

March 5th KTE

Embryo Coculture

Yves J R Ménézo (1), Edouard Servy (2), Anna Veiga (3), André Hazout (1), Kay Elder (4)

1- UNILABS France, 55 Rue St Didier 75116 Paris France

2- The, Servy Institute for Reproductive Endocrinology, 812 Chaffee Avenue, 30904 Augusta Ga, USA

3- Servei de Medicina de la Reproducció, Institut Universitari Dexeus, Gran Via Carlos III, 71-73 08028 Barcelona

4-Bourn Hall Clinic – Bourn, Cambridge CB23 2TN United Kingdom

Keywords: Coculture, embryo, blastocyst, growth factors, free radical scavengers

Summary:

During the 1970s, domestic animal biotechnology i.e embryo transfer in farm animals, was confronted with the problem of embryonic developmental arrest observed in vitro, especially during the cycle in which MZT cycle (Maternal to zygotic transition) takes place. In farm animals, obtaining blastocysts is mandatory, as transfer at earlier stages results in expulsion of the embryo from the vagina. In humans, the first attempts to obtain blastocysts with classical culture media were disappointing, and the use of a coculture strategy was naturally tempting: the first significant results of successful blastocyst development were obtained in the early 1980s, using trophoblastic tissue as a feeder layer in order to mimic an autocrine embryotrophic system. The next supporting cell systems were based on oviduct epithelial cells and uterine cells in order to achieve a paracrine effect. Non-hormone dependence was then demonstrated with the use of prepubertal cells, and finally with the use of established cell lines of non-genital origin (African Green Monkey Kidney, Vero cells). The embryotrophic properties are linked to features of “transport epithelia”. Vero cells have been extensively used in human ART, and most of our knowledge about the human blastocyst was gathered with the use of this technology. Coculture is still in current use, but with systems that employ autologous uterine cells. Results following the use of this technology in human ART are superior to those observed with the use of sequential media. The benefit is linked to the release of free radical scavengers and growth factors by the feeder cells. In animal

biotechnology, an important part of the “precious embryos” i.e. those resulting from cloning technology, involves coculture with BRL (buffalo rat liver cells) or Vero cells.

1. Introduction:

The first mammalian embryo culture systems used in domestic animal biotechnology were dependent upon coculture with feeder cells in order to achieve embryo transfer. Embryo transfer in farm animals can only be performed at the blastocyst stage: transfer at earlier stages of development (after IVF and short duration of culture), prior to day 5, results in embryos being expelled into the vagina. Embryo culture was confronted with the problem of developmental arrest during the cell cycle that corresponds to genomic activation: MZT (maternal to zygotic transition), the shift that occurs from maternal control of metabolism via mRNAs and proteins stored during oocyte maturation, to activation of the zygote genome to direct further embryonic development. This transition occurs at the 2-4 cell stage in the mouse, 4 to 8 cells in human, 8 to 16 cells in bovine. The transition occurs during an extended cell cycle, the longest during early embryo development, which lasts for 24 hours before the first embryonic transcripts appear. The embryo then becomes autonomous. Dynamic in vivo conditions are always difficult to reproduce in vitro, and early culture systems yielded blastocyst formation rates that barely reached 50%. Therefore, coculture, defined as the simultaneous culture of somatic cells together with the embryo, was a logical intermediary step. Bovine embryo co-culture began in the early 1980's, using trophoblastic vesicles (1-2) to mimic an autocrine embryotrophic system. This protocol was the first to allow in vitro culture from the one-cell stage to blastocyst, with live calves delivered after transfer. The second supporting cell systems used oviduct epithelial cells (3), in order to achieve a paracrine effect. This type of cell layer was first employed in the monkey by Goodeaux et al (4) using uterine cells and in the human by Bongso (5-6) using homologous oviduct epithelial cells. We were then able to demonstrate that the embryotrophic effect was not hormone-dependent, by using prepubertal oviduct in mouse and in the bovine (7) and non-genital established cell lines in the human (8). Vero (Green monkey kidney) cells have the same embryonic origin (mesonephros) as the uterus and oviduct, and have been extensively used in human cell culture systems. Vero, as well as BRL (Buffalo rat liver) are still used for bovine embryo production. We will discuss the mechanisms whereby coculture can improve embryo quality.

1.1 Prolonged culture time:

The transfer of any embryo before it has undergone genomic activation is a “blind process”, even when kinetic and morphological features have been assessed. The morula stage corresponds to a full remodeling of cells, so transfer at the blastocyst stage is a logical strategy. Moreover, uterine motility is decreased after D5, once the blastocyst stage has been reached (9). Blastocyst transfer as a selection process has been questioned even within some teams (10-11-12), but this concept is now fully recognised. In terms of cytology, it allows the selection of healthy embryos in translocation carriers (13). This positive selection effect, together with the relatively poor efficacy of PGD/PGS has led the utility of PGD to be contested (14). Moreover, an embryo’s cytogenetic status can evolve in a positive manner, with “self correction” between early cleavage stages and blastocyst. An increasing number of teams are using now blastocyst biopsy for PGD (15-16), following our design of this technology (17). However, as in any biological process, this selection is not perfect, as can be observed by the fact that trisomies 13, 18 and 21 occur naturally. Nonetheless, when a SET “single embryo transfer” is indicated, the decision must be blastocyst transfer.

1.2 Different technologies

Early embryonic development is bi-phasic. The first cleavage divisions are completely driven by maternal reserves stored during oocyte growth and maturation, especially for the final “presentation/preparation” of maternal mRNAs as condensed polyadenylated clusters, ready for translation. This first phase is of necessity the most fragile: any attrition in the storage of protein and mRNA required for housekeeping mechanisms, i.e. cell division homeostasis, redox potential regulation, energy metabolism, protection against external insults (ROS, pH etc.) or DNA repair will result in either developmental arrest or apoptosis. It is also now evident that very early anomalies may lead to delayed embryonic problems (18; see also large offspring syndrome). The long period of time required for MZT presents a problem, and every supporting system that can avoid delays in this transition allows a certain level of reserves to be kept until the blastocyst stage. The presence of supporting feeder cells may also partially rescue borderline embryos that would have been arrested in vitro, allowing them to pass this critical stage. However, this view is only schematic, as some of the mRNAs stored during maturation are used at the blastocyst stage.

Somatic cells as feeders can be used as cell suspensions, pieces of tissue (trophoblast) or in monolayers. Coculture is a 3-partner relationship: culture medium, cells and embryo. The first assays of these systems began in the early 1980’s with the fantastic development of embryo

transfer for animal breeding, especially in bovine, and in particular after in vitro maturation and in vitro fertilization proved to be successful. The use of trophoblastic cells (D18 embryo) to culture one-cell embryos demonstrated that the trophic factors are not hormone-dependent (1-2). This hypothesis was then confirmed by transferring embryos into prepuberal oviduct in mouse (*in vivo* and *in vitro*) and in bovine (7-19), as well as by coculture with de-synchronized tubal or uterine cells (20). It should be mentioned here that coculture with human endometrial cells represents a de-synchronization, as the uterine biopsies are performed during the luteal phase, when there is an abundance of material (i.e. uterine cells). Although there is apparently no hormonal prerequisite, it is also clear that not all types of cells are equally efficient. Fibroblasts are poor feeder layers for embryo culture, for two basic reasons: fibroblasts continue to divide and overgrow, leading to culture medium depletion, generally associated with a decrease in pH (acidification), which is incompatible with the embryo's needs and capacity to manage an acidic pH (21). The second point is that the quality and specificity of growth factors released by fibroblasts do not have counterpart receptors on the embryo. In fact, the ability of cells to sustain embryonic development is limited to epithelial transporters (22). For this reason we used Vero cells initially, an established cell line that is easy to handle and manipulate and highly controlled, since these cells are used for vaccine production (23). Vero cells can be bought with a certificate of safety from the ATCC (American Tissue Culture Collection, Rockville Maryland) or from the WHO library, at a passage of around 121: each batch has a documented passage number. They can be easily frozen and thawed, and can conveniently be used routinely in an IVF unit. Tumorigenicity of the cells has never been described before passage 149 (24). Most of the information related to human blastocyst formation is based on information gathered from Vero cell coculture: from paternal and maternal effects (25-26) to cryopreservation (27-28). Autologous co-culture, using endometrial biopsies (29-30, see figure 1) or oviduct cells (5-6) started a little later in human IVF.

A European laboratory (Laboratoires Genevrier, Sophia Antipolis, France) is now offering the opportunity for autologous coculture from uterine biopsies to be proposed for patients in European IVF units: Endocell, the first commercial ready-to-use kit designed for embryo coculture up to the blastocyst stage. Endometrial biopsies are frozen in a previous cycle, and the plated cells are sent back to the IVF lab at the time of the corresponding patient treatment procedure (Figure 2 and 3. with permission). Cumulus cells have also been proposed as feeder cells for co-culturing human embryos (31), but although this technique is relatively easy and attractive in principle, the results obtained were contested, and the technique has not been

sustained. Problems encountered with the use of non-established cells as feeder layers include difficulty in obtaining sub-passages after trypsinization, rapid ageing, and attrition of their trophic capacities is generally observed quite rapidly – the systems are “one shot”.

1.3 Why still Coculture now:

The evidence has confirmed that embryo coculture systems improve embryo quality and result in better pregnancy rates than conventional culture systems (32-33-34-35). The blastocyst expansion appears quicker, on day 5.5 post *in vitro* insemination (Figure 4), synthesis of hCG and hatching are initiated earlier (Vero Cells:8-36,).The largest study demonstrating this positive effect has been demonstrated by the Spanish group of IVI in Valencia (autologous uterine cells, 30-34-35) the group at Cornell University (New York) using uterine cells (32-33), and our group with the group of institute Dexeus in Barcelona (Spain) and the Servy institute for reproductive endocrinology in Augusta (Georgia), all three of us using Vero cells. The impact of this effect is even more important when coculture is performed for difficult cases, when culture with conventional media and SET (single embryo transfer) is recommended. It appears that coculture with Vero or uterine cells yield very similar results. In our experience we found a major difference in terms of freezing between co-cultured blastocysts and the ones cultured in sequential media. (37-38)

Most of the criticism levelled against coculture and blastocyst transfer is not valid: the sex ratio is not modified (1014 males /945 females: 51.7%), and the birth weight of babies is not increased (39-40). The latter could have been a source of concern, considering the large offspring syndrome described in farm animals.

It has been repeatedly said that prolonged culture time increases the incidence of monozygotic twinning. We demonstrated that this consequence is typically due to the effect of sequential media that has insufficient protection from ROS- induced apoptosis (41-42); monozygotic twinning is not increased in coculture systems. The cells of the feeder release sulphur derived anti-oxidants such as hypotaurine, the natural free radical scavenger in the embryo environment (43-44), We observed that therapeutic abortion rates are decreased for co cultured blastocysts (less than 1%, 12/1971) and there is also a low incidence of stillbirths and death <3mths (9/1968).

Using a very large cohort of oocytes, Dominguez et al. (35) found that coculture yielded a highly significant difference in blastocyst formation rates, 56.0 vs 45.9 %, $p < 0.01$. In their

March 5th KTE

oocyte donation programme, where the maternal effect (26) is minimal, the difference is even greater: 70.5% vs 56.4%. ($p < 0, 0001$). In term of pregnancy and implantation rates the data follow the same pattern: pregnancy rates 39.1% vs 27.5%, implantation rates 33.3% vs 20.9%. Again, the results obtained with Vero cells are similar (45: patients with repeated failures of implantation): 39.3 PR per retrieval with cocultured blastocyst transfer.

In fine, a meta analysis (46) concluded that there is “an overall statistically significant effect of coculture on the implantation rate, clinical pregnancy rate, and ongoing pregnancy rate”.

1.4 Coculture and blastocyst freezing:

In order to develop a universal method of calculating a programme's success rate, the overall outcome must be clearly defined. Freezing rate per surplus (non-transferred) embryo is a mandatory consideration, as well as the take home baby rate per frozen embryo, i.e the percentage of extra births added by the cryopreservation program. This factor is rarely mentioned the published literature (37-38). It is usually assumed that prolonged culture time decreases the number of supernumerary embryos available for cryopreservation; as mentioned previously, coculture increases the blastocyst formation rates compared to conventional media. Moreover, the blastocysts obtained are more cryo-resistant, as a result of enhanced protection against ROS-induced damage. Release of sulphur-derived anti-oxidants by the feeder cells (47) prevents the formation of peroxidised lipids in the membranes. These prostaglandin-like endoperoxides distort the shape of the membrane and create fracture zones during freezing and thawing (48). The increase in cryotolerance may be also related to the higher number of cells per blastocyst (49), related to LIF produced by the feeder layer (50). This means that the take home baby rate per frozen embryo can easily be 10 times higher than that observed for early stage embryos (37-38). In addition to the effect of increased cryo-resistance, there is a selection effect: a very high number of early stage embryos have chromosomal anomalies (51). In our blastocyst program with Vero cells, freezing routinely resulted in exactly 25.4% extra babies per year. The protocol used has been described elsewhere (28-52). It must be noted here that blastocysts obtained on Day 7 can lead to pregnancy and birth only when they are transferred to recipients prepared for Day 5 transfer. In our experience fresh transfers on Day 7 never led to pregnancy, as if the “implantation window is closed”.

1.5 How can we explain the positive effect of coculture?

Coculture systems have been proposed to exert their effect through 2 different mechanisms: removal of toxic compounds and /or secretion of embryotrophic factors. We will attempt to clarify these assertions.

The metabolism of oxygen is very important for the embryo. ROS production by embryos has been repeatedly reported: the three main ROS are produced, i.e. O_2^- , H_2O_2 and OH° . An increased production of these ROS is observed for in vitro produced embryos vs. those conceived in vivo. ROS production is observed to rise in the mouse at the time of fertilisation, and they are considered to be at least partly responsible for developmental arrests at the G2/M phase of the second cell cycle, the time of MZT, maternal to zygotic transition (53). ROS are endogenously produced by various metabolic pathways (oxidative phosphorylation, glucose in excess, NADPH and Xanthine Oxidases etc... 44). ROS have been shown to be deleterious for the preimplantation embryo: they induce DNA strand breaks, mitochondrial alterations, lipid peroxidation etc... The consequences are numerous, and they lead to developmental arrests, sometimes via apoptosis. H_2O_2 is a strong inducer of apoptosis in the embryo (54), and is the most deleterious embryo "killer". Moreover, it is rarely mentioned that culture media itself can generate ROS when not well designed, and especially when not very well protected against ROS. There are important differences between different types of culture media in term of spontaneous ROS production (55), and protection from ROS effects is not obvious: for example glutathione, the universal ROS scavenger, does not penetrate embryo membranes, and although vitamin C can be protective it can also be pro-oxidant. Clearly, feeder cells are able to generate and release protective sulphur-derived antioxidants (47). Oviduct, uterine and Vero cells possess the cysteine sulfinic acid pathway (43-47) allowing the synthesis of hypotaurine and taurine, important protective agents in the genital tract *in vivo*. This exogenous protection reinforces endogenous protection. Vero cells may also counteract unknown detrimental effects, such as those present in a hydrosalpinx environment. (56).

1.5.1 Growth factors: Maternal transcripts and proteins are present in finite quantities in the oocyte, dependent upon the quality of oocyte maturation and especially in relation to the age of the female. The same growth factors that allow the oocyte to gain its competence *i.e.* ability to pass the critical cycle of MZT and allow full development, must be active during the first three days of culture (57). It is important to avoid any delay in the initiation of embryonic MZT. Growth factors act in a harmonious and synergistic manner, interacting with metabolic

pathways via activation and inhibition. If we consider what is secreted by the different feeder cells used in coculture, a few may be of importance. Using microarray analysis, we have assessed the presence of growth factor receptor transcripts in the oocyte (58), and their signalling pathways: these are presented in figure 5. Coculture cells that express leukaemia inhibitory factor (LIF) enhance blastocyst development in vitro in mouse (59), in the bovine (60) and in the ewe (61) where a peak of LIF is also observed prior to ovulation. Uterine cells and Vero cells secrete LIF and interleukin 6 (62-63). GP130, the common receptor for LIF and IL6, as well as LIF receptor beta are expressed in the oocyte. The signalling pathways are also expressed. The same observation can be made for Platelet activating factor (PAF) and platelet derived growth factor (PDGF). The mitogenic effect of EGF/TGFbeta, has been demonstrated in the mouse (64), but although these are secreted by Vero cells, it is not clear that all feeder cells release such growth factors. TGF beta receptors 1 and 3, as well as all the Erbs, members of the EGF receptor family, are highly expressed on the human oocyte. *In vivo*, other growth factors such as growth hormone are present, and corresponding receptors on the human oocyte can also be found (65). However, maintaining perfect synchrony and regulation of differentiation may require both positive and negative effects. Growth factors are redundant and affect gene regulation, but the precise intervention of each is still unknown. For example, one function of apoptosis is to eliminate cells that are damaged by any induced stress. The concept of adding one specific growth factor in order to inhibit apoptosis in culture media can be contested; although the number of blastocysts may be increased, this is accompanied by cytogenetic anomalies (66) The complexity, the cross linking and the redundancy of all the pathways involved has to be taken into consideration. Most of the growth factors involved in regulating development will be synthesised in the embryo later, post genomic activation, but the exogenous growth factors still regulate embryonic development and differentiation (Figure 6).

1.6 Conclusions: Coculture systems, past and future

The benefit of coculture systems has been a matter of controversy since the time that they were first used in embryo culture. Irrespective of the type of feeder layer, the main criticisms have been:

- 1- *The risk of contamination.* Since the time of Pasteur, we have known that there is no spontaneous generation of bacteria and viruses. In a well-equipped laboratory with trained biologists, the risk of contamination is no higher than in classical IVF. The

technology is even safer when established cell lines are used, rather than autologous cells. Established lines have a quality control that is guaranteed by ATCC or WHO. Training of technicians and biologist in cell culture is of course mandatory, but this should in any case be a part of routine IVF training. In the year 2000, the French government mandated that coculture with Vero Cells was not recommended, especially because of lack of training within the IVF teams. This may be reversed if the system is developed through a pharmaceutical company that will furnish pre-plated petri dishes (Nunc). Naturally the supplier will have to maintain a very high level of quality control of the cells (number of sub-passages, stock library etc...). One laboratory is also preparing autologous uterine cells on request, prepared from a clinical biopsy that is taken in a cycle prior to the IVF treatment cycle; the plated cells are returned ready for use (Endocell, Laboratoire Genevrier, Sophia Antipolis, France: figures 4 and 5).

- 2- *Safety of the procedure for the babies:* the first large scale blastocyst transfer programmes were set up using coculture on Vero cells (67). The first babies were born in France from fresh blastocysts in March 1988, and in November 1988 in the USA (Augusta Reproductive Biology associates Augusta Georgia). The first births from frozen blastocysts were in the fall of 1988 in France, and in the spring of 1989 in the US. The babies are now 24 years of age, well past the age of puberty, and no specific problem has been identified, including overweight, shift in sex ratio. etc.. (39-40). Therefore, although the technology of blastocyst culture may have been contested, blastocyst transfer would no longer be challenged.
- 3- *Time consuming:* Organization of cell culture systems is simply a matter of planning. We have worked with coculture systems for 10 years without problems, and our system has been described in post-graduate courses at American Society for Reproductive Medicine (ASRM) meetings. However, if the cells can be bought ready for use, this aspect is no longer relevant.

To the question "Is there still a place for coculture in IVF?" - The answer is yes: it is not an obsolete concept. All of the careful analyses have demonstrated superiority in terms of results. Compared with sequential media systems, successful coculture has features that are unique. First of all, it is both active and dormant at the same time: it represents a state that is static, without overgrowth that will result in media degradation, but at the same time is an evolving dynamic system, releasing antioxidants and growth factors, a situation that may be considered to mimic in vivo conditions. This is especially important, since some sequential media that

lack so-called “essential amino acids” (especially methionine) can increase the risk of imprinting anomalies and anomalies linked to DNA damage (68-69). Saturation /overwhelming of embryo DNA repair capacity leads at best to developmental arrest, or at worst to a tolerance for mutations that might subsequently lead to malignant cell transformation. Coculture may help to provide a better understanding of the embryo’s interactions with its environment, and thus help in improving conventional culture media. Early preimplantation development is very far from being understood, and improvements in our various concepts surrounding it are still required.

2. Materials

2.1 Culture Flasks : Tissue culture dishes, Becton Dickinson, Falcon 3001 (35X10mm), Tissue culture dishes Falcon 3002 (60X15mm), Culture plates multiwell Falcon 3047, Culture flasks Falcon 3014^E. Centrifuge conical graduated tubes Falcon 2095. Plastic filters 0,45 micron Flowpore D2645

2.2 Media: B2 Medium Eurobio France; HEPES buffered Medium 199 Flow Laboratories 1223454); Hanks balanced solution w/o Ca and Mg Flow laboratories (1810454)

2.3 Vero Cells: The cells were either obtained from ATCC (American Type culture collection) or WHO collection (UK), at sub passage 120 to 134, in a frozen tube containing 25 million cells. A certificate of quality including all the tests for viruses and electron microscopy is given

2.4 Calf Serum: Rhone Merieux, France (origin:New Zealand)

Reagents: Trypsin: GIBCO (0.25% solution: O6607072E) and SIGMA (T0134); EDTA: SIGMA ED4SS

2.5 Quality control: Chlamydia (BioMérieux Kit 5532/1) and Mycoplasma (BioMérieux Kit 4240/2)

2.6 Freezing: Air Liquide, Liquid nitrogen programmable machine (Minicool AS 25); DMSO Merck 2951; Cryovials NUNC

3. Methods: Preparation of Vero Cells

3.1 Programmed handling of the cells: careful organization of the cell culture system is essential, as shown in Diagram 1.

- 3.1.1 From the frozen cells, flasks are seeded with $2-3 \times 10^6$ cells, in B2 medium with 15% fetal calf serum (FCS) reaching confluence within 4 days ($6-8 \times 10^6$ cells/ flask).
- 3.1.2 After trypsinization (0.025% in Calcium- free Hanks balanced salt solution), the cell suspension is split into three parts: one is used to seed a new flask (in B2 + 15% FCS), one is frozen, and the remaining part is used to seed wells, at a concentration of 100000 cells per well (seeding in B2 with 7.5% fetal calf serum). Confluence was reached in wells after 3 days.
- 3.1.3 Trypsinization was performed in 0.25% HEPES buffered medium TC199 containing EDTA (0.11%). The cells must not be passaged repeatedly: we observed that growth slows down after four sub passages, and it proved better to freeze fourth passage cells and seed new flasks with a previously frozen aliquot .

3.2 Freezing Vero Cells for Co-culture

Methods of cell freezing and thawing have been previously described (47-70).

- 3.2.1 The cells were placed in 2mL cryo-vials (Nunc) and frozen in B2 medium containing 10% DMSO.
- 3.2.2 Cells were frozen with the following temperature gradient: $-5^{\circ}\text{C}/\text{min}$ from ambient temperature to -20°C (hold for 5 min), then $-1^{\circ}\text{C}/\text{min}$ to -30°C , then $-2^{\circ}\text{C}/\text{min}$ to -60°C and then plunge into liquid N_2 .
- 3.2.3 After several sub-passages, the cells were tested for Chlamydia and Mycoplasma as an internal quality control. No contamination was ever detected.

3.3 Preparation of feeder layers

- 3.3.1 Co-culture plates must be incubated at an alkaline (pH 7.4), bicarbonate containing medium. B2, Earle's and TCM 199 are considered as optimal coculture media for human and bovine, goat and sheep cloned embryos, suitable for the needs of both the epithelial cells and the embryos
- 3.3.2 All the plates for the following week are prepared every Friday afternoon, according to the estimated number of cycles scheduled for that week.
- 3.3.3 The cells were seeded in B2 medium alone, at a concentration of approx. 15000 cells per well.

3.3.4 The medium is changed for all the plates on Monday morning: the unattached cells are removed by washing. The medium is then changed again when coculture is initiated, immediately before the embryos are deposited on the layer.

3.3.5 The embryos are plated on the cells on day 2, at the 2 to four cell stage. The coculture medium is not changed until embryo transfer and /or freezing.

3.3.6 All plates must be discarded after use, or after one week post seeding if not used.

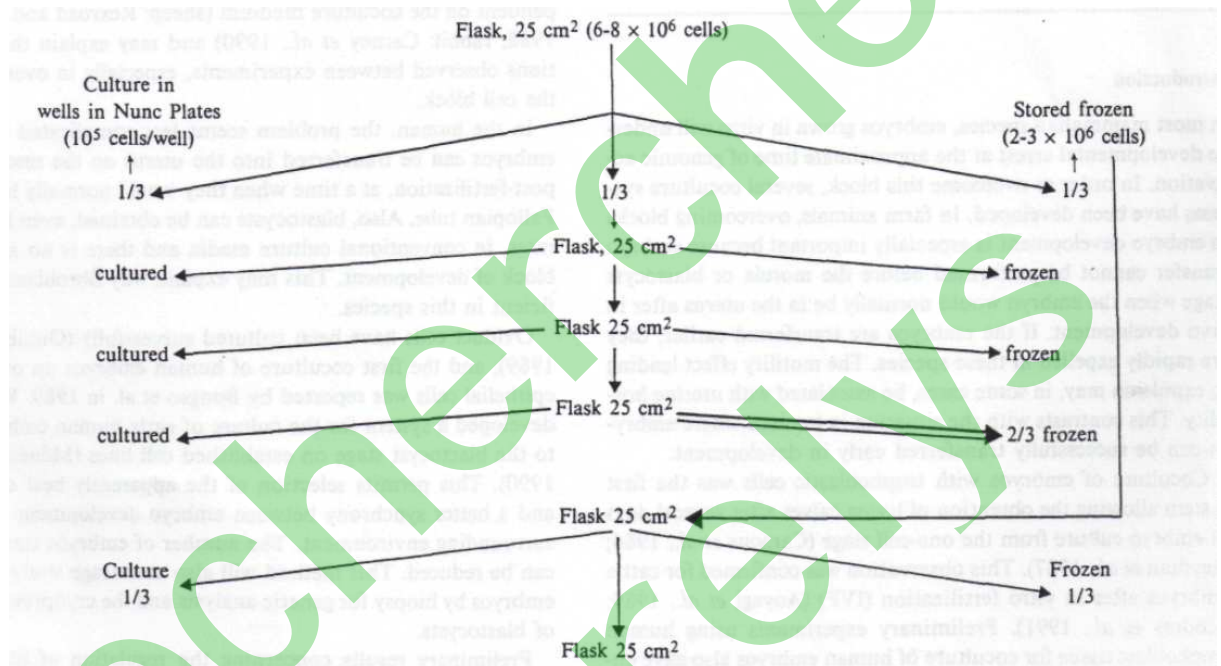


Diagram 1: Manipulation of Vero cells in a large scale embryo coculture program

3.4 Coculture with autologous uterine cells

3.4.1 A 19-15 mG biopsy is performed during the luteal phase in a previous ovarian cycle (day 22 to 24 of the cycle). Two options are then possible:

a) freeze the biopsy with DMSO according to the protocol described, then thaw the sample during the treatment cycle, and trypsinize (0.25% in Hepes buffered TCM199, 10 times more concentrated than for established cell lines); well seeding is performed 3-5 days before the expected coculture attempt.

b) the biopsy can be trypsinized (0,25% in HBSS) immediately using the classical protocol with 0.25% trypsin.

The primary cell culture is then set up after seeding; when the cells are confluent, the primary culture is trypsinized and the cells are frozen.

Thawing of the cells and seeding of the wells will be performed 3 days before the expected the coculture time.

3.4.2 Primary cultures cannot be used more than 2-3 times. Currently in France, IVF units are using a commercial system (Endocell) instead of an in-house process. The full experimental procedure with details of all reagents and controls has been published elsewhere (70).

4. Notes:

4.1 If required, the purity and the quality of the epithelial cells can be tested via immunochemical techniques, i.e. by testing the keratin content of the cells. The presence of keratin filaments confirms that the cells are epithelial in nature.

4.2 Detachment of the cells from the plastic support always indicates that there is a problem. This may sometimes happen in primary cultures that have a borderline contamination (e.g. Chlamydia, especially in uterine cells). Detachment can also happen if the culture medium is not well buffered (alkaline: bicarbonate), if a toxic compound is present, or if there is de-regulation of CO₂ in the incubator.

References

- 1-Camous S, Heyman Y, Meziou W *et al.* 1984. Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. *J. Reprod. Fert* , 72, 479-485.
- 2-Heyman Y, Ménézo Y, Chesne R *et al.* 1987. In vitro cleavage of bovine and ovine early embryos: improved development using coculture with trophoblastic vesicles. *Theriogenology* , 27, 59-68.
- 3-Gandolfi F, Moor RM (1987) Stimulation of early embryonic development in the sheep by co-culture with oviductepithelial cells. *J Reprod Fertil* 81: 23-8
- 4-Goodeaux LL, Voelkel SA, Anzalone CA *et al.* 1989 The effect of rhesus epithelial cell monolayers on in vitro growth of Rhesus embryos. *Theriogenology* 39:187
- 5-Bongso A, Soon-Chye N, Sathananthan H *et al.* 1989 Improved quality of human embryos when co-cultured with human ampullary cells. *Hum Reprod.*;4:706-13.
- 6-Bongso A, Ng SC, Fong CY, *et al.* 1992.Improved pregnancy rate after transfer of embryos grown in human fallopian tubal cell coculture. *Fertil Steril.* 58:569-74
- 7-Ménézo Y, Hamidi J, Khatchadourian C *et al.* (1989) The murine prepuberal oviduct supports early embryo development in vitro. *Develop Growth Differ* 31:551-5
- 8-Ménézo Y, Guerin JF, Czyba JC (1990) Improvement of human early embryo development in vitro by coculture on monolayers of Vero cells. *Biol Reprod* 42: 301-6
- 9-Fanchin R, Ayoubi JM, Righini C, *et al.* 2001 Uterine contractility decreases at the time of blastocyst transfers. *Hum Reprod.*;16:1115-9
- 10-Kolibianakis EM 2004 Day 5 embryo transfer does not enhance reproductive outcome compared to D3 transfer using the current culture systems; in *ESHRE pre congress course Special interest groups "Embryology"* Berlin pp 47-52
- 11-Kolibianakis EM., Zikopoulos K, Verpoest W *et al.* 2004 Should we advise patients undergoing IVF to start a cycle leading to a day 3 or a day 5 transfer? *Hum Reprod.* 19, :2550-4.
- 12-Papanikolaou EG, Camus M, Kolibianakis EM *et al.* 2006 In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. *N Engl J Med.* 354:1139-46
- 13-Ménézo Y, Chouteau J, Veiga A 2001 in vitro fertilization and blastocyst transfr for carriers of chromosomal translocation *Eur J Obstet Gynecol Reprod Biol.*96: 193-5
- 14-Fauser BC 2008 Preimplantation genetic screening: the end of an affair? *Hum Reprod.* 23:2622-5.

March 5th KTE

15-Veiga A, Gil Y, Boada M, *et al* 1999 Confirmation of diagnosis in preimplantation genetic diagnosis (PGD) through blastocyst culture: preliminary experience. *Prenat Diagn.* 19:1242-7

16-McArthur SJ, Leigh D, Marshall JT, *et al.* 2008 Blastocyst trophectoderm biopsy and preimplantation genetic diagnosis for familial monogenic disorders and chromosomal translocations. *Prenat Diagn.* ;28:434-42

17-Veiga A, Sandalinas M., Benkhalifa M *et al.* 1997 Laser blastocyst biopsy for preimplantation diagnosis in the human. *Zygote*, 5, 351-354.

18-Fernández-Gonzalez R, Moreira PN, Pérez-Crespo *et al.* 2008. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behaviour of adult offspring. *Biol Reprod.* 78:761-72.

19-Papaioannou VE, Ebert KM.1986 Development of fertilized embryos transferred to oviducts of immature mice. *J Reprod Fertil.* 76:603-8

20-Thibodeaux JK, Menezo Y, Roussel DJ *et al.* (1992) Coculture of in vitro fertilized bovine embryos with oviductal epithelial cells originating from different stages of the estrous cycle *J. Dairy Sci* 75: 1448-55

21-Dale B, Menezo Y, Cohen J, *et al.*1998 Intracellular pH regulation in the human oocyte. *Hum Reprod.*;13:964-70

22-Scodras JM, Pollard JW, Betteridge KJ 1991 Effect of somatic cell type on bovine embryonic development in coculture. *Theriogenology* 35: 269

23-Simizu B, Terasima T 1988 *VERO cells, Origin, properties and BioMedical applications* Chiba university Japan ed. , Soft Science Publications pp 1-234

24-Levenbook IS, Petricciani JC, Elisberg BL 1988 Tumorigenicity of Vero cells in *VERO cells, Origin, properties and BioMedical applications* Chiba university Japan ed. , Soft Science Publications, p 183-193

25-Janny L, Menezo YJ.1994 Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. *Mol Reprod Dev.* 38:36-42.

26-Janny L. and Ménézo Y. (1996.) Maternal age effect on early human embryonic development and blastocyst formation. *Mol. Reprod. Develop.*,**45**, 31-37.

27-Ménézo Y, Nicollet B, Herbaut N *et al.* (1992b) Freezing co-cultured human blastocysts. *Fertil. Steril* 58: 977-80

28-Ménézo Y. and Veiga A. 1997 Cryopreservation of blastocysts. In Gomel V., Leung P.C.K. Editors *In Vitro Fertilization and Assisted Reproduction*, 10th World Congress of in Vitro Fertilization and Assisted Reproduction, Vancouver, Monduzzi, P. 49-53

29-Jayot S, Parneix I, Verdaguer S, *et al* 1995 Coculture of embryos on homologous endometrial cells in patients with repeated failures of implantation. *Fertil Steril.*;63:109-14

March 5th KTE

- 30-Simón C, Mercader A, Garcia-Velasco J *et al.* 1999. Coculture of human embryos with autologous human endometrial epithelial cells in patients with implantation failure. *J Clin Endocrinol Metab.* 84:2638-46.
- 31-Quinn P, Margalit R 1996 Beneficial effects of coculture with cumulus cells on blastocyst formation in a prospective trial with supernumerary human embryos. *J Assist Reprod Genet.* 13:9-14.
- 32-Barmat LI, Liu HC, Spandorfer SD, *et al.* 1998 Human pre-embryo development on autologous endometrial coculture versus conventional medium. *Fertil Steril.*;70:1109-13
- 33-Barmat LI, Liu HC, Spandorfer SD, *et al.* 1999 Autologous endometrial co-culture in patients with repeated failures of implantation after in vitro fertilization-embryo transfer. *J Assist Reprod Genet.* 16:121-7
- 34-Mercader A, Garcia-Velasco JA, Escudero E *et al.* (2003) Clinical experience and perinatal outcome of blastocyst transfer after coculture of human embryos with human endometrial epithelial cells: a 5-year follow-up study. *Fertil Steril* 80: 1162-8
- 35-Dominguez F, Gadea B, Mercader A *et al.* (2010) Embryologic outcome and secretome profile of implanted blastocysts obtained after coculture in human endometrial epithelial cells versus the sequential system. *Fertil Steril* 93: 774-82
- 36-Turner K, Lenton EA 1996 The influence of Vero cell culture on human embryo development and chorionic gonadotrophin production in vitro. *Hum Reprod*; 11:1966-74.
- 37-Ménézo Y.2004 Cryopreservation of IVF embryos: which stage? *Eur J Obstet Gynecol Reprod Biol.* 113 Suppl 1:S28-32
- 38-Ménézo YJ.2004 Blastocyst freezing. *Eur J Obstet Gynecol Reprod Biol.* 2004 Jul 1;115 Suppl 1:S12-5
- 39-Ménézo YJ, Chouteau J, Torelló J, *et al.*1999 Birth weight and sex ratio after transfer at the blastocyst stage in humans. *Fertil Steril.*;72:221-4.
- 40-Ménézo Y, Veiga A, Pouly JL (2000) Assisted reproductive technology (ART) in humans: facts and uncertainties. *Theriogenology* 53: 599-610
- 41-Ménézo Y, Sakkas D (2002) Monozygotic twinning: is it related to apoptosis in the embryo? *Hum Reprod* 17:247-8
- 42-Cassuto G, Chavrier M, Menez Y.2003 Culture conditions and not prolonged culture time are responsible for monozygotic twinning in human in vitro fertilization. *Fertil Steril.*;80:462-3.
- 43-Guérin P, Ménézo Y 1995 Hypotaurine and taurine in gamete and embryo environments: de novo synthesis via the cysteine sulfinic acid pathway in oviduct cells. *Zygote*, 3:333-43

March 5th KTE

44-Guérin P, El Mouatassim S, Ménéz Y 2001 Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update*. 7:175-89

45-Ménéz Y, Sakkas D, Janny L 1995 Coculture of human embryo: factor affecting human blastocyst formation in vitro. *Micr. Res Tech*, 32: 50-56

46-Kattal N, Cohen J, Barmat LI. 2008 Role of coculture in human in vitro fertilization: a meta-analysis. *Fertil Steril*. 90:1069-76.

47-Ouhibi N, Hamidi J, Guillaud J *et al.* (1990) Co-culture of 1-cell mouse embryos on different cell supports. *Hum Reprod* 5: 737-43

48-Pryor WA, Stanley JP, Blair E (1976) Autoxidation of polyunsaturated fatty acids: II. A suggested mechanism for the formation of TBA-reactive materials from prostaglandin like endoperoxides. *Lipids* 11: 370-9

49-Vlad M, Walker D, Kennedy RC. 1996 Nuclei number in human embryos co-cultured with human ampullary cells. *Hum Reprod*. 11:1678-86

50-Carnegie JA, Morgan JJ, McDiarmid N, *et al* 1999 Influence of protein supplements on the secretion of leukaemia inhibitory factor by mitomycin-pretreated Vero cells: possible application to the in vitro production of bovine blastocysts with high cryotolerance. *J Reprod Fertil*. 117:41-8

51-Iwarson E, Lundqvist M, Inzunza J *et al.* 1999 A high degree of aneuploidy in frozen-thawed human preimplantation embryos. *Human genet.*, 104: 376-382

52-Kaufman RA, Ménéz Y, Hazout A, *et al.* 1995 Cocultured blastocyst cryopreservation: experience of more than 500 transfer cycles. *Fertil Steril*. 64:1125-9

53-Nasr-Esfahani MM, Johnson MH. 1991 The origin of reactive oxygen species in mouse embryos cultured in vitro. *Development*. 113: 551-60

54-Pabon JE Jr, Findley WE, Gibbons WE. 1989 The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. *Fertil Steril*. 51: 896-900

55-Martín-Romero FJ, Miguel-Lasobras EM, Domínguez-Arroyo J *et al.* (2008) Contribution of culture media to oxidative stress and its effect on human oocytes. *Reprod Biomed Online* 17: 652-61

56-Kim YB, Ahn SH, Chang DY, *et al.* 2002 Vero cell co-culture counteracts the detrimental effects of hydrosalpinx fluid on the development of mouse embryos in vitro. *J Korean Med Sci*. 17:217-9

57-Ménéz Y, Elder K ; 2011 The enhancers of oocyte competence in *Oocyte Maturation and Fertilization: A Long History for a Short Event*, E Tosti and R Boni ed, Bentham publisher, pp 64-70

March 5th KTE

58-Ménézo Y Jr, Russo G, Tosti E, *et al.* .2007 Expression profile of genes coding for DNA repair in human oocytes using pangenomic microarrays, with a special focus on ROS linked decays. *J Assist Reprod Genet.*;24:513-20

59-Kauma SW, Matt DW. 1995 Coculture cells that express leukemia inhibitory factor (LIF) enhance mouse blastocyst development in vitro. *J Assist Reprod Genet.*;12:153-6

60-Fukui Y, Saito T, Miyamoto A *et al* 1994 Effect of Leukaemia inhibiting factor on in vitro development of parthenogenetic bovine morulae *Theriogenology* 42, 1133-1139

61-Ptak G, Lopes F, Matsukawa K, *et al.* 2006 Leukaemia inhibitory factor enhances sheep fertilization *in vitro* via an influence on the oocyte. *Theriogenology* 65:1891-9.

62-Desai NN, Goldfarb JM. 1996 Growth factor/cytokine secretion by a permanent human endometrial cell line with embryotrophic properties. *J Assist Reprod Genet.*;13:546-50.

63-Desai N, Goldfarb J.1998 Co-cultured human embryos may be subjected to widely different microenvironments: pattern of growth factor/cytokine release by Vero cells during the co-culture interval. *Hum Reprod.*,13:1600-5

64-Paria BC, Dey SK. 1990 Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. *Proc Natl Acad Sci U S A*;87:4756-60

65-Ménézo YJ, el Mouatassim S, Chavier M, *et al.*2003 Human oocytes and preimplantation embryos express mRNA for growth hormone receptor. *Zygote.* 11:293-7

66-Paula-Lopes FF, Hansen PJ.2002 Apoptosis is an adaptive response in bovine preimplantation embryos that facilitates survival after heat shock. *Biochem Biophys Res Commun.*;295:37-42

67-Ménézo Y, Hazout A, Dumont M *et al.* (1992a) Coculture of embryos on Vero cells and transfer of blastocysts in humans. *Hum Reprod* 7 (Suppl 1): 101-6

68-Ménézo Y, Elder K, Benkhalifa M *et al.* 2010a DNA methylation and gene expression in IVF. *Reprod Biomed Online.* 20:709-10.

69-Ménézo Y, Dale B, Cohen M 2010b DNA damage and repair in human oocytes and embryos: a review. *Zygote.* 18:357-65

70-Ouhibi N, Benet G, Ménézo Y 1991Fetal bovine pithelial cell monolayes : method of culture and identification. *J Tissue Cult Meth,* 13: 289-294

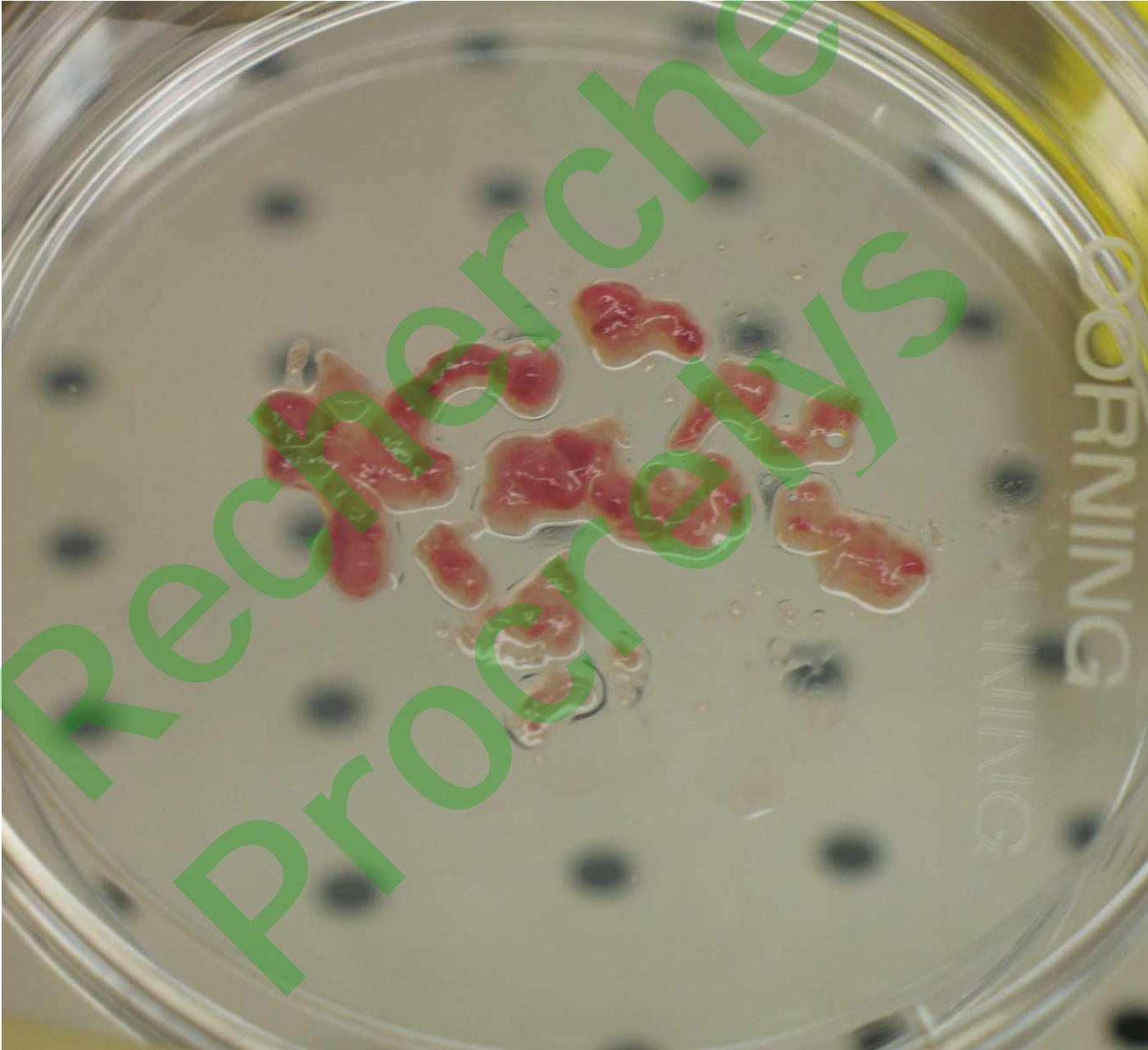


Figure 1 : Fresh biopsy of endometrial cells (25mG)



Endocell
Cellules autologues d'endomètre humain

Centre de FIV :



Usage autologue / autogène

Condition
conser

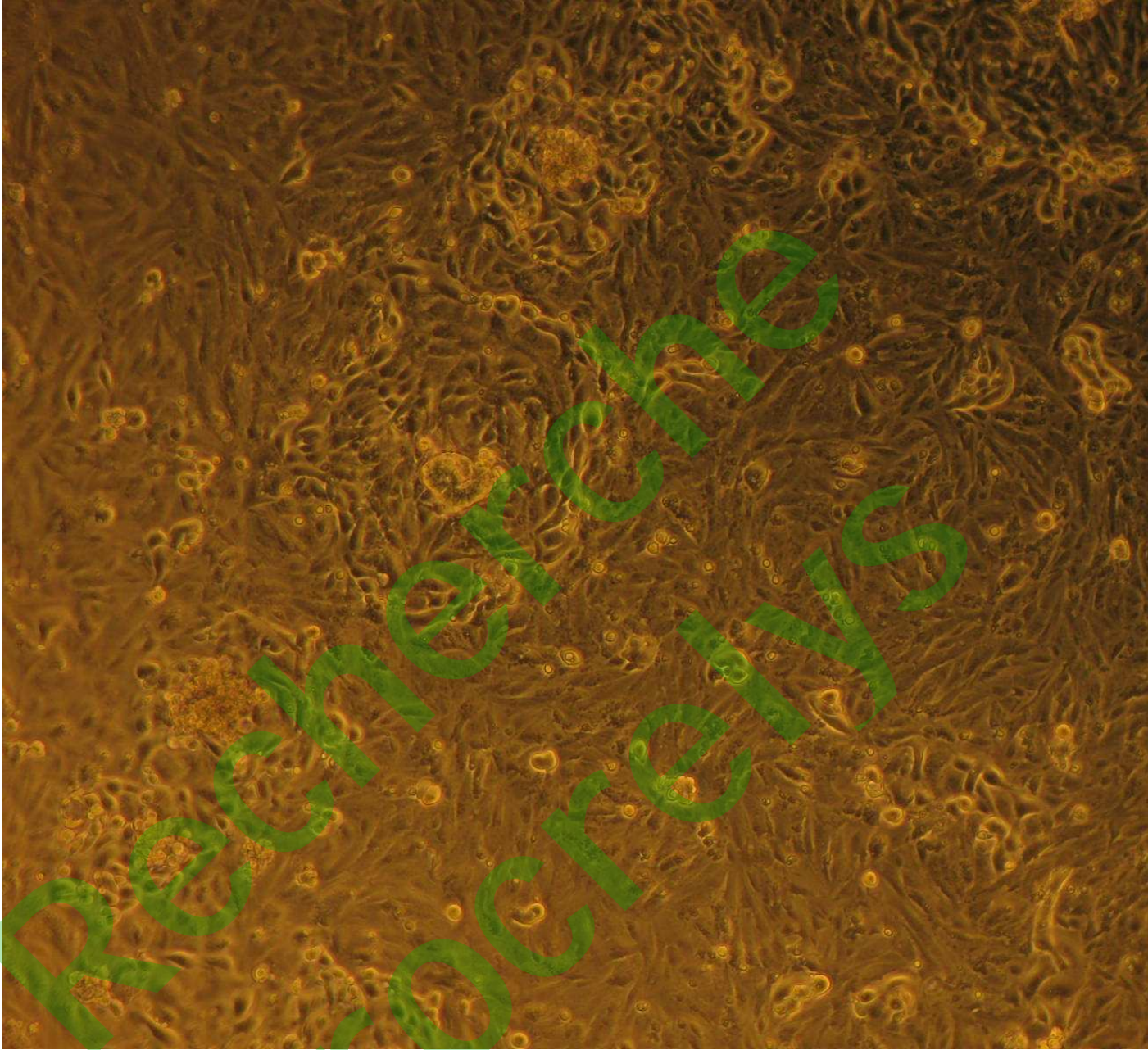
5

March 5th KTE

Figure2: A 4-well plate in a sterile blister with the autologous uterine cells ready for use (Endocell, with permission)

Recherche
Procrelys

Figure 3



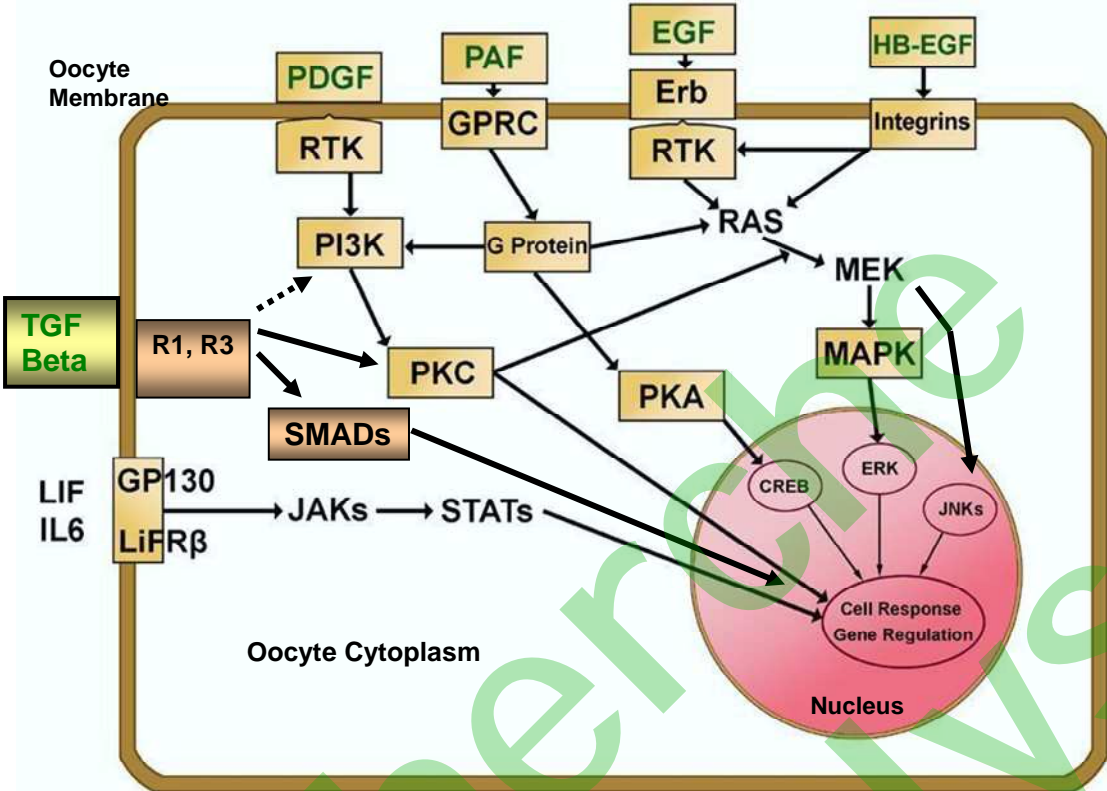
March 5th KTE

Legend Figure 2 :

Figure 3 : A Well covered with uterine cells (*Endocell*, with permission). The uterine biopsy has been sent one or 2 months previous the IVF cycle. The plated wells are sent under a sterile blister “ready for use”

Recherche
Procrelys

Figure 5



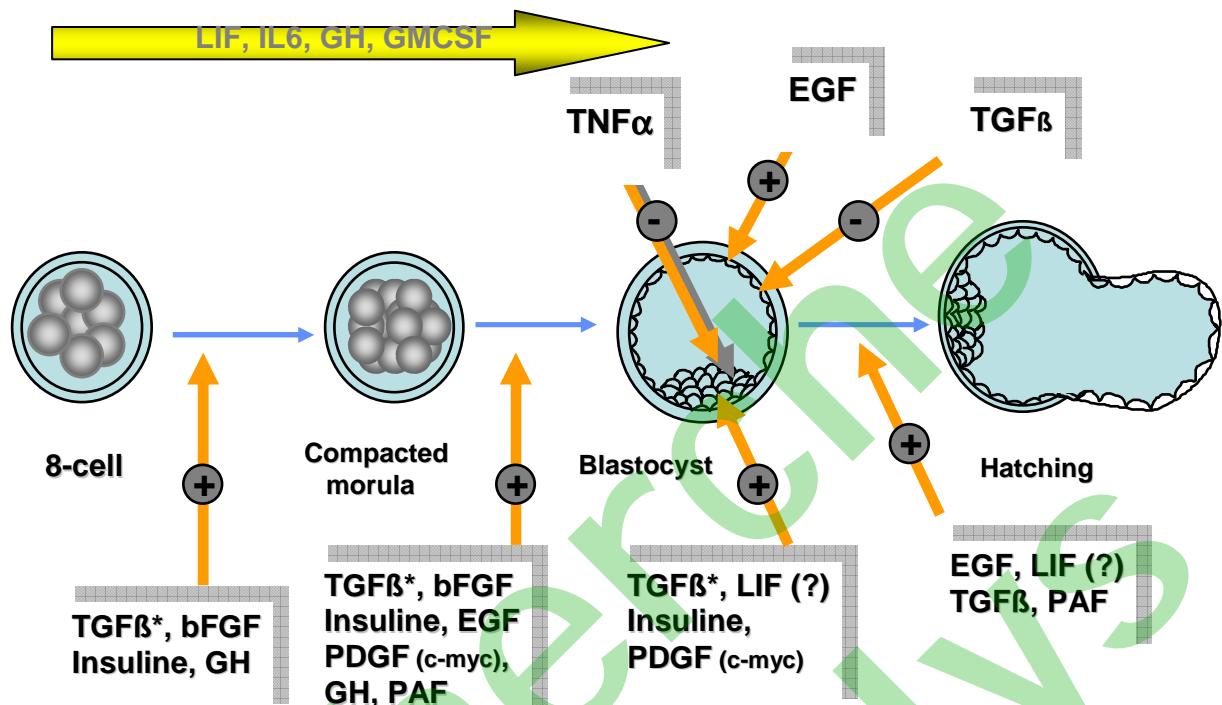
Research Procreans

Embryo coculture Legend Figure 5:

Growth factors released by feeder cells and impact on early preimplantation development: the diagram shows receptors and their interaction in signalling mechanisms for cell growth, differentiation and apoptosis. mRNA transcripts for all of the molecules and their signalling pathways have been identified by microarrays on pools of oocytes (for protocol see Ménéz et al. 2007)

CREB	cAMP-response element-binding protein
EGF	Epithelial Growth factor (TGF alpha)
ERK	Extracellular Signal-regulated Kinase
GP130	Glycoprotein 130
HB	Heparin Binding
IL6	Interleukin 6
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
LIF	Leukaemia Inhibiting Factor
MAPK	Mitogen -activated protein kinase (Serine threonine protein kinase)
MEK	MAP-Erk Kinase, extracellular-signal-regulated kinase (Serine threonine protein kinase)
PAF	Platelet activating factor
PDGF	Platelet derived growth factor
PI3K	Phospho-inositol 3 phosphate
PKA	PhosphoKinase A
PKC	PhosphoKinase C
RAS	(abbreviation of RAt Sarcoma), small GTPases
RTK	Receptor Tyrosine Kinase
SMAD	homologue of drosophila protein, mothers against decapentaplegic (MAD)
STAT	Signal Transducer and Activator of transcription
TGF beta	Transforming Growth factor beta

Figure 6: Growth factors and preimplantation development, post MZT



- Hyaluronic acid may act in synergy ;
- Role of Interleukines
- LIF/Receptor betaGP130

+ Activator, - inhibitor
 MZT Maternal to Zygotic transition

EGF Epithelium Growth factor (TGF alpha)
 GH Growth hormone
 PAF Platelet activating factor
 TGF beta: transforming growth factor beta

b FGF: basic fibroblast growth factor
 LIF Leukaemia inhibiting factor
 PDGF: Platelet derived growth factor
 TNF alpha : Tumour necrosis factor