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Endometriotic cell culture contamination and authenticity: a source of bias in *in vitro* research?

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STUDY QUESTION: Are the primary cell cultures and cell lines used in endometriosis research of sufficient quality?

SUMMARY ANSWER: Primary cells used in endometriosis research lack purity and phenotypic characterisation, and cell lines are not genotypically authenticated.

WHAT IS KNOWN ALREADY: The poor reproducibility of *in vitro* research and the lack of authenticity of the cell lines used represent reasons of concern in the field of reproductive biology and endometriosis research.

STUDY DESIGN, SIZE, DURATION: In the present study, past *in vitro* research in the field of endometriosis was systematically reviewed to determine whether the appropriate quality controls were considered. In addition, we explored the performance of Paired Box 2 (Pax2) as an endometrium specific marker in endometrial and endometriotic primary cell cultures; we also characterised the most diffused endometriosis cell lines with respect to important markers including the short tandem repeat (STR) profile.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Literature review part: almost 300 published protocols describing the isolation and creation of primary cell cultures from endometriosis were reviewed. Wet-lab part: primary cells isolated from 13 endometriosis patients were analysed by immunohistochemistry, immunofluorescence and FACS for the expression of Pax2. Cell lines Z11 and Z12, the most diffused endometriosis cell lines, were characterised with respect to the expression of Pax2, steroid hormone receptors and STR profile.

MAIN RESULTS AND THE ROLE OF CHANCE: From the literature review work, we underscored the lack of sufficient cell purity and phenotypic characterisation of primary cell cultures, which present high risk of contaminations from surrounding non-endometriotic tissues. Past work based on the use of cell lines was reviewed as well, and it emerged that cell line authentication was never performed.
In an effort to address these weaknesses for future research, we present data on the performance of Pax2, a suitable marker to exclude ovarian (and other non-endometrial) cell contaminations from primary cell cultures; STR profiles of cell lines Z11 and Z12 were analysed and indicated that the cells were authentic. These profiles are now available for authentication purposes to researchers wishing to perform experiments with these cells.
A quality control pipeline to assure sufficient quality of *in vitro* research in the field of reproductive biology and endometriosis is proposed. We encourage scientists, research institutes, journal reviewers, editors and funding bodies to raise awareness of the problem and adopt appropriate policies to solve it in the future.

LARGE-SCALE DATA: STR profiles of cell lines Z11 and Z12 are deposited at the Cellosaurus database—web.expasy.org.

LIMITATIONS, REASONS FOR CAUTION: There may be additional markers suitable to assess cell quality.

WIDER IMPLICATIONS OF THE FINDINGS: Future *in vitro* research in endometriosis and the reliability of outcomes can be improved by using the recommendations presented in this study.

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Key words: endometriosis / endometrium / cell culture / quality control / short tandem repeat

Introduction

In vitro cell cultures are valuable and widely used models to dissect molecular and cellular mechanisms of normal and diseased conditions. These methods have contributed to important advances in drug discovery and other biomedical research and are expected to continue to do so in the future, thanks also to the recent technological advances of *in vitro* research that allows 3D culturing, refined medium, oxygen/CO_2 and nutrient supply and exchange and high-throughput formats (Vinci *et al.*, 2012).

During the last 10–15 years, however, numerous publications and the experience of cell-culture repositories found that 18–36% of cultures used for experimental research contained contaminating species or cell types. This, together with the alarmingly low reproducibility of preclinical research, induced the scientific community to become increasingly more aware of the necessity of rigorous standardisation and characterisation of *in vitro* tools (Korch *et al.*, 2012; Yu *et al.*, 2015; Fusenig *et al.*, 2017; Voelkl *et al.*, 2018).

Although these aspects are well recognised in the field of oncology research, where most journals currently require robust quality controls of cell authenticity prior to publication, this is less acknowledged in the field of reproductive biology. A paradigmatic example is represented by the case of endometriosis, a benign gynaecological disorder characterised by the presence of endometrial-like tissue at ectopic locations, i.e. outside the uterus, like the ovary—also referred to as ovarian endometrioma—and other peritoneal organs. With an estimate of around 176 million women during their reproductive age affected (endometriosis.org), endometriosis bears important burdens for the patient personal life (being the disease associated with chronic pain and infertility), for the society and healthcare system (the estimated costs range around 10 000€ per patient per year) and research in endometriosis drives a considerable portion of the financial resources the pharma industry dedicates to women health (Simoens *et al.*, 2011; De Graaff *et al.*, 2013; Vercellini *et al.*, 2014; De Graaff *et al.*, 2015; De Graaff *et al.*, 2016).

A substantial part of preclinical research in endometriosis is conducted through *in vitro* tools, either primary cell cultures or cell lines. Primary endometriosis cell cultures are isolated directly from fresh tissues of donor patients, and, in most cases, ovarian endometriosis is used because of the abundance of surgical material available. Cell lines are derived from primary cells that acquired (or are manipulated to obtain) the ability to proliferate for an unlimited period of time, can be repeatedly passaged and reliably recover from cryopreservation. Overall, no clear quality standards are recommended or followed to perform such *in vitro* research.

In order to evaluate what criterions of quality are currently adopted in endometriosis research, and what the potential existing biases are within, we first systematically reviewed published protocols used to isolate primary cells as well as the use of cell lines in endometriosis research. After identifying the potential risks of bias in current *in vitro* research practice, we propose an essential pipeline of quality controls that should be undertaken prior to perform *in vitro* experimental research in endometriosis and in reproductive biology in general.

Materials and Methods

Ethical statement

Procedures were conducted in accordance with ethical standards, national guidelines and international guidelines according to the Declaration of Helsinki and were approved by the local ethical authorities (METC-14-4-003 and protocol no. 726906), and subjects provided written informed consent.

Patients and human material

Two different types of specimens have been used, i.e. paraffin-embedded tissue sections for immunohistochemistry and fresh tissue-derived cell cultures for immunocytochemistry, immunofluorescence and fluorescence-activated cell sorting (FACS). Patient characteristics are summarised in Supplementary Table SI.

Tissue sections for immunohistochemistry

One normal endometrial sample and two deep infiltrative endometriotic samples were anonymously obtained from the Maastricht Tissue Bank (Maastricht University Medical Centre). Two ovarian samples were obtained from the pathology archives of the Maastricht University Medical Centre.

Fresh tissue

Normal endometrium and endometriotic samples were obtained from 13 patients undergoing laparoscopy between December 2016 and April 2017 at the San Raffaele Hospital.

Eutopic endometrial cell isolation and culture

Samples of uterine endometrium were obtained using an endometrial biopsy curette. Upon removal, specimens were transported to the laboratory in sterile vials containing Ham's F-10 solution. To isolate cells, our previously developed protocol was used (Vigano *et al.*, 1993). In short, tissue was gently minced into small pieces of 1 to 2 mm^3 and washed in fresh medium to remove mucus or debris. Thereafter, it was incubated for 2 h at 37°C in a shaking water bath in 10 ml Ham's F-10 solution containing 0.1% collagenase (w/v). At the end of the incubation, cells clumps were mechanically disaggregated by aspiration through a Pasteur pipette. After several washings, the cell suspension was digested in a 0.05% trypsin solution (v/v) for 3–5 min.

For mixed stromal and epithelial cell cultures, cells were washed twice in Ham's F-10 supplemented with 10% foetal calf serum and antibiotics and allowed to adhere selectively to tissue culture plates. They were cultured as monolayers in 1 ml of Ham's F-10 with 10% foetal calf serum, 2 mmol/l L-glutamine, antibiotics and 2.5 µg/ml fungizone in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. When the cell culture reached confluence, cells were either resuspended and fixed or directly fixed on the culture plate with 100% cold methanol.

For isolated stromal or epithelial cell cultures, single stromal cells were separated from large clumps of epithelium by a 10-min period of differential sedimentation at unity gravity. The top 8 ml of the medium, containing predominantly stromal cells, was then slowly removed, and the cells were collected by centrifugation (200*g*). The stromal-enriched fraction was washed twice in complete medium and allowed

to adhere selectively to 25-cm^2 tissue culture dishes for 15 min at 37°C in 5% CO_2. Thereafter, non-attached epithelial cells still present were removed and a purified stromal preparation was obtained on the surface of the culture dishes. Cells were cultured as monolayers in 1 ml of Ham's F-10 with 10% foetal calf serum, 2 mmol/l L-glutamine, antibiotics and 2.5 µg/ml fungizone in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. When the cell culture reached 60–80% confluence, cells were either resuspended and fixed or directly fixed on the culture plate with 100% cold methanol.

For epithelial cell isolation, the bottom 2 ml of sedimentation media was collected, washed and layered over 10 ml of fresh medium for two more 5-min sedimentation periods. Dissociation of glands in single cells or very small clumps was achieved by digesting the pellet with 4 ml of a 0.05% trypsin-0.02% EDTA solution for 3–5 min with constant aspiration through a Pasteur pipette. Digestion was stopped by adding the medium supplemented with foetal calf serum, and cells were washed twice by centrifugation. Final purification of epithelial cells was obtained by selective plating of any remaining stromal cells onto plastic substrate.

Cells were cultured as monolayer in 1 ml of Ham's F-10 with 10% foetal calf serum, 2 mmol/l L-glutamine, antibiotics and 2.5 µg/ml fungizone in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. When the cell culture reached 60–80% confluence, cells were either resuspended and fixed or directly fixed on the culture plate with 100% cold methanol.

Ectopic endometrial cell isolation

To isolate cells from ectopic tissue, the protocol derived from Ryan *et al*. was used (Ryan *et al*., 1994). Endometriosis cultures were prepared from biopsies of ovarian endometrioma cyst linings. All biopsies were collected in the operating room under sterile conditions. The tissue was rinsed with phosphate-buffered saline (PBS), and cyst lining was dissected free from underlying parenchyma, minced and digested with collagenase 0.1% at 37°C in 5% CO_2 for 90 min. At the end of the incubation, cell clumps were mechanically disaggregated by aspiration through a Pasteur pipette and allowed to adhere selectively to tissue culture plates. Glands and stroma were not further separated. They were cultured as monolayers in 1 ml of Ham's F-10 with 10% foetal calf serum, 2 mmol/l L-glutamine, antibiotics and 2.5 µg/ml fungizone in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. When the cell culture reached 60–80% confluence, cells were resuspended and fixed with 100% cold methanol.

Endometriosis cell lines

Cell lines Z11 and Z12 were kindly gifted by Prof. Starzinski-Powitz and were maintained as described earlier (Zeitvogel *et al*., 2001).

Immunohistochemistry

Pax2 protein expression in ovarian, endometrial and endometriotic tissue sections was determined using immunohistochemistry and anti-human Pax2 rabbit monoclonal antibody clone EP3251 (Abcam ab229318; Cambridge, UK) as described earlier (de Graaff *et al*., 2012). In short, antigen was retrieved with Tris–EDTA buffer (pH 9.0), followed by incubation with the primary antibody (1:1000 diluted). To visualise antibody binding, the Dako REAL EnVision Detection System (Glostrup, Denmark) was used. The sections were briefly counter stained in haematoxylin, dehydrated and sealed in Entellan (Merck, New Jersey, USA).

Immunocytochemistry and immunofluorescence

Immunofluorescence was performed on eutopic endometrial stromal and epithelial cells fixed on the culture plate. Cells were incubated overnight at 4°C with Pax2 monoclonal antibodies clone EP3251 (1:900; Abcam ab229318; Cambridge, UK), washed in PBS and incubated for 1 h at room temperature with FITC-labelled swine anti-rabbit secondary antibodies.

Immunocytochemistry was performed on resuspended and fixed eutopic endometrial stromal and epithelial cells. Cells were concentrated using cytospin centrifugation, and Pax2 antibody (1:500) was applied overnight at 4°C, followed by detection with the Chemate Envision and 3,3-diaminobenzidine solution (Dako, Glostrup, Denmark).

Fluorescence-activated cell sorting (FACS)

FACS analysis was performed on cells resuspended and fixed with methanol. After rinsing with PBS, cells were incubated with Pax2 monoclonal antibodies clone EP3251 (1:50; Abcam: ab229318; Cambridge, UK) for 2 h at room temperature, followed by incubation with phycoerythrin-conjugated goat anti-rabbit secondary antibody (1:20; Sigma-Aldrich, Zwijndrecht, The Netherlands) for 1 h at room temperature. Analyses were performed on a FACSCalibur (BD Biosciences, CA, USA) and analysed using the CellQuest software (BD Biosciences, CA, USA). Forward and sideward light angle scattered cells were gated to exclude cell debris/aggregates, and the percentage of positive cells was measured using as cutoff for background unstained cells/secondary antibody only stained cells.

STR profile

For STR profiling, genomic DNA was isolated from cells (2×10^6) using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) and was analysed through AmpFlSTR™ Identifiler™ PCR Amplification Kit (Thermo Fisher Scientific, Warrington, UK) according to the manufacturer's instructions.

Results

Approaches to primary ectopic endometrial cell isolation: a view on the past

A systematic review of the literature was used to evaluate the methodologies employed to isolate cells, especially regarding the assessment of cell culture purity and phenotypic fidelity. PubMed database (www.ncbi.nlm.nih.gov) was searched up to October 2018 for articles published in English language using the search terms 'endometriosis and cell culture', 'endometrioma and cell culture', 'endometriotic cells', 'endometriotic cell' and 'endometrioma and cell isolation'. A total of 4140 articles were retrieved and screened by an independent reviewer (EA), and 294 were included in the analysis (Supplementary Table SII). Only original articles involving the use of primary cell cultures derived

from human endometriotic tissues were included, while case reports, reviews and animal studies were excluded.

Isaacson and co-workers (Isaacson *et al.*, 1989) first described the isolation of ectopic endometrial cells from ovarian endometrioma and peritoneal implants to establish primary cell cultures. These authors referred to a previously published method (Satyaswaroop *et al.*, 1979) used to isolate primary cells from the eutopic endometrium. Such method was based first on tissue digestion with collagenase to obtain a suspension of single cells or small clumps followed by serial filtrations to separate epithelial and stromal components. Two subsequent studies established endometriotic cell cultures using endometrioma and peritoneal implants (Matthews *et al.*, 1992) or only endometrioma tissue (Ryan *et al.*, 1994). The methods for cell isolation did not substantially differ with respect to the protocol adopted by Isaacson and co-workers (Isaacson *et al.*, 1989), but the authors included a step to remove contaminating leukocytes. Ryan *et al.* (1994) exploited the fact that stromal cells adhere earlier than leukocytes (selective adhesion) and confirmed the absence of leukocytes by staining with the specific markers CD45, CD3 and CD11b, whereas Matthews *et al.* (1992) used Percoll density gradient solution. Both studies further used cytokeratin and vimentin as epithelial and stromal cell markers, respectively, to determine the purity of their cultures.

The protocols in these 'pioneering' studies (Satyaswaroop *et al.*, 1979; Isaacson *et al.*, 1989; Matthews *et al.*, 1992; Ryan *et al.*, 1994) were used by the majority of the papers published afterwards to isolate endometriotic cells. About half of the studies we reviewed either used a protocol developed for eutopic endometrial cell isolation (Satyaswaroop *et al.*, 1979) or did not cite any reference methodology, thus suggesting that authors may have overlooked the implications of working with different tissue sources (eutopic versus ectopic endometrial samples). The pioneering protocol for ectopic cell isolation by Ryan *et al.* (1994) served as model for the remaining second half of the studies that adopted only minor modifications and the addition of differential sedimentations for epithelial and stromal cell separation in some studies. A network of retrieved references and their interconnections is illustrated in Supplementary Figure S1.

Potential biases of past protocols: ovarian parenchyma cell contaminants and the choice of the chemicals and procedures

The 294 published studies described in the previous paragraph used different markers in order to characterise the purity of ectopic endometrium-derived cell cultures (Table I). Importantly, 84 studies did not assess cell culture purity, while the majority of the studies (158) used cell type-specific markers like vimentin and cytokeratin, and in some cases other markers to exclude the presence of leukocytes and endothelial and smooth muscle cells. These markers are useful to determine the efficiency of the epithelial/stromal separation and contamination by other cell types, but do not inform about potential contamination by cellular species of different tissue origins.

Most studies used ovarian endometrioma for primary cell isolation ($n = 192/294$), and to a lesser extent, peritoneal implants (Supplementary Fig. S1). Ovarian endometrioma (as well as peritoneal ectopic implants) are closely opposed to the underlying tissue (Muzii *et al.*, 2007; Sanchez *et al.*, 2014a; Sanchez *et al.*, 2014b; Sanchez *et al.*, 2015), and it is practically not possible to accurately separate the ovarian parenchyma from the ovarian endometriosis macroscopically. Therefore, the assessment of contaminations with cells originated from the tissue surrounding endometriosis is necessary prior to proceeding with the experiments. Nevertheless, none of the markers mentioned above can evaluate the presence of cells of non-endometrial origin surrounding endometriosis, especially the ovarian stroma. In our systematic search, out of the 192 studies that used ovarian endometriosis and 102 studies that used peritoneal or mixed peritoneal/ovarian samples for primary cell isolation, such quality control was undertaken by 50 studies only, using the endometrium-specific marker CD10 and/or prolactin secretion upon progestogen stimulation (Table I). Although these quality controls represent an improvement compared with studies where no such characterisation was performed, they still present limitations: CD10 has low specificity since it shows a variable expression in activated leukocytes and endothelial cells and it is present on stromal but not on epithelial cells. Prolactin secretion cannot be used to assess the cell purity, since non-endometrial cells may not secrete prolactin and would remain hidden. In this context, Yagyu and co-workers described that the level of prolactin produced by ectopic cells was lower than that of eutopic cells, underscoring the possibility that peritoneal fibroblasts might have contaminated the culture (Yagyu *et al.*, 2005). Moreover, prolactin is produced by stromal cells and therefore cannot be used for epithelial cell culture purity assessment. Two other studies used FSH-receptor as a negative marker (Zhang *et al.*, 2015; Zhang *et al.*, 2016). The FSH receptor may be a potential test for contamination by granulosa cells or ovarian stroma, but since it is also variably expressed by endometrial cells, it therefore lacks sufficient specificity (Sacchi *et al.*, 2018).

Therefore, based on our systematic review of the literature, the risk of contamination by cells derived from the tissue surrounding the endometriotic tissue used to establish cell cultures is very high, and the presence of such contaminants should be excluded/quantify prior to proceeding with the *in vitro* experiments.

Besides the molecular and histologic purity of the material used to derive primary cells, additional important aspects that can introduce extra potential biases are the reagents and procedures used for *in vitro* protocols, starting from the method used to disaggregate the tissue and ending with the culture conditions and supplements added. The strategies and the materials used to separate cells in human

Table I Overview of the markers used to characterise the purity of cell cultures upon isolation from ectopic (endometriotic) tissues among the 294 studies reviewed.

Specificity	Markers
Stromal cells	Vimentin, Thy-1, CD13
Epithelial cells	Cytokeratin, EpCAM, CD9, E-Cadherin
Leukocytes	CD45, CD3, CD11b, CD56, CD14, CD68
Endothelial cells	Von Willebrand factor, CD31, CD146
Smooth muscle cells	Alpha-smooth muscle actin, smoothelin
Mesenchymal cells	CD63, CD90, CD105, nestin
Endometrial cells	CD10 (stroma only)
Endometrial cells	FSH receptor
Endometrial cells	Prolactin production

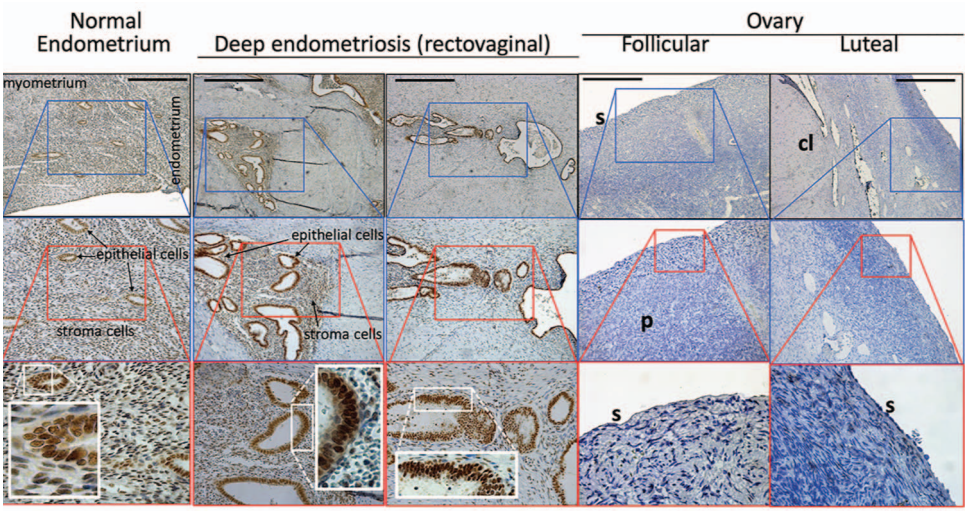

Figure 1 Immunohistochemistry of Pax2 on endometrium and ovary. Pax2 levels in normal endometrium (left), endometriosis (rectovaginal infiltrative lesion, middle) and ovarian tissue (right) were determined by immunohistochemistry. Pax2 shows strong nuclear staining in both stroma and epithelial cells of endometrial origin, whereas ovarian structures (surface epithelium (S), parenchyma (P), corpus luteum (CL) and follicles—not in figure) are devoid of any Pax2 expression. Bar scale: 0.5 mm.

tissues were derived from protocols optimised in several other species (Cherny *et al.*, 1990). Enzymes such as collagenase, trypsin, pancreatin, hyaluronidase and DNAse were used to disaggregate the tissue and dissociate endometrial cell subtypes. Most studies on endometriotic cell isolation (that referred to the aforementioned 'pioneering' studies) made used 0.25–2% collagenase solution, which was also shown to be as efficient as a triple enzyme solution (0.1% trypsin, 0.1% hyaluronidase and 0.05% collagenase) in separating stromal cells from glands (Varma *et al.*, 1982). Separated cells can be further purified by a variety of combinations of mechanical separation techniques including pipetting, differential centrifugation, filtration and density sedimentation. Critical for the separation methods are differences in the speed of sedimentation in a tube and in the easiness of attachment/detachment of cultured cells to the plastic (Sharpe-Timms *et al.*, 2002). Filtration through sieves of different sizes allows single cells to be separated from aggregates of cells. The cell population retained on the sieve consists mostly of glandular epithelial cells while the cell population that passes through the sieve consists mostly of stromal and blood cells. However, such filtration does not exclude that a proportion of stromal single cells are retained on the sieve together with glands (Isaacson *et al.*, 1989). Therefore, procedures involving differential sedimentation and selective adhesion to the plastic have been added to or have replaced the filtration procedure in order to increase the recovery of the stroma cell population and the purity of epithelial cells (Vigano *et al.*, 1993).

Once cells are sufficiently purified and characterised, the choice of the culture medium and the supplements can have important effects of the phenotype of the cells grown *in vitro*. Media with high nutrient, vitamin and glucose contents like Dulbecco's modified Eagle's medium (DMEM, simple or supplemented with Nutrient Mixture F-12, DMEM-F12) or Ham's Nutrient Mixture F-10 were most commonly used by authors, and these media were further supplemented with 5–15% foetal bovine/calf serum. This last can be a great source of variability, since strong batch to batch variations in the content of micro/macro nutrients, growth factors and cytokines exist. Experienced laboratories purchase large quantities of a serum and characterise each batch thoroughly for the effect on the used cells.

Paired-box 2 is a suitable marker to assess endometriotic cell purity

Based on our systematic search of the literature, we underscore the lack of sufficient molecular characterisation of the primary material used to establish the cultures, which bear the risk of non-endometrial cell contamination, and we emphasise the importance of adopting rigorous standard operating procedures and protocols (some recommendations are given at the end of the discussion). We next explored whether any already known marker could serve as quality control to assess cell purity and quantify the contamination of primary cultures with non-endometrial cells. Paired-box 2 (Pax2) is a transcriptional regulator of the paired-box family and is widely expressed during the development of both ductal and mesenchymal components of the urogenital system (Blake *et al.*, 2014). Pax2 immunostaining is strongly associated with the Müllerian duct, urinary tract, uterovaginal canal, uterine tube, uterine corpus and uterine cervix and is absent in the ovary (https://www.proteinatlas.org; http://www.pathologyoutlines.com/; Tong *et al.*, 2007; Tung *et al.*, 2009; Uhlen *et al.*, 2015).

Due to such high expression in both the epithelial and stromal components of endometrial cells and endometriosis, and to its absence in the tissues surrounding the lesions at ectopic locations (in particular in the ovary), Pax2 was proposed already in the past as a marker to distinguish endometriotic tissues from other contaminants (de Graaff *et al.*, 2012).

In order to demonstrate here that Pax2 can be used as a marker of endometriotic tissue, we confirmed by immunohistochemistry the specificity of Pax2 for endometrium and endometriosis where strong

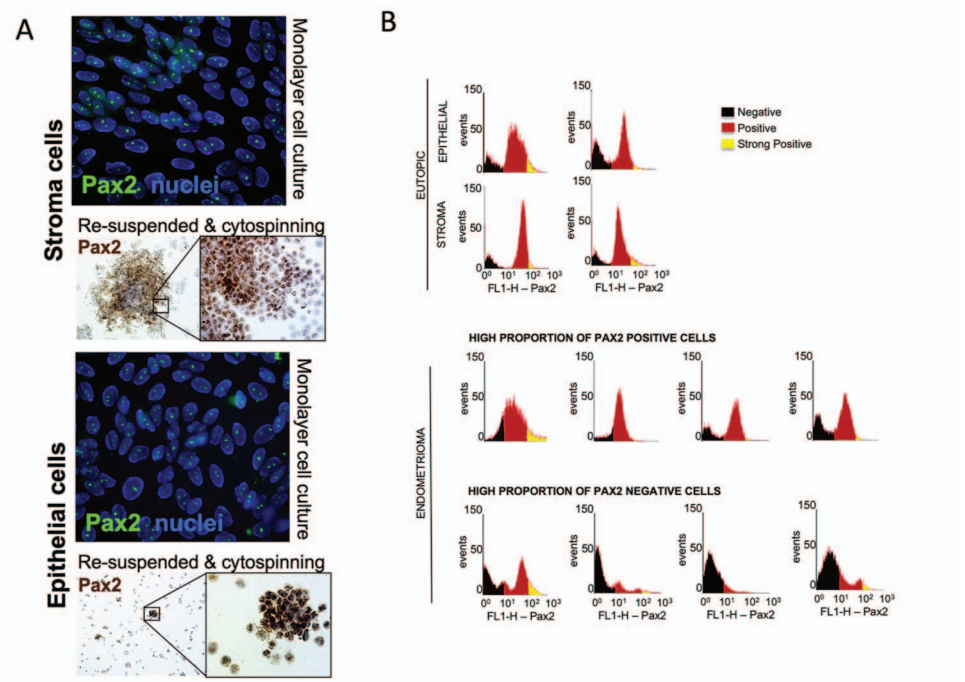

Figure 2 Pax2 expression in isolated eutopic and ectopic primary cells. (**A**) Adherent cells were stained for Pax2 by immunofluorescence (large panels, Pax2 is green). Resuspended cells were attached to a glass (cytospin) and stained for Pax2 using immunocytochemistry (ICC; small panels). Both eutopic stromal and epithelial cell compartments show strong Pax2 expression levels. (**B**) Cells were resuspended, fixed in methanol and subjected to fluorescence-activated cell sorting (FACS) to assess for cell purity. Cells isolated from the eutopic endometrium (top histograms) are Pax2 positive (shown for two out of the three patients analysed). In contrast, cells isolated from endometriomas (eight samples shown out of nine analysed) show that the proportion of Pax2-negative cells is variable across patients.

nuclear immunoreactivity was observed (Fig. 1). In contrast, no Pax2 expression was seen in the ovarian parenchymal tissue/surface cells (Fig. 1).

To further prove the suitability of Pax2 as an endometrial marker, we isolated primary cells from eutopic and ectopic tissue obtained upon surgery from 13 women with endometriosis (patient characteristics are given in Supplementary Table SI). Eutopic endometrial cells were isolated using our previously developed protocol (Vigano *et al.*, 1994), while ectopic endometrial cells were derived according to the procedure used in one of the pioneering studies (Ryan *et al.*, 1994), with the exception that glands and stroma were then analysed together because our aim was not the purity of the endometrial cell populations (i.e. stroma versus epithelial) but the potential contamination by cell types from the tissue surrounding endometriosis. Avoiding any procedure to isolate specific cell types should allow to exclude the possibility to have introduced a technical selection of the contaminating cells.

Strong Pax2 expression was confirmed in stromal and epithelial cells isolated from eutopic tissue and cultured *in vitro* (Fig. 2A). Fluorescence-activated cell sorting (FACS) analyses were used to quantify the proportion of Pax2 positivity and negativity among primary cells isolated from independent patients using eutopic or ectopic material. The majority of stroma and epithelial cells isolated from eutopic tissue were clearly Pax2-positive (Fig. 2B, upper panel). In contrast, endometrioma-derived cell cultures showed variable proportions of Pax2-negative cells (Fig. 2B, lower panel).

Endometriotic cell lines: lack of genotypic reference for authenticity controls

A number of immortalised cell lines deriving from endometriosis have been established by either forcing cells to survive through a cell crisis or by the introduction of one or more oncogene(s) (Table II). The starting cells used for immortalisation are primary cell cultures; hence, the criteria reported earlier about procedural biases and possible contaminations should be considered also in case of producing new cell lines. Most cells were obtained upon collagenase digestion of the human tissue (see Table II) and maintained in DMEM-F12, with one exception, EEC16/ECC16TERT, which were isolated by mechanical brushing of the superficial endometriotic lesion on the ovarian surface and were maintained in a specific medium optimised for ovarian surface cells (Brueggmann *et al.*, 2014; Lawrenson *et al.*, 2014).

With regard to the molecular purity of cells, two groups (Bouquet de Joliniere *et al.*, 1997; Zeitvogel *et al.*, 2001) excluded the presence of contaminating immune, mesothelial or epithelial cells in their developed cell lines, and cells EEC16 were explored by whole expression profiling (RNA sequencing), which allows thorough phenotypic characterisation of generated lines and verification of the similarities to the (patho)physiology of the tissue of origin (Brueggmann *et al.*, 2014; Lawrenson *et al.*, 2014). Further characterisation with respect to karyotyping, steroid hormone receptor expression or steroid hormone response or the changes the cells undergo throughout culture passages was assessed to various extents in the different studies (Table II).

Table II Overview of the endometriosis cell lines created and their main features.

Cell line (Ref)	Cell type*	Site of origin#	Method of isolation and immortalisation**	Contaminations##	Purity$ —extra features	Deposited@	STR
FbEM-1 (Bouquet de Joliniere, Validire, Canis, Doussau, Levardon and Gogusev, 1997, Gogusev *et al.*, 2000)	EP	Per & Ov	- Collagenase - Serial passaging (spontaneous)	vWF (−), CD3 (−), CD20 (−), CD45 (−) CEA (−), Ca125 (−) EMA (−), HLA (+) Desmin (−), Collagen IV (−)	CK (+), Vim (+) ERα (−), ERβ (−) PR (+), AR (−)—Karyotype	CVCL_0Q76	No
Emosis-CC/TERT *other cells* (Bono *et al.*, 2012)	EP	Ov	- Collagenase—CCND1, Cdk4, hTERT	none reported	CK (+), CD10 (−) FSP1 (−) ERα (+/−)& PRB (+/−)& PRA (+/−)&	no	no
EEC16 (Brueggmann, Templeman, Starzinski-Powitz, Rao, Gayther and Lawrenson, 2014)	EP	Ov	- Brushed ovarian surface - Non-immortalised	None reported	CK (+), Vim (+) E-cad (−) ERα (−) N-cad (−) RNA profiling 3D-culturing	No	No
EEC16 TERT (Lawrenson, Lee, Torres, Lee, Brueggmann, Rao, Noushmehr and Gayther, 2014)	EP	EEC-16	- Non-applicable - hTERT	None reported	CK (−), Vim (+) - EMT (+)^^ 3D-culturing	No	No
CRL-7566 (Owens *et al.*, 1976)	EP	Ov	- Collagenase - Non-performed$$			CVCL_0936 ATCC^	yes
hEM-5B2 (Chen *et al.*, 2016)	EP	Ov	- Collagenase - SV40-T	None reported	ERα (+)&& ERβ (+)&& PR (+)&&	No	
11Z, 12Z *other cells* (Zeitvogel, Baumann and Starzinski-Powitz, 2001)	EP	Per	- Collagenase + dispase - SV40-T	CD31 (−), vWF (−) CD3 (−), CD45 (−) CD68 (−) FVIIIa (−) Calretinin (−) clone TE7 (−) Desmin (−)	CK (+) Vim (+) ERα (+)& ERβ (+)& PR (+)&	CVCL_0Q72 CVCL_0Q73	Here
22B *other cells* (Zeitvogel, Baumann and Starzinski-Powitz, 2001)	ST	Per	- Collagenase + dispase - SV40-T	None reported	CK (−) Vim (+)	No	No
No name (Boccellino *et al.*, 2012)	EP/ST	DE	- Collagenase - hTERT	None reported	CK (+) CD10 (−) ERα (+)& ERβ (+/−)& PR (+)& - Karyotype	No	No

Continued

Table II Continued.

Cell line (Ref)	Cell type*	Site of origin#	Method of isolation and immortalisation**	Contaminations##	Purity$—extra features	Deposited@	STR
Clo03 *other cells* (Akoum *et al.*, 1999)	EP	Per	- Collagenase - SV40	None reported	CK (+) Vim (+) - Karyotype	No	No

*EP: epithelial; ST: stromal
#Per: peritoneal lesion; Ov: ovarian endometrioma; DE: deep endometriosis lesion
**Genetic modifications used for cell immortalisation: CCND1: cyclinD; Cdk4: cyclin-dependent kinase 4; hTERT: human telomerase reverse transcriptase; SV40-T: virus VS-40 large T-antigen; SV40; complete virus
##Markers used for contamination assessment:
- Endothelial markers: vWF (Von Willebrand factor); FVIIIa (Factor VIIIa), CD31
- Vascular marker: Collagen IV
- Leukocyte markers: CD3 (T cells), CD20 (B cells), CD45 (general leukocytes), HLA: human leukocyte antigen
- Macrophage marker: CD68
- Mesothelial cell marker: calretinin, clone TE7
- Neoplastic cell marker: EMA (epithelial membrane antigen), CEA (carcinoembryonic antigen), Ca125
$Markers used for purity assessment:
CK: cytokeratin; Vim: vimentin; E-cad: E-cadherin; N-cad: N-cadherin; CD10: endometrial stroma cell marker; FSP1: fibroblast (stroma cell) marker; ERα: estrogen receptor-α; ERβ: estrogen receptor-β; PR: progesterone receptor; AR: androgen receptor.
@Databases: Cellosaurus database ((Bairoch, 2018), web.expasy.org) given as 'CVCL' number; ATCC (atcc.org)
^This cell line was discontinued by ATCC due to slow grow rate and difficulties to maintain the cell in the repository (ATCC, personal communication by Charlotte Williams, ATCC Technical Specialist, LGC Standards, UK).
^^Authors determined functionally the occurrence of EMT (epithelial to mesenchymal transition).
&Expression was detected at various levels in different cell clones.
&&Expression level was determined by reverse transcriptase PCR.
$$The line senesces after few passages.

In addition to this, since immortalised cell lines are kept in culture for long periods of times and may accidentally enter in contact with other lines growing in the same incubator/lab, the assessment of the genotypic authenticity by short-tandem-repeat (STR) prolife (or other unique genotypic features like SNPs) is needed. This aspect becomes of foremost importance when cell lines are exchanged between laboratories and used for different experiments worldwide, like in the case of the cell line CRL-7566 (ATCC deposited), or for epithelial cell lines Z11 and Z12 (Zeitvogel *et al.*, 2001). Supplementary Tables SIII–SVII show the distribution of the different cell lines to various laboratories. Strikingly, the STR profile for cell line authentication is available for cell line CRL-7566, only.

Since several scientists in the field of endometriosis consider and use Z11 and Z12 cell lines as models for endometriosis, and since these cells are diffused and used at laboratories other than the laboratory that created the cells, we performed a series of extra quality controls. Z11 and Z12 lines were kindly gifted by Prof. Starzinski-Powitz (Wolfgang-Goethe Frankfurt University, Germany).

STR profile (Fig. 3A) showed no identity of Z11 and Z12 with other deposited cell lines and confirmed that these cell lines are authentic and non-contaminated by other existing cell lines. Next, we also determined the expression of Pax2 (strong nuclear positivity), ERα (expressed in both nucleus and cytoplasm) and PR, which was not detectable in our hands (Fig. 3B).

Discussion

In the present study, we critically analysed past *in vitro* research in the endometriosis field to determine whether the appropriate quality controls were considered. Through a systematic review of past literature, we underscore in particular the lack of sufficient cell purity and phenotypic characterisation in the use of primary cell cultures, which presents a high risk that cultures were contaminated by cells from surrounding non-endometriotic tissues. We also emphasise the lack of reference deposited data for genetic authentication of the cell lines used in endometriosis research. In an effort to contribute addressing these weaknesses for future research, we present data on the performance of Pax2 as a quality control marker that can help exclude ovarian (and other non-endometrial) cell contaminations from cell cultures; we also characterise the most diffused endometriotic cell lines with respect to important markers including the STR profile.

In vitro research is a valuable tool in biomedical investigations that allows us to perform experiments in a simple and easily controlled environment. In recent years, however, the poor reproducibility—and even the false findings—of previous experimental evidences became a reason of concern within the scientific community, since it causes delays in the translation of scientific research into applications and practice or, even worse, can drive resources in the wrong directions (Korch *et al.*, 2012; Yu *et al.*, 2015; Fusenig *et al.*, 2017; Voelkl *et al.*, 2018).

The reliability of data obtained from *in vitro* investigations is strongly affected by the use of non-authentic or phenotypically incorrect material. In oncology research, several journals, funding agencies and research institutes require quality controls to phenotypically characterise and genetically authenticate the cells used for *in vitro* experiments. Currently, several online databases that comprehensively

A

Mark	Z11	Z12
Amel	X,X	X,X
D5S818	12,12	12,12
FGA	21,24	23,24
D19S433	12,13	13,14
vWA	15,17	17,17
TPOX	8,10	11,12
D18S51	16,18	13,15
D3S1358	15,17	14,17
THO1	9,10	9,9.3
D13S317	9,9	12,13
D16S539	9,9	13,13
D2S1338	20,20	19,19
D8S1179	13,15	10,10
D21S11	29,33.2	28,30.2
D7S820	8,12	9,12
CSF1PO	11,12	11,13

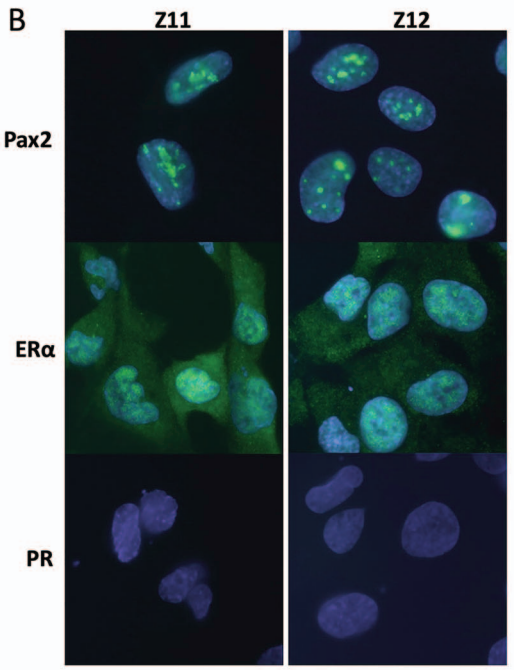

Figure 3 Characterisation of Z11 and Z12 endometriosis cell lines. (**A**) STR profile of Z11 and Z12 cell lines (the profile is deposited and publicly available at: ExPASy, web.expasy.org). (**B**) Expression of Pax2 (positive), ERα (positive) and PR (negative) in Z11 and Z12 cell lines.

report the STR profile of common cell lines are freely available. One of the first developed is offered by the German Cancer Research Centre (DKFZ; www.dsmz.de), but also ATCC (atcc.org) and Cellosaurus database (Bairoch, 2018; web.expasy.org) give similar services and provide easy algorithm-based tools to determine the purity or the identity of a cell line simply by inputting its STR profile online. Nowadays, most journals have mandatory requirements for cell line authentication prior to publishing (Lichter *et al.*, 2010; Masters, 2012; Yu *et al.*, 2015). However, these same aspects are less recognised in the field of reproductive biology. In endometriosis research, over 15 000 papers are retrievable in PubMed since 2000. Through a systematic search, we found that over 290 studies made use of primary cells isolated from patient biopsies. These articles have been cited 12 355 times, a number that highlights their potential ability to divert research toward certain paths. After cross-referencing the articles included, it emerged that two protocols published between 1992 and 1994 (Matthews *et al.*, 1992; Ryan *et al.*, 1994) and specifically developed for ectopic endometrial cell isolation were used directly or indirectly as a reference for cell culture development methods by many of the other papers, with poor evolution of characterisation strategies over time, while the rest of the studies either did not cite a reference method, or used a methodology developed for eutopic (i.e. non-endometriotic) endometrial cell isolation as a reference. None of the protocols does assure the absence of contaminants originated from the tissues surrounding endometriosis. The lack of an appropriate biomarker specific for endometrial cells has been most likely one of the reasons for such lack of quality control. Few studies used CD10 as endometrial biomarkers, which, while efficiently used by pathologists to detect ectopic endometrial stroma in clinically difficult cases, is not specific enough. Other authors used prolactin secretion or FSH-R expression, but also these markers lack sufficient specificity (see earlier).

We show here the suitability of Pax2 as an endometrial specific marker. Pax2 was characterised previously by our team as an endometrial marker, highly expressed in the nucleus of both ectopic and eutopic cells (stroma and epithelium) and absent in the tissues surrounding the endometriotic lesions (de Graaff *et al.*, 2012). In this study, we demonstrated the specificity of Pax2 towards endometrial cells and the negativity for ovarian structures that are very close to the ovarian endometriotic tissues frequently used for primary cell culture establishment. We also demonstrated that staining with Pax2 is a straightforward and reliable way to determine and quantify the presence of contaminating cells in primary endometriotic cell cultures.

In endometriosis research, due to the benign nature of the disease, immortalised cell lines are use less frequently than primary cell cultures. Nevertheless, at least eight different teams published about the establishment and characterisation of endometriotic cell lines and over 400 papers referred to the original articles describing the creation of these cell line (Supplementary Table SIII). In addition, over 50 papers described results generated with these lines and these papers have been cited over 800 times (Supplementary Tables SIII–SVII). Again, these numbers underscore the impact that these tools can have on research. In particular, since their generation, cell lines Z11 and Z12 have been circulating through labs to generate new publications, but the need of genetic authentication was disregarded by all authors and no STR profile was publicly available till now. Through STR analyses, we confirmed that Z11 and Z12 cells are authentic and

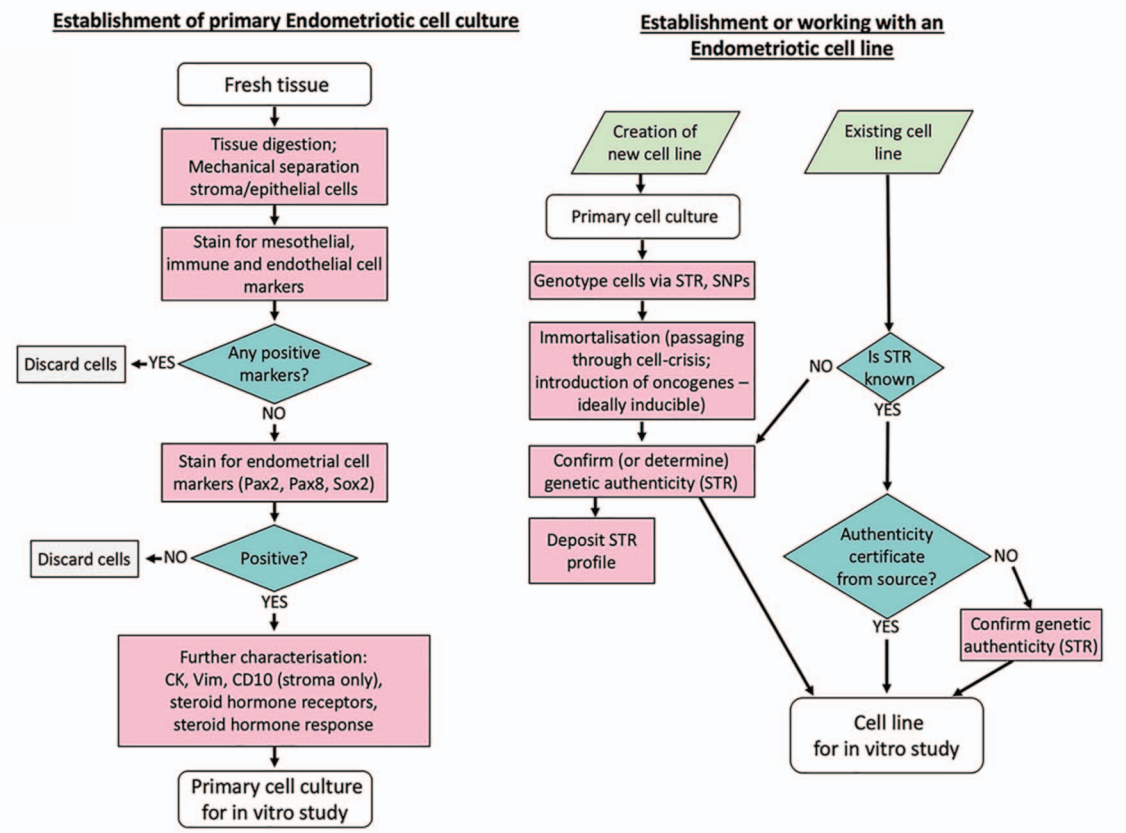

Figure 4 Flowchart of recommended quality controls. Proposed flowchart for quality control assessment in endometriosis *in vitro* research. The creation of primary cell cultures from ectopic tissues (left) should confirm the absence of mesothelial cells (negativity for calretinin, clone TE7), endothelial and vascular cells (negativity for Von Willebrand factor, factor VIIIa, CD31, collagen IV, α-smooth muscle actin), immune cells (negativity for CD3, CD20, CD45, CD68, human leukocyte antigen). Subsequently, endometrial origin of cells should be confirmed by Pax2 positivity (also Pax8 and Sox2—www.proteinatlas.org (Uhlen *et al.*, 2015))—may be good candidate markers (though they were not thoroughly tested by us) followed by further characterisation regarding the expression of steroid hormone receptors and epithelial and stroma markers. With regard to cell lines, established cell lines obtained from non-certified suppliers should be always authenticated by STR profile, whereas the creation of a new line should be always accompanied by description of deposition in public databases of the authentic STR profile.

not contaminated by other common cell lines. Their STR profiles are now available for genetic authentication purposes to other scientists (Fig. 3A, and Cellosaurus database, web.expasy.org). In our hands, these cells expressed Pax2 and ERα, but, in contrast to other reports, they were PR-negative, suggesting the presence of possible batch-to-batch variations, or variability due to the culture methods and protocol. However, since signalling initiated by both ERα and PR is necessary for endometrial physiology, it is of foremost importance that cells are thoroughly characterised prior to each experiment for the maintenance of the proper phenotype and for their receptor status.

In a strive to define guidelines to improve the quality of endometriosis research in future, we present a pipeline of quality controls, based on marker expression for phenotypic quality and genetic authentication profiling, that we recommend authors should undertake in order to guarantee good standards of their results based on primary cells or cell lines. Such pipeline is illustrated in Fig. 4, and it should not be underestimated the fact that an efficient teamwork including the surgeon, the pathologist and the researchers is important to assure good quality of the initial clinical material.

In addition to this, other technical aspects (although they were not directly addressed in the present study), should be considered to assure the fidelity of cell phenotype and the reproducibility of the results. Phenotypic characterisation of cells and lines and characterisation of their signalling pathways by assessing the expression of large panels of genes—as performed for cell lines Z11, Z12 and B22 (Banu *et al.*, 2008) and line EEC16 TERT (Lawrenson *et al.*, 2014)—or by global expression profiling (Brueggmann *et al.*, 2014) are extremely powerful tools to verify the fidelity of cells to the original tissue phenotype. In this respect, 3D cultures as spheroids or organoids were shown to better recapitulate than monolayer cell cultures the endometrial pathophysiology (Brueggmann *et al.*, 2014) and should be considered as preferred *in vitro* method when possible. Also, the choice of the ideal culture media, the foetal calf/bovine serum batch characterisation, the regular cell passaging (that should not exceed the number of 3–4 for primary cells), the regular refreshment of cultured lines (after $\pm$10 passages) to avoid the excessive accumulation of chromosomal aberration and the mycoplasma testing should all be part of the standard workup in a cell culture laboratory. In addition to this, cell cultures (in

particular primary) consist of heterogeneous populations of cells that could be differentially favoured to grow during *in vitro* conditions. As a result, some populations may prevail over the others during passaging. Therefore, it is highly recommended to characterise (primary) cells during passages to document eventual phenotypic changes. Finally, in experiments where the steroid signalling (and other hormones) is explored, media without phenol red and supplemented with foetal calf/bovine serum that is deprived of (steroid) hormones have to be used.

In conclusion, *in vitro* research allowed important breakthroughs in medical sciences and it is likely to continue doing so in future, due to the impressive technological advances of *in vitro* tools, the use of high-tech culture systems like on-chip cultures or three-dimensional systems and the development of synthetic scaffold, novel hydrogels, novel biomaterials and high-throughput platforms for drug screening and personalised medicine (Vinci *et al.*, 2012; Madl *et al.*, 2018; Ronaldson-Bouchard *et al.*, 2018; Vianello *et al.*, 2019). To make best use of these opportunities, professionals should be aware of the potential biases associated with *in vitro* models, both in terms of authenticity of the material used and with respect to the study design and research questions that can be answered by *in vitro* research.

We encourage all professionals involved in the field of reproductive biology, researchers, research institutes, reviewers and editors and journals—the ultimate stakeholder in ensuring experimental quality and rigorousity—to raise awareness of these aspects and potential biases and to adopt and request proper quality controls.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

S.X., B.D. and E.G. performed the experiments and acquired and analysed the data; P.V. and A.R. supervised and coordinated the project; A.R., P.V. and E.A. discussed the results; A.R., P.V. and E.A. drafted the manuscript. All authors read and approved the manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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