBA-positive germ cells, sections were double stained with DBA and anti-POU5F1 (Fig. 1*h-j*) or anti-NANOG (Fig. 1*k-m*). Most of the cells expressing POU5F1 (Fig. 1*h*) were DBA-positive (Fig. 1*i*). An example of a cell expressing POUF1 that is DBA-negative is shown by the white arrow (Fig. 1*h-j).* The POU5F1 expression was detected in most of the DBA-positive cells, but some of the POU5F1-positive cells were not shown the DBA signal (Fig. 1*h-j*). The expression of NANOG was also detected in the seminiferous

tubules (Fig. 1*k*). Some of the NANOG-positive cells were DBA-positive and some were not (Fig. 1*j-m*). Some of the NANOG-negative cells were also DBA-positive (Fig. 1*k-m*), indicating that DBA and NANOG expression were not coincident in germ cells of the prepubertal bovine testis.

***Cultivation and characterization of bovine gonocytes***

Bovine gonocytes were isolated and enriched by Percoll centrifugation (Fig. 2*a*). When the isolated cells were cultured on a GN-coated dish, they formed cell clumps at 1 day of culture (Fig. 2*b*) and formed mouse ES-like colonies by 3-4 days (Fig. 2*c*), which became compacted around 6-7 days (Fig. 2*d*) and gradually enlarged during the culture period. Most of these colonies were stained with germ cell-specific markers (DBA, Fig. 2*e* and DDX4, Fig. 2*g*) and stem cell-specific markers (POU5F1, Fig. 2*f* and NANOG, Fig. 2*h*), suggesting that gonocyte colonies in culture still have a stem cell potential. In the following passages, the colonies gradually decreased in number and disappeared by 5-7 passages.

***Binding of gonocytes to DBA and different ECM matrices***

The binding of gonocytes to different ECM matrices and DBA was examined at 4 hr after cell plating (Fig. 3). The average number of testicular cells was significantly higher on the PPL plates (192.0 ±14.7 cells, $p<0.01$) and lower on the LN plates (79.0 ±9.6 cells) (Fig. 3*a*) with compared to the GN and DBA plates. The average numbers of testicular cells on the GN and DBA plates were similar (98.3 ±22.6 cells and 104.6 ±9.1 cells, respectively) (Fig 3*a*). In the case of Sertoli cells, which are identified by staining for VIMENTIN, about equal numbers of cells bound to each of the different ECM matrices, and non-positive cells were significantly increased on the PLL plates compared to other palates (Fig 3*a*). Although the number of attached testicular cells was highest on the PLL plates, it is interesting that the number of gonocytes was significantly higher on the DBA plates (4.21% ±0.49) than on the GN (2.03% ±0.59, $p<0.05$) and LN plates (0.75% ±0.43, $p<0.01$), but not significantly different from the number of cells on the PLL plates (2.08 ±0.52) (Fig. 3*b*). Cells

that adhered to the DBA and ECM plate were detected by a germ-cell marker (DBA) and a Sertoli-cell marker (VIMENTIN) (Fig. 3*c*). Gonocytes were stained only with DBA and Hoechst 33342. Cells that adhered to the DBA plate also expressed UCHL1 and had a large nucleus stained with Hoechst 33342. However, VIMENTIN-positive cells were not stained with DBA and had a small nucleus.

***Colony formation on the DBA and ECM plates***

Freshly collected cells were cultured on plates coated with DBA and different ECMs for 5 days (Fig. 4*a* and *b*) and the numbers of colonies were estimated. The number of colonies on the DBA (15.8 ±1.5) and the PLL plates (14.0 ±4.4) were significantly greater than the number of colonies on the GN (6.0 ±0.4) and the LN plates (2.0 ±0.4) (Fig 4b). However, these colonies gradually disappeared on most plates around 7 days of culture.

After 12 hr of positive selection of attached testicular cells followed by 5 days culture, colonies were observed on the DBA, GN and PLL plates, but not on the LN plates (Fig. 4*c*). Interestingly, more colonies formed on the DBA plate than on the ECM plates (Fig. 4*c* and *d*). The average number of colonies on the DBA plates (126.5 ±7.5) was significantly higher than the numbers of colonies on the GN, LN and PLL plates (72.5 ±0.5, $p<0.05$; 0, $p<0.001$; 33 ±13.0, $p<0.01$, respectively) (Fig. 4*d*). On the other hand, the proliferation of somatic cells was effectively suppressed on the DBA plate, but not on the ECM plates (Fig. 4*c*). When isolated gonocytes were pretreated with 30$\mu g/ml^{-}$ DBA and then cultured on the DBA plates (D30_DBA) and GN plates (D30_GN), the average number of colonies were significantly decreased in both the GN (11.0 ±1.0, $p<0.001$) and DBA (30.0 ±2.0, $p<0.001$) plates (Fig. 4 *c* and *d*). Additionally, the growth of somatic cells on the DBA was strongly suppressed on the DBA plates, but was not on the GN plates (Fig. 4*c*). These results show that GalNAc residues on the surface of gonocytes were associated with cell adhesion and colony formation of gonocytes on the DBA plates.

***Characterization of gonocytes on the DBA plate.***

Three-dimensional colonies on the ECM matrices were double stained with anti-UCHL1 and anti-VIMENTIN. Most of the colonies on the DBA, GN and PLL plates were positive for UCHL1, and some of the cells in the colonies were partially positive to anti VIMENTIN (Fig. 5a). On the LN plate, few of the colonies were UCHL1-positive, while most of the colonies were VIMENTIN-positive (Fig. 5a).

To estimate the stem-cell characteristics of gonocytes on the DBA plate, colonies that formed at 5 days of culture were double stained with germ-cell markers (DBA and UCHL1) and stem-cell markers (NANOG and POU5F1). Most of the colonies were strongly positive for DBA staining and were co-localized with UCHL1, and also were positive for NANOG and POU5F1 with DBA staining (Fig. 5B).

***RT-PCR analysis***

Testes tissues and cultured cells were subjected to semi-quantitative RT-PCR analysis to identify stem cell-specific transcripts such as *NANOG, POU5F1, SOX2, C-MYC* and *REX1* (Fig. 6A and B). In the testis section, most of the transcripts with the exception of *C-MYC* were detected and the expression level of *NANOG* was strongly detected compared to other transcripts. Transcripts of these genes were also detected in cultured cells, but the expression patterns of transcripts were markedly different on the different ECM matrices and DBA. *C-MYC* transcripts were more abundant in most of the cultured cells than in freshly collected testicular cells, while but *NANOG* transcripts were less abundant in the cultured cells. Among the different ECM matrices and DBA, the expression levels of *POU5F1* and *UCHL1* were markedly increased on the DBA plate, and *SOX, C-MYC* and *REX* transcripts on the DBA plate were considerably up-regulated compared to the other plates, but the expression level of *NANOG* was relatively low. On other hand, on the LN plate, the expressions of *NANOG* and *C-MYC* transcripts were weak, while the expressions of *POU5F1* and *SOX2* transcripts were not be detected.

## Discussion

One of the unique biological features of gonocytes is their adhesion to the basement membrane of the seminiferous tubule. This study investigated the effects of ECMs and DBA on the adhesion and growth of gonocytes and on their stem cell characteristics in culture.

Germ cells usually require feeder cells for their survival, proliferation and maintenance in cultures (Nagano *et al.* 2003). However, it was later revealed that feeder cells are not essential because they can be replaced with ECM molecules such as laminin (Kanatsu-Shinohara *et al.* 2005). The present study indicated that ECM molecules were not effective at enriching or purifying gonocytes from the prepubertal testis (Fig. 3). In addition, ES-like colony formation from gonocytes was not stimulated by the ECM molecules, but was stimulated by the presence of DBA after DBA-positive-cell selection (Fig. 4). ECM molecules have been used as a component of the culture medium for various types of cells. The requirement of ECM on cell survival and growth varies depending on cell types; for instance, laminin is suitable for the culture of post-migratory primordial germ cells (PGCs) (Garcia-Castro *et al.* 1997), gelatin is suitable for muscle cells and endothelial cells (Richler and Yaffe 1970; Folkman *et al.* 1979) and poly-L-lysine is suitable for neuronal cells (Yavin and Yavin 1980). In the present experiment, testicular cell cultures after positive cell selection resulted in different cell populations on each ECM plate (Fig. 4C). For example, cells grown on the DBA plates mainly consisted of gonocytes with ES-cell like morphology, and cells grown on the LN plates mainly consisted of VIMENTIN-positive and epithelial-type cells, indicating that they are Sertoli cells (Herrid *et al.* 2007). Therefore, the cell type-specific growth pattern of testicular cells including gonocytes may be affected by ECM molecules or DBA, which are closely associated with the cell surface molecules, suggesting that ligands for the cell surface molecules are essential components for cell adhesion and regulate physiological features of gonocytes in culture.

DBA, which recognizes α- and β-linked GalNAc residues (Kamada *et al.* 1991; Klisch *et al.* 2008), has been used to detect gonocytes and SSCs in domestic species such as pig (Goel *et al.*

2007) and cattle (Ertl and Wrobel 1992; Izadyar *et al.* 2002; Herrid *et al.* 2007). DBA has also been used to enrich germ cells by magnetic-activated cell sorting (MACS) (Herrid *et al.* 2009), indicating that it can be a ligand for the surface glycan epitopes of germ cells. The specific affinity of the terminal GalNAc residues for their ligands may be associated with the cell surface interaction of gonocytes. Similarly, a terminal carbohydrate, such as mannose (Huang and Stanley 2010) and *N-acetylglucosamine* (GlcNAc) (Akama *et al.* 2002), may be involved in the interaction between germ cells and Sertoli cells, indicating that the binding of germ cells to Sertoli cells depends on the terminal carbohydrate. Although these reports suggest that terminal carbohydrates on the surface of germ cells are associated with the cell adhesion, there is no evidence that terminal GalNAc residues are involved in the adhesion activity in the testis. At the beginning of this study, we hypothesized that GalNAc residues on the surface of gonocytes in the bovine testis that are specifically recognized by DBA affect cell survival and expansion *in vitro*. Our finding that the number of adhered gonocytes was significantly higher on the DBA-coated plate than on the ECM-coated plates (Fig. 3b), indicates that DBA can support the cell adhesion associated with cell survival and cell growth in cultured gonocytes.

The results shown in Fig. 4 indicate that the DBA-coated plates support the binding of gonocytes to the plates and result in the increased number of colonies. GalNAc residues on the surface of gonocytes are a part of Sda-glycotopes on glycoproteins, which are associated with cell surface interactions (Klisch *et al.* 2011). The surface interaction of terminal glycan epitopes such as *N-acetylglucosamine* (GlcNAc)-terminated N-linked glycans, which are combined with proteins or lipids, was found to affect the adhesion and differentiation of gonocytes on Sertoli cells in mouse (Akama et al. 2002). Similarly, O-linked glycoproteins on mouse ES cells, which also have GalNAc residues and are recognized by DBA, are associated with the transition of the cells to a pluripotent state (Nash *et al.* 2007). The finding that masking of the terminal GalNAc residues of gonocytes by DBA pretreatment suppressed colony formation on both GN and DBA plates (Fig. 4c and d) indicates that the proliferation and adhesion of gonocytes can be stimulated by terminal GalNAc residues. Since structural changes of glycoproteins on a cell surface can affect cell-cell interactions and signal transduction (Dennis *et al.* 2009; Varki and Lowe 2009), the formation

of a GalNAc-DBA complex on gonocytes may affect cell growth, cell survival and colony formation in culture. On the other hand, the proliferation of somatic cells on the DBA plates was suppressed (Fig. 4c), and this may provide a suitable condition for efficient colony formation.

The ability to maintain germ cells in culture depends on the presence of supporting cells that are associated with reconstruction of the niche microenvironment (Wu *et al.* 2011). Sertoli cells are key somatic cells that secrete growth factors, such as glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF), which are critical factors for the self-renewal and colony formation of germ cells in mice (Meng *et al.* 2000; Kubota *et al.* 2004). The presence of Sertoli cells in cultures is known to improve the growth of germ cells (Koruji *et al.* 2009; Mohamadi *et al.* 2011). However, the flat cells surrounding the colonies of gonocytes in this experiment were mainly Sertoli cells on the LN plate that did not support colony formation (Fig. 4), while the DBA plates that suppressed the growth of somatic cells supported colony formation (Fig. 4). The absence of colonies on the LN plates was considered to be due to the extensive growth of testicular somatic cells that inhibited the proliferation of germ cells (Kanatsu-Shinohara *et al.* 2005). The higher number of colonies on the DBA plates than on the ECM plates (Fig. 3) suggests that a proper stimulation of somatic cells including Leydig cells, Sertoli cells and endothelial cells, which are necessary for survival and proliferation of germ cells (Aponte *et al.* 2008), supports colony formation of gonocytes on the DBA plates.

Colonies of bovine gonocytes have stem cell potential, as identified by the expression of stem cell-specific genes (*NANOG* and *POU5F1*) (Fujihara *et al.* 2011). The colony formation of testicular cells in culture depends on the presence of germ cell populations (Aponte *et al.* 2008), and these cell populations were strongly associated with the expression of NANOG and POU5F1 (Fig. 5*b*). Transcripts of other pluripotency-related genes such as *SOX2* and *REX1* were expressed in 3 month-old bovine testis (Fig. 6*a*). The expression patterns of these genes depended on the culture plates, indicating that adhesion molecules on the plates were associated with the characteristics, including stem cell potential of germ cells in culture. The expression of most of the pluripotency-related genes (*POU5F1*, *SOX2*, *REX1* and C-*MYC*, but not *NANOG*)

was considerably increased on the DBA plates. The expression of these genes may be required for the survival and proliferation of gonocytes. In pig, up-regulation of pluripotency-related gene in gonocytes in primary culture was shown to stimulate the proliferation and stem cell potential of the gonocytes (Goel *et al.* 2009). Gonocytes have been considered to be in a mitotically quiescent state and their proliferation could be initiated by altering their characteristic in culture (Kanatsu-Shinohara *et al.* 2005). The finding that the expression of germ-cell marker *UCHL1* was markedly increased on the DBA plates indicates that the germ cells were enriched on the DBA plates. These results suggest that a culture system using the DBA-coated plates for bovine gonocytes can provide a suitable microenvironment for supporting the proliferation and survival of germ cells.

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522
523 **Figure Legends**

524 **Figure 1.** Characterization of bovine germ cells in the testis at 3 months of age. (*a*-*d*) Germ cells in
525 the testis stained with germ-cell markers (DBA and UCHL1). Dashed lines show the basement of the
526 seminiferous tubules in the testis sections. (*a*) Some of the DBA signals were observed on the
527 gonocytes. (*b*) The same sample stained with Hoechst 33342. (*c*) UCHL1 expression was strongly
528 detected in the cytoplasm and nucleus of gonocytes. (*d*) The same sample stained with Hoechst 33342.
529 (*e*-*g*) Co-immunolocalization of specific markers for germ cells (DBA and UCHL1) in the bovine
530 testis. (*e*-*f*) UCHL1 expression was observed in most of the DBA-positive cells (green yellows), but
531 was observed in only some of the DBA-negative cells (white arrows). These images were merged
532 after double-immunostaining (*g*). (*h*-*j*) Double-immunostaining of DBA and POU5F1. (*h*-*i*) The
533 expression of POU5F1 was detected on the nucleus of gonocytes in most of the DBA-positive cells
534 (green arrows). A few POU5F1-positive cells were negative for DBA (white arrows). Merged
535 POU5F1-staining images (*j*). (*k*-*m*) Double-immunostaining of DBA and NANOG. (*k*-*i*) NANOG

expression was strongly detected on the nucleus of germ cells, some of which were partially positive for the DBA signal (green arrows). The expression of NANOG was observed in some DBA-negative cells (white arrows). DBA signals were detected in some NANOG-negative cells (red arrows). The image of NANOG-staining was merged with the DBA-staining image after double-immunostaining (*m*). Bar = 20㎛.

**Figure 2.** Cultivation of bovine gonocytes *in vitro*. A) Gonocytes from the bovine testis were collected by three-step enzymatic digestions and were cultured on gelatin-coated dishes. (*a*) Freshly collected testicular cells contained gonocytes (red arrows) and testicular somatic cells (black arrows). (*b*) Gonocytes with a larger diameter formed cell clumps (red arrows) 1 day after culture, whereas somatic cells (asterisk in the white dashed circle) did not form clumps in 1 day cultures. (*c*) These clumps formed mouse embryonic stem (ES) cell-like colonies at 3-4 days, (*d)* and were enlarged during 6-7 days of the culture period. (*e* and *g*) ES cell-like colonies expressed germ cell markers (DBA (*e*) and DDX4 (*g*)), pluripotency markers (POU5F1 (*f*) and NANOG (*h*)). Bar = 50㎛

**Figure 3**. Binding affinity of gonocytes to different ECM components and DBA. Freshly collected cells were seeded on culture dishes previously coated with different ECM components and DBA, and were incubated for 4 hr. Attached cells on the dishes were stained with antibodies raised against a germ-cell marker (UCHL1) and a Sertoli-cell marker (VIM: VIMENTIN). (*a*) Numbers of cells positive for VIM (n=4) and UCHL1 (n=4) were counted and were analyzed using graph-based visualization (mean ±SEM). Neg. (yellow bars) indicates somatic cells without staining signals. (*b*) Proportion of UCHL1-positive germ cells after culture on ECM- (n=4) and DBA-coated (n=5) plates (mean ±SEM). (*c*) Cells were double stained to identify gonocytes cultured on ECM- and DBA-coated plates at 4 hr after plating. Attached cells were stained with a germ-cell marker (DBA) and a Sertoli cell marker (VIM) on cover-glasses coated with DBA (a´), gelatin (GN) (b´), laminin (LN) (c´) and poly-L-Lysine (PLL) (d´). On the DBA-coated glass plate, germ cells were stained only with UCHL1 and overlaid with a Hoechst 33342-stained image (e´ and f´, red arrows), and somatic cells

were stained only with VIM (e´ and f´, white arrows). H: Hoechst 33342. Bar = 50$\mu$m

*P < 0.05, **P < 0.01 *vs.* DBA, ††P < 0.01 *vs.* PLL, ANOVA and Tukey's *post-hoc* test, respectively.

**Figure 4**. Colony formation of gonocytes cultured on different ECM and DBA plates (*a* and *b*) and after (*c* and *d*) positive germ cell selection. (*a*) Freshly collected testicular cells were cultured for 5 days on ECM (gelatin, GN; laminin, LN; and poly-L-lysine, PLL) and DBA plates. (*b*) Estimated numbers of colonies on ECM (GN, LN and PLL) and DBA plates (n= 4; mean ±SEM). The DBA and PLL plates had significantly more colonies than the other ECM plates (GN and LN). Bar = 50$\mu$m. (*c*) Freshly collected gonocytes were divided into two groups; one for DBA-nontreated group, in which gonocytes were simply cultured on DBA or ECM plates, and another for DBA-pretreated group, in which gonocytes were pretreated with DBA (30$\mu$g/ml$^{-}$) and then cultured on DBA (D30_DBA) or GN (D30_GN) plate. The growth patterns of gonocytes on the ECM and DBA plates were different. (*d*) Estimated numbers of colonies on the ECM and DBA plates after 5 days of culture (n= 4; mean ±SEM). The DBA plates had significantly more colonies than the GN the PLL and LN plates. Colony formation of gonocytes was significantly decreased after pre-treatment with DBA (30$\mu$g/ml$^{-}$) on both the GN plates (DBA30_GN) and DBA (DBA30_DBA) plates. Bar = 50$\mu$m.

**P < 0.01, ***P < 0.001 *vs.* DBA, †P < 0.05, ††P < 0.01 *vs.* PLL, ##P < 0.01 vs. GN, ANOVA and Tukey's *post-hoc* test, respectively.

**Figure 5**. Immunocytochemical characterization of ES cell-like colonies in primary culture. (*a*) Colonies that appeared on the ECM and DBA plates were stained with a germ-cell marker (UCHL1) and Sertoli cell marker (VIM: VIMENTIN). Colonies on the DBA, GN and PLL plates were positive for UCHL1 and some of the colonies expressed VIMENTIN. On the LN plate, the UCHL1 signal was weak and the VIMENTIN signal was strong. (*b*) Double immunocytochemical staining was performed to identify the stem-cell potential of colonies. DBA-positive colonies were positive for

589 UCHL1 and were also positive for stem-cell markers (POU5F1 and NANOG). All images were
590 merged with the image of Hoechst 33342-staining. GN: gelatin; LN: laminin; PLL: poly-l-lysine;
591 VIM: VIMENTIN. Bar = 20㎛.

592

593 **Figure 6**. RT-PCR analysis of pluripotency-related genes in germ cells cultured on the ECM and DBA
594 plates. (*a*) PCR products on an agarose gel stained with ethidium bromide-staining. (*b-g*) Estimates
595 of numbers of transcripts of a germ cell-specific gene UCHL1 (*b*) and pluripotency-related
596 genes *POU5F1* (*c*), *C-MYC* (*d*), SOX2 (*e*), REX1 (*f*) and NANOG (*g*). The DBA plate had
597 significantly more *UCHL1* and *POU5F1* transcripts than the ECM matrix plates. In addition,
598 *C-MYC*, *SOX2* and *REX1* transcripts were high among the ECM plates. The *C-MYC* gene was
599 strongly expressed on the GN and DBA plates, but was not detected in 3-month old testis. Transcript
600 levels were normalized to the abundance of *β-ACTIN* (BACT)transcripts. GN: gelatin; LN: laminin;
601 PLL: poly-l-lysine.

602

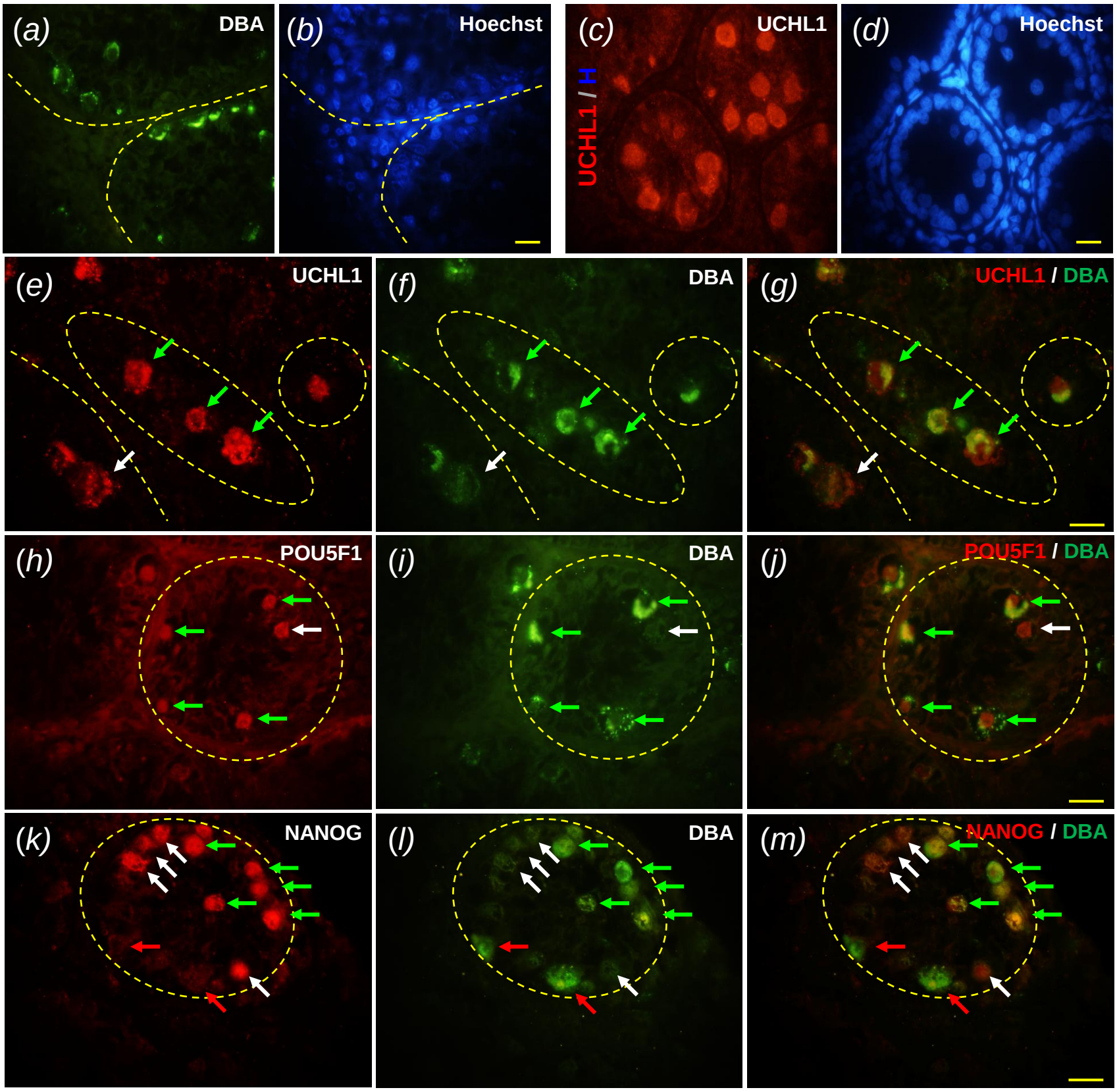
603 **Table 1. RT-PCR primer sequences used in this study.**

| Gene | | Primer Sequence (5´-3´) | Product Size (bp) | GenBank Accession no. |
|---|---|---|---|---|
| *POU5F1* | F | GGTTCTCTTTGGAAAGGTGTTC | 314 | NM_174580.2 |
| | R | ACACTCGGACCACGTCTTTC | | |
| *NANOG* | F | GACACCCTCGACACGGACACT | 153 | NM_001025344.1 |
| | R | CTTGACCGGGACCGTCTCTT | | |
| *SOX2* | F | GTTTGCAAAAGGGGGAAAGT | 200 | NM_001105463.1 |
| | R | GAGGCAAACTGGAATCAGGA | | |
| *REX1* | F | GCAGAATGTGGGAAAGCCT | 209 | XM_003584155.1 |
| | R | GACTGAATAAACTTCTTGC | | |
| *UCHL1* | F | ACCCCGAGATGCTGAACAAAG | 236 | NM_001046172.1 |

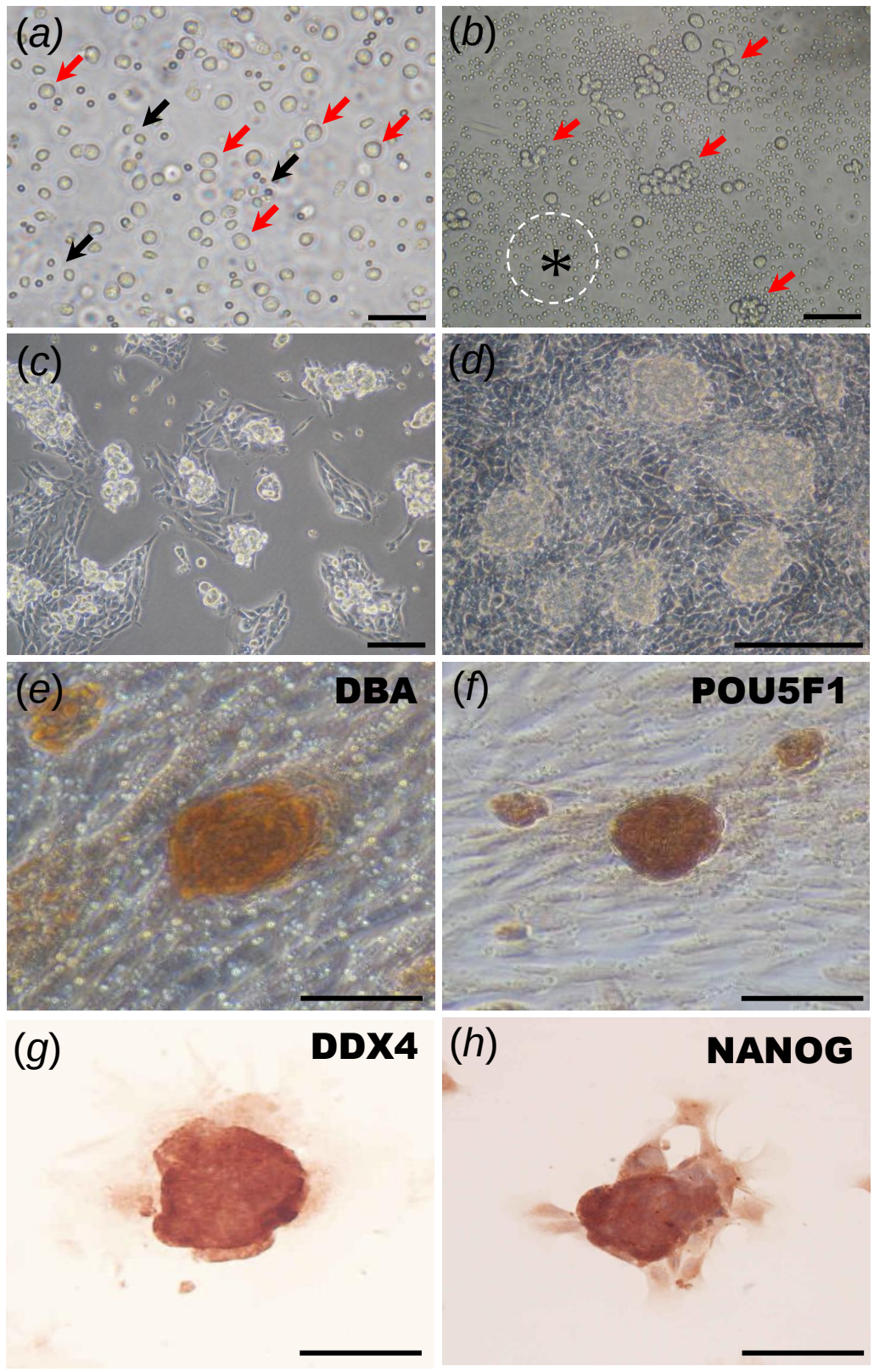
| | | | | |
|---|---|---|---|---|
| | R | CCCAATGGTCTGCTTCATGAA | | |
| *C-MYC* | F | AGAGGGCTAAGTTGGACAGTG | 346 | NM_001046074.2 |
| | R | CAAGAGTTCCGTATCTGTTCAAG | | |
| *BACT* | F | TCCCTGGAGAAGAGCTACGA | 364 | NM_173979.3 |
| | R | ACATCTGCTGGAAGGTGGAC | | |

604

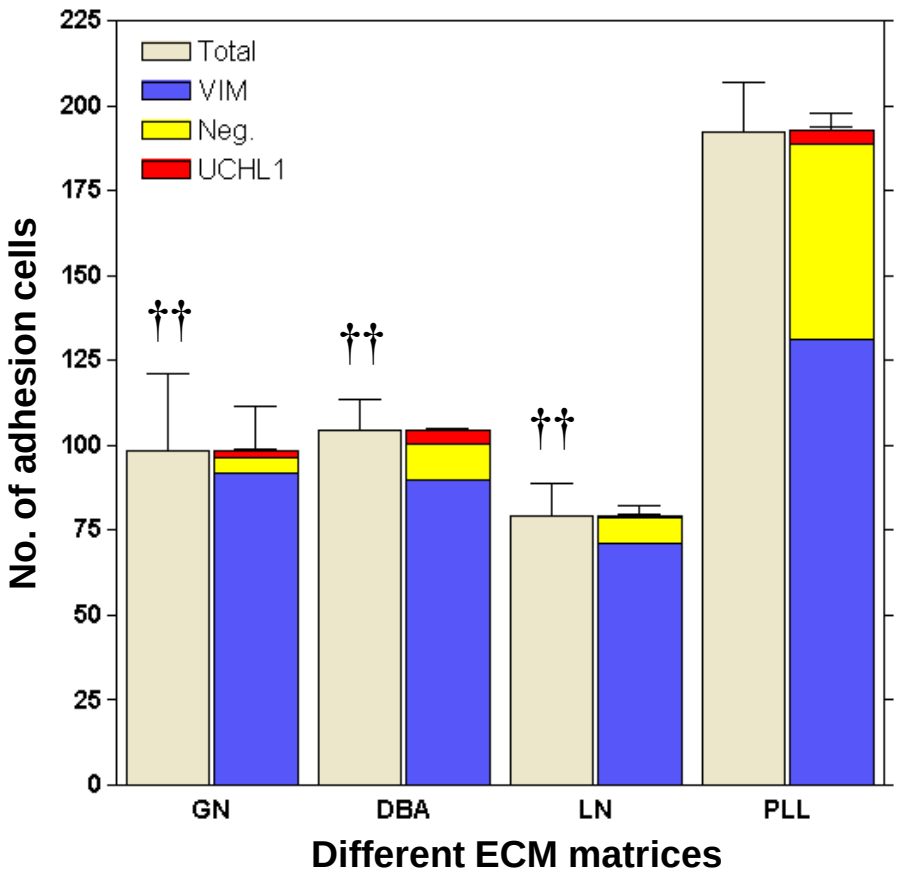
# Figure 1

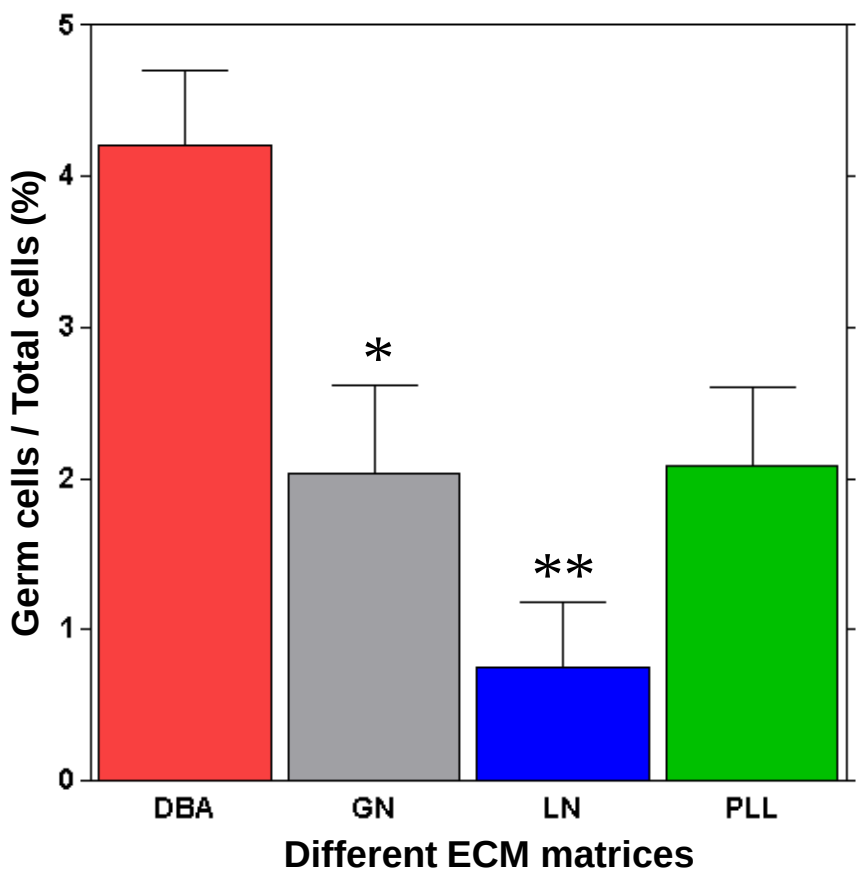

(a)
DBA
(b)
Hoechst
(c)
UCHL1
UCHL1 / H
(d)
Hoechst
(e)
UCHL1
(f)
DBA
(g)
UCHL1 / DBA
(h)
POU5F1
(i)
DBA
(j)
POU5F1 / DBA
(k)
NANOG
(l)
DBA
(m)
NANOG / DBA

# Figure 2

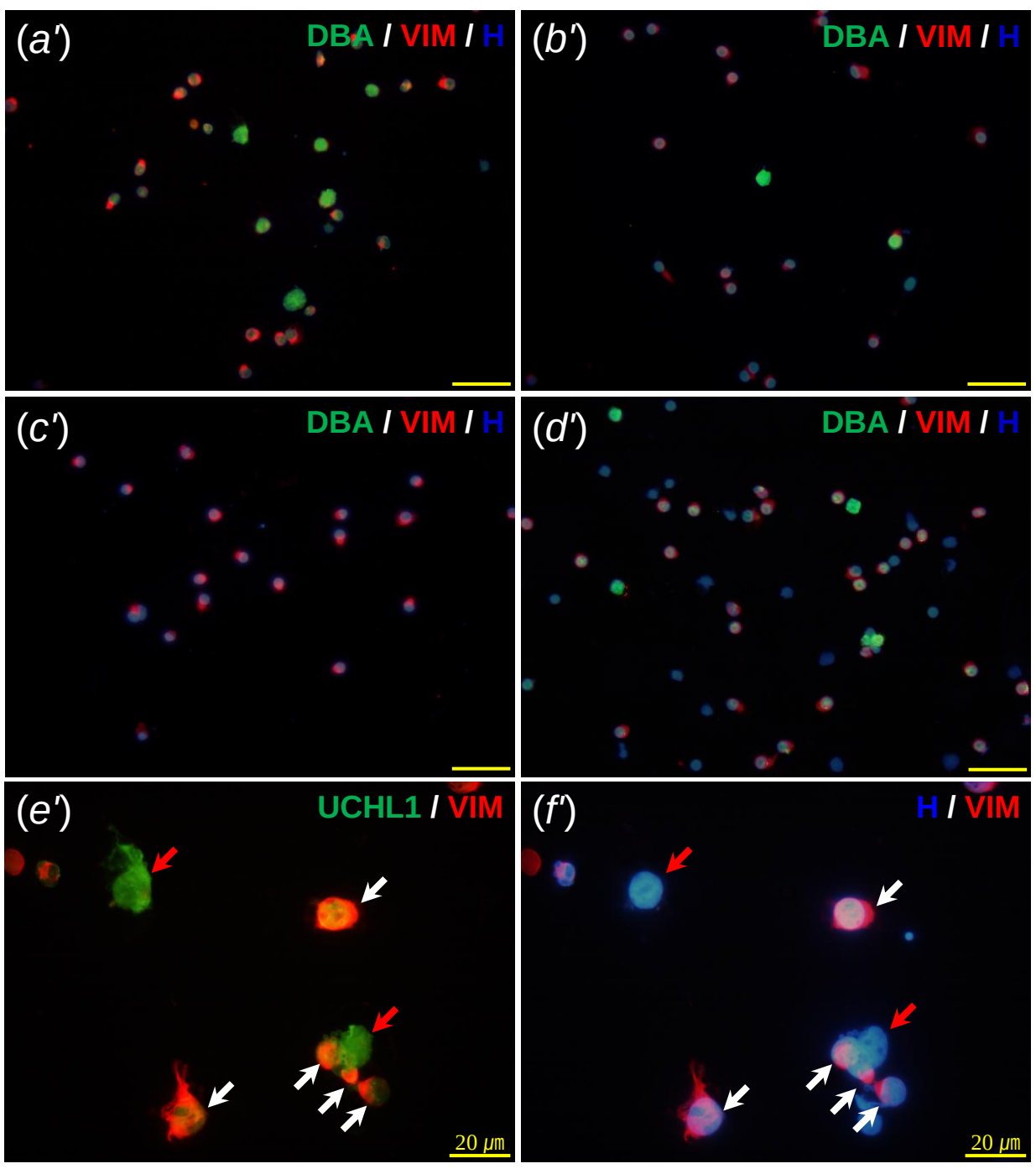


# Figure 3


(a)
Total
VIM
Neg.
UCHL1
No. of adhesion cells
225
200
175
150
125
100
75
50
25
0
††
††
††
GN
DBA
LN
PLL
Different ECM matrices

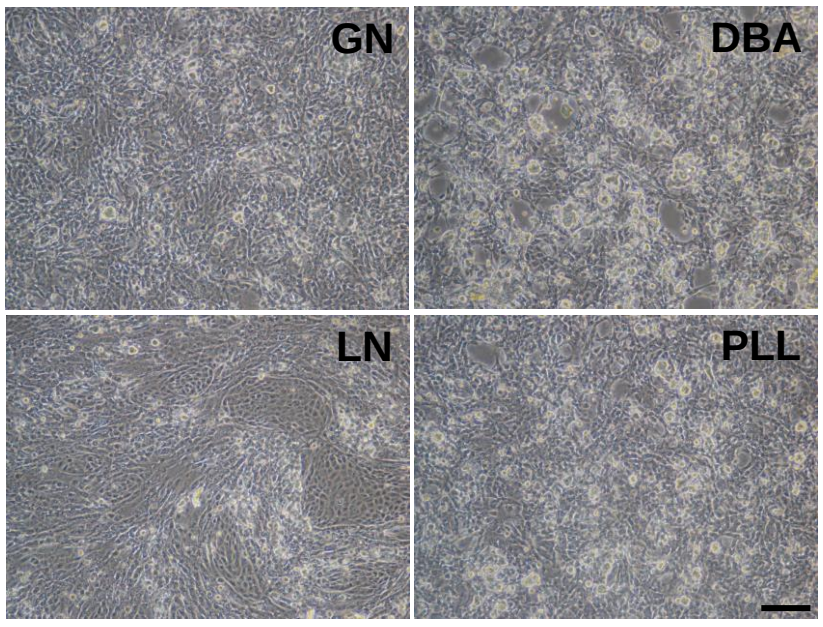

(b)
Germ cells / Total cells (%)
5
4
3
2
1
0
*
**
DBA
GN
LN
PLL
Different ECM matrices

(c)

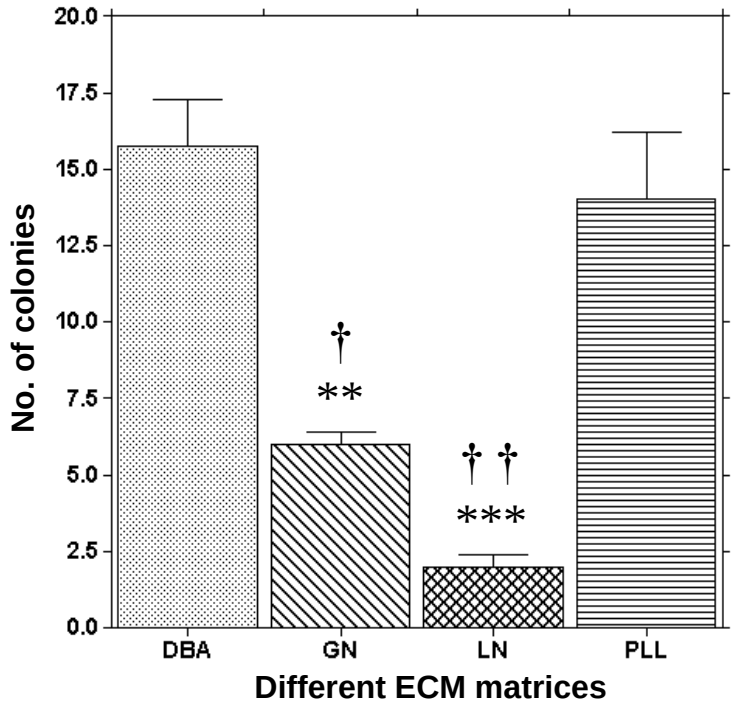

(a') DBA / VIM / H
(b') DBA / VIM / H
(c') DBA / VIM / H
(d') DBA / VIM / H
(e') UCHL1 / VIM
(f') H / VIM
20 μm
20 μm

# Figure 4

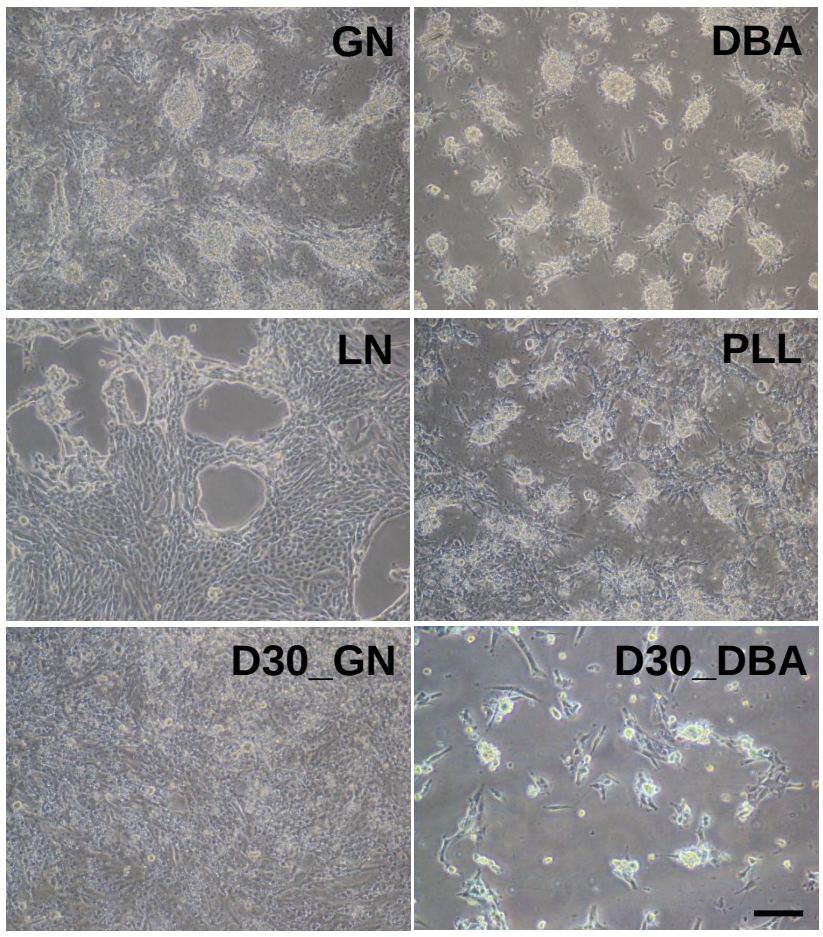
(*a*)



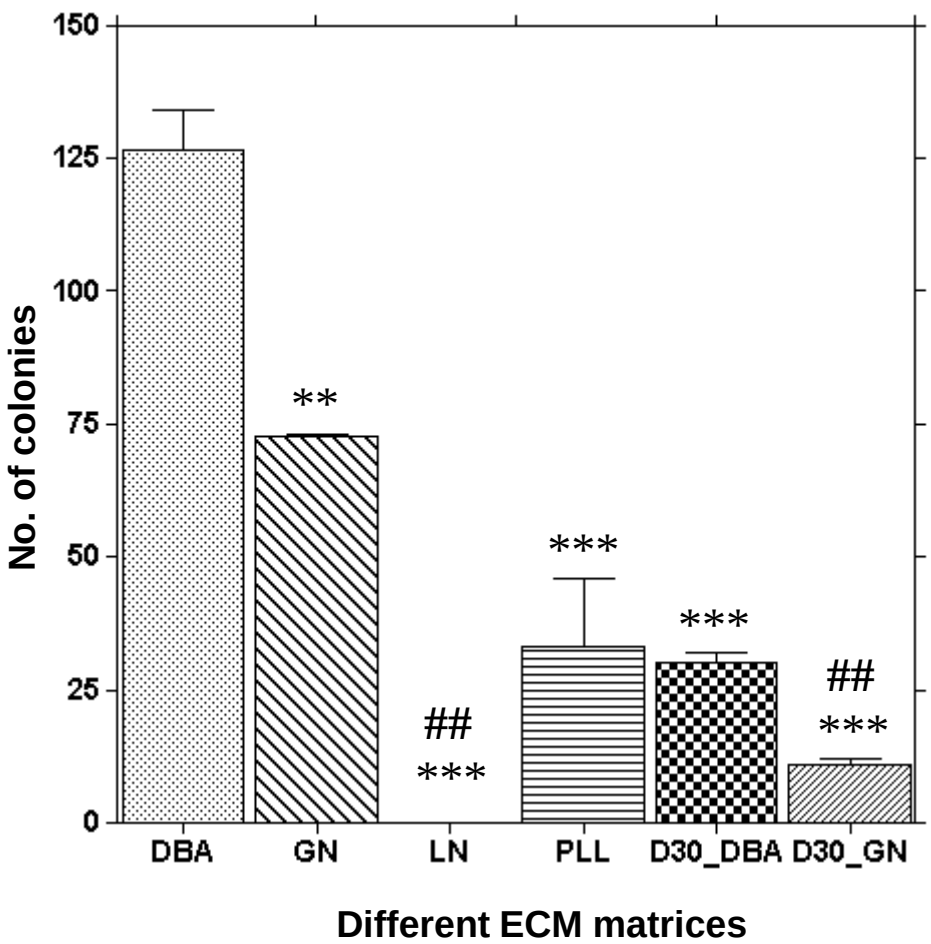

(*b*)



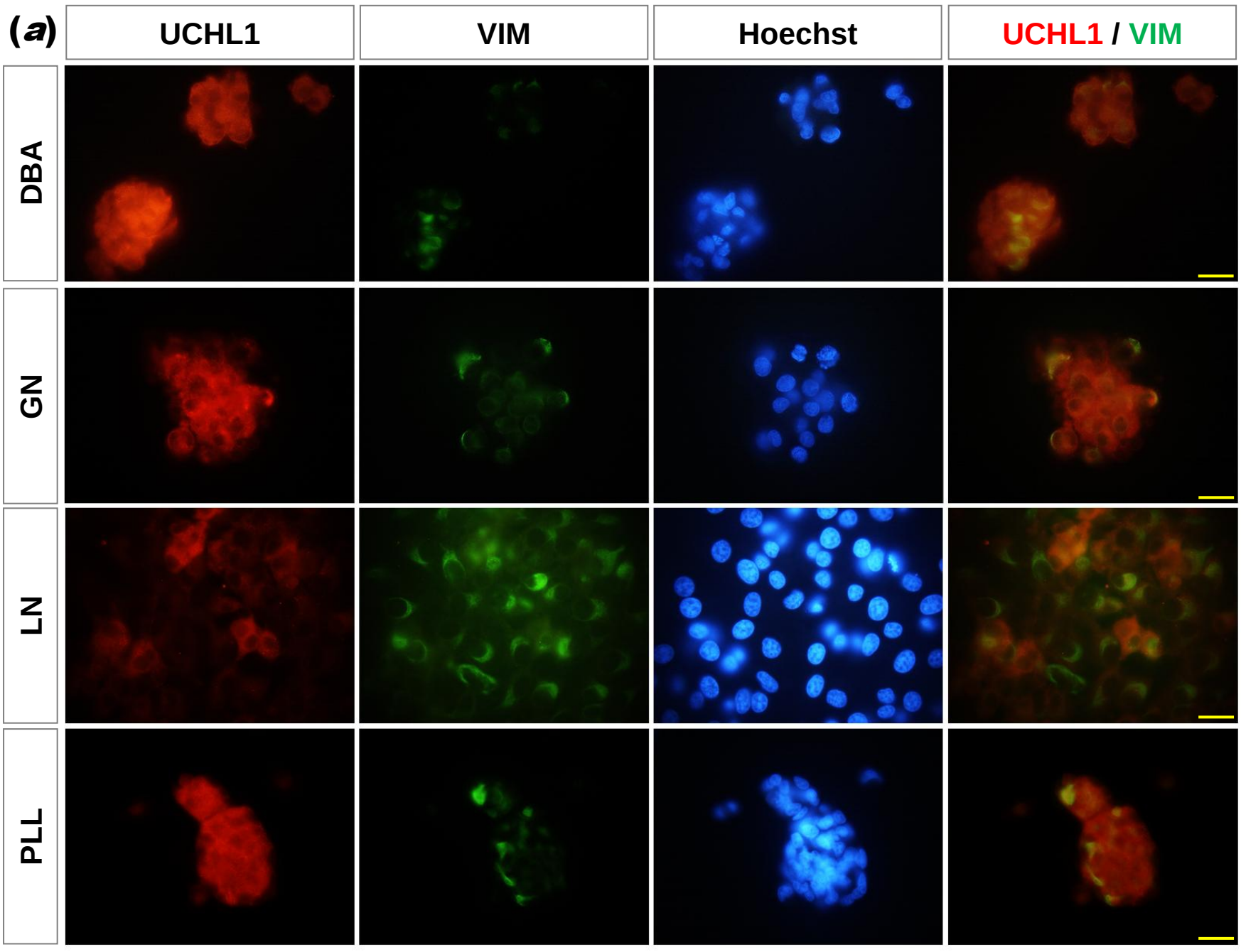

(*c*)

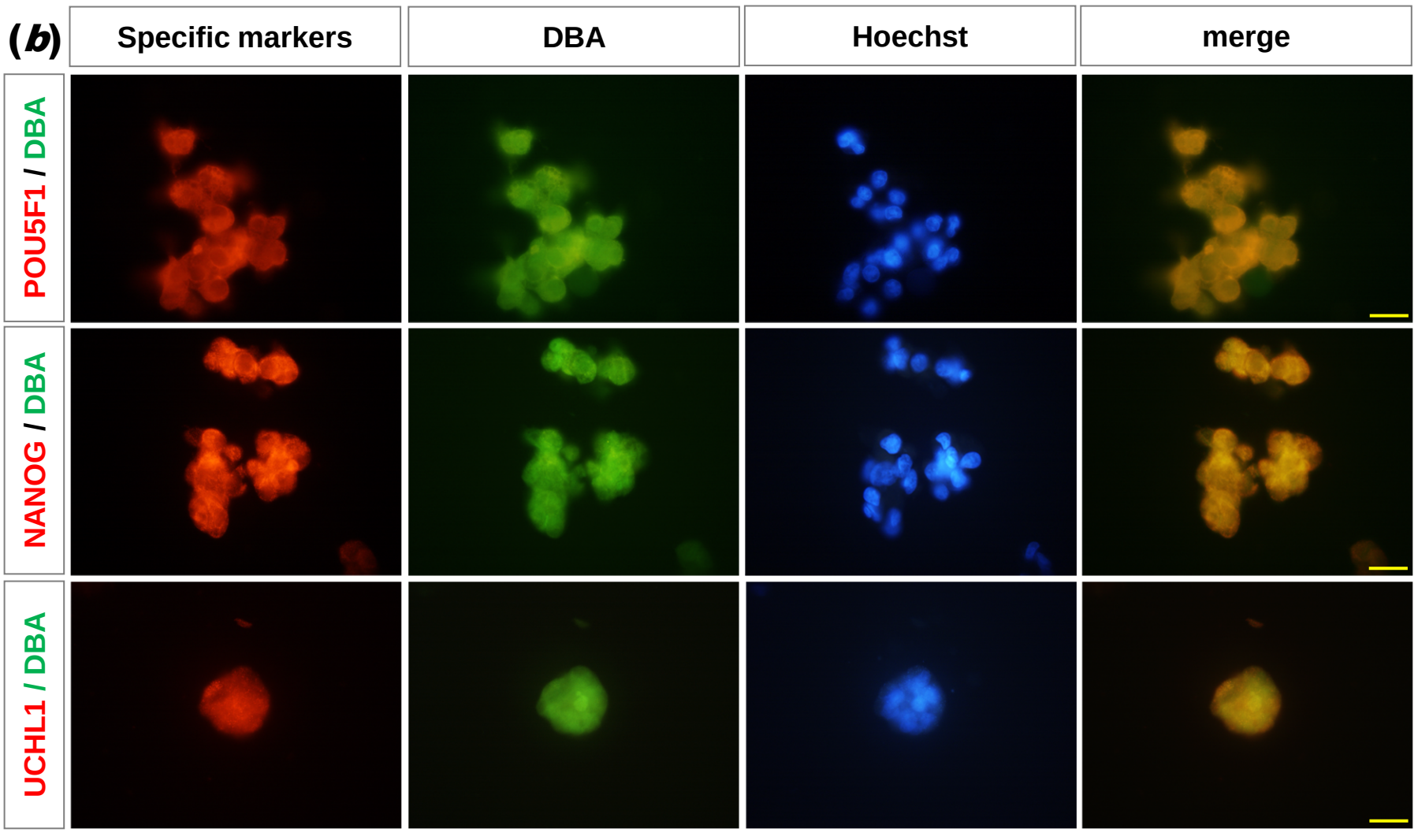



(*d*)

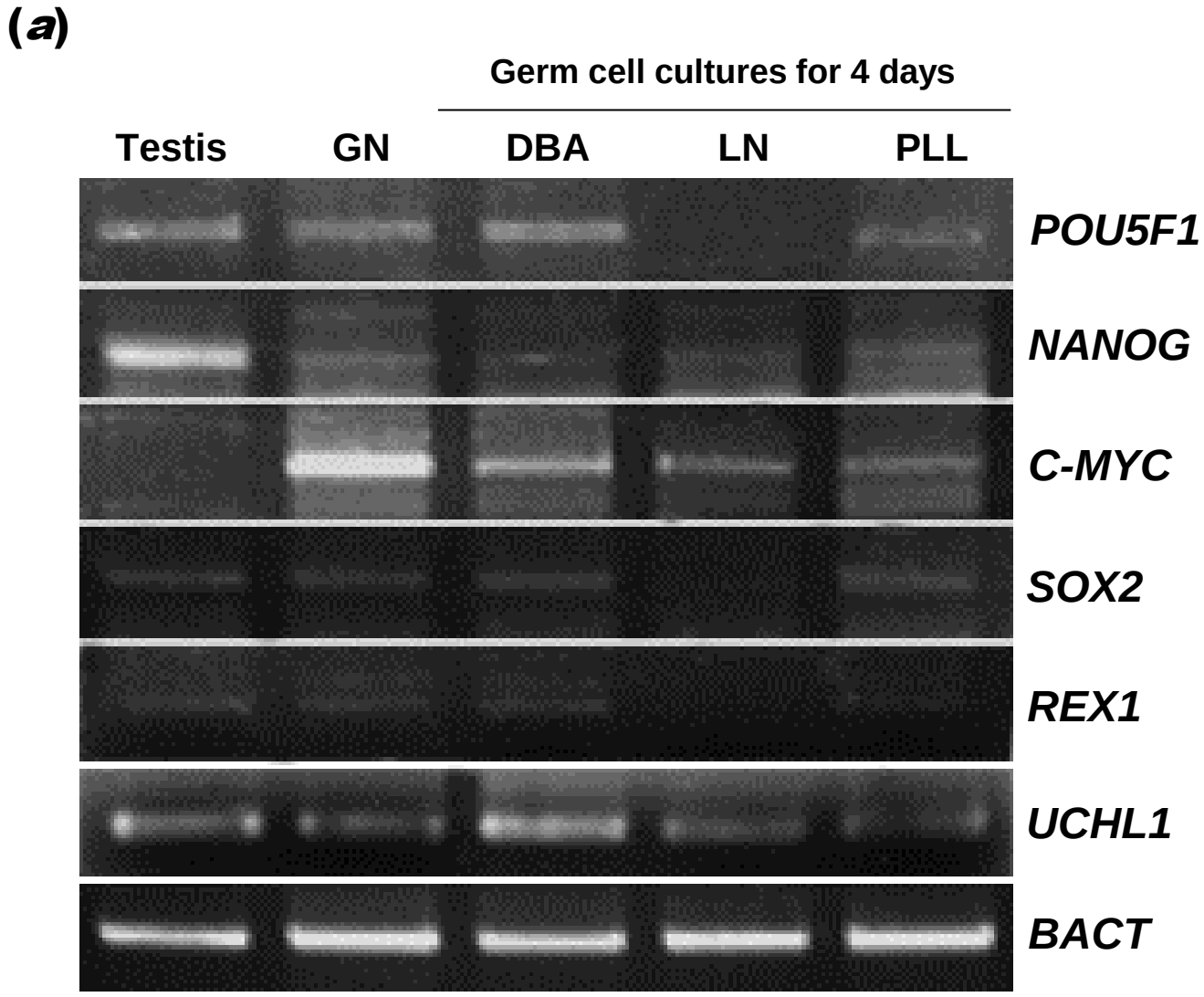


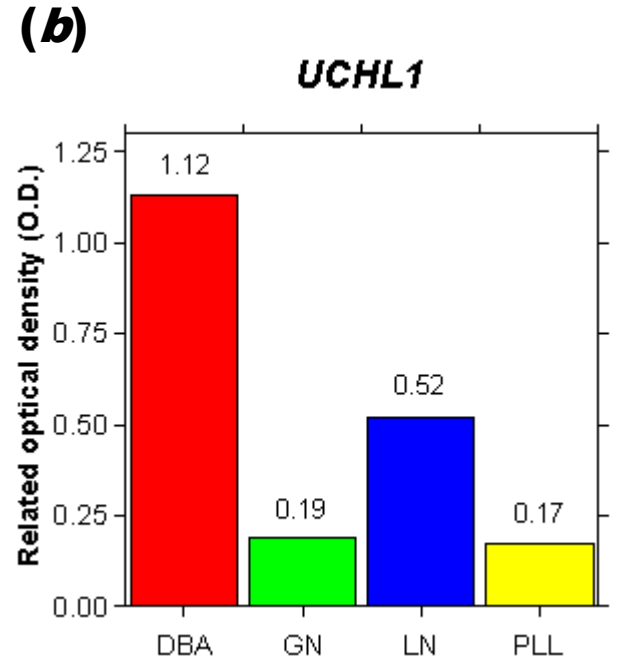
# Figure 5

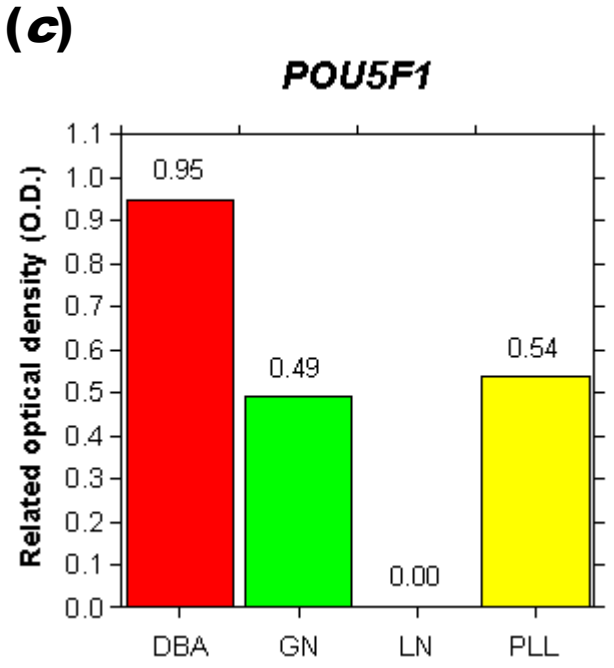

(a)
UCHL1
VIM
Hoechst
UCHL1 / VIM
DBA
GN
LN
PLL

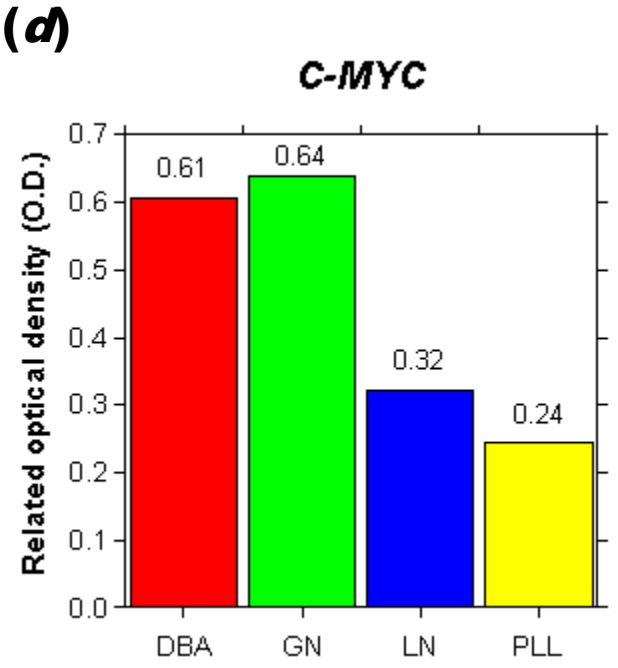

(b)
Specific markers
DBA
Hoechst
merge
POU5F1 / DBA
NANOG / DBA
UCHL1 / DBA

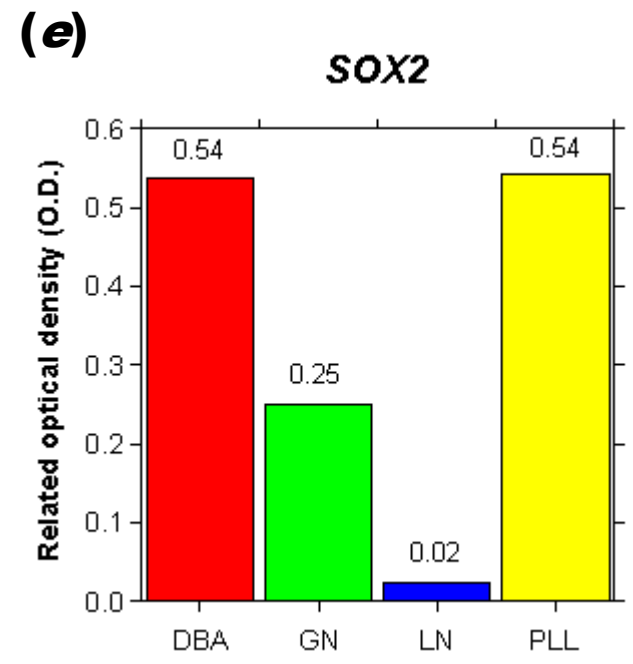
# Figure 6

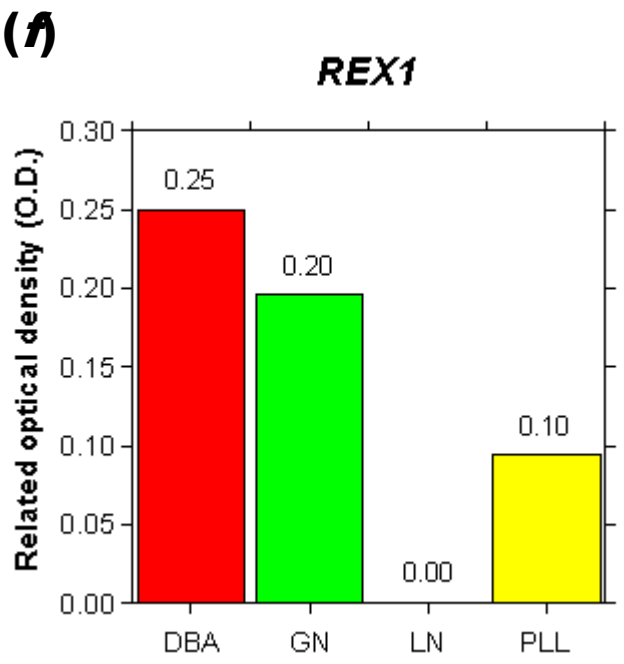

(a)
Germ cell cultures for 4 days
Testis
GN
DBA
LN
PLL
POU5F1
NANOG
C-MYC
SOX2
REX1
UCHL1
BACT

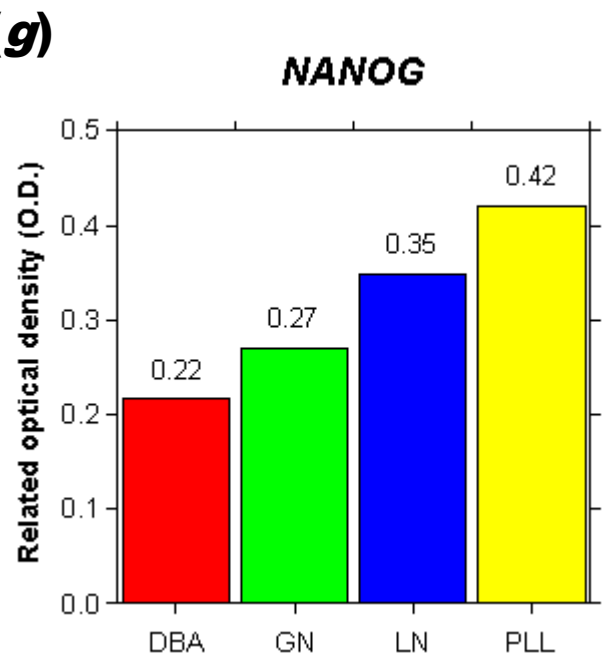

(b)
UCHL1
Related optical density (O.D.)
1.12
0.19
0.52
0.17
DBA
GN
LN
PLL


(c)
POU5F1
Related optical density (O.D.)
0.95
0.49
0.00
0.54
DBA
GN
LN
PLL


(d)
C-MYC
Related optical density (O.D.)
0.61
0.64
0.32
0.24
DBA
GN
LN
PLL


(e)
SOX2
Related optical density (O.D.)
0.54
0.25
0.02
0.54
DBA
GN
LN
PLL


(f)
REX1
Related optical density (O.D.)
0.25
0.20
0.00
0.10
DBA
GN
LN
PLL


(g)
NANOG
Related optical density (O.D.)
0.22
0.27
0.35
0.42
DBA
GN
LN
PLL