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Human Embryonic Stem Cell Protocols

Second Edition

Edited by

Kursad Turksen

Humana Press

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Preface

Embryonic stem (ES) cells, and more specifically human ES cells, remain promising tools for understanding questions of lineage commitment and for exploring potentialities for regenerative medicine applications.

In this second edition, I have tried to bring together a number of new protocols that will extend the topics and reflect new developments since publication of the first edition.

I would like to take this opportunity to thank all the contributors for very graciously providing their protocols for this volume. Without them and their willingness to share protocol details, this new volume would not have materialized.

I would also like to thank Dr. John Walker, the Editor-in-Chief of the Methods in Molecular Biology series, for his continued support.

Patrick Marton, the Editor of the Methods in Molecular Biology series at Springer, also deserves thanks for always being available to answer my questions, for patiently listening to my suggestions, and for supporting this volume during its maturation stages. Thanks also to David Casey for his invaluable help during the production stages of this volume.

Kursad Turksen

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Contents

Chapter 1

Human Embryo Culture and Assessment for the Derivation of Embryonic Stem Cells (ESC)

A. Henry Sathananthan and Tiki Osianlis

Abstract

The culture and critical assessment of early human embryos during the first week of human development are reviewed for the derivation of ESC. Both normal and abnormal features are assessed by phase contrast microscopy of whole embryos and in serial sections of fixed material by light and electron microscopy (TEM). Normal embryos follow a time table of development and have equal blastomeres with minimal fragmentation and nuclear defects. Abnormal embryos show more fragmentation and nuclear aberrations such as micronucleation and multinucleation, reflected by aneuploidy, polyploidy, and mosaicism. The selection of normal embryos and the hardiest of embryos that survive to blastocysts is recommended for the derivation and culture of ESC.

Key words: Human, preimplantation embryos, blastocysts, culture, assessment, ESC, TEM.

1. Introduction

Theoretically it is possible to derive human ESC from any embryonic cleavage stage (2-cell to 8-cell) prior to blastulation – **Figs. 1.1** and **1.2** (1, 2). This is because the blastomeres have not yet differentiated to other cell lineages and are believed to be "totipotent" – each cell capable of giving rise to a complete individual, which is now debatable (3). Recently, the morula (~32 cells) has been used to produce ESC (4). However, morulae have internalized cells within those at the surface, destined to become ICM and trophoblast in blastocysts. Hence most of these early embryonic cells seem to have the potential to generate ESC including cells of the extra-embryonic cell lineages.

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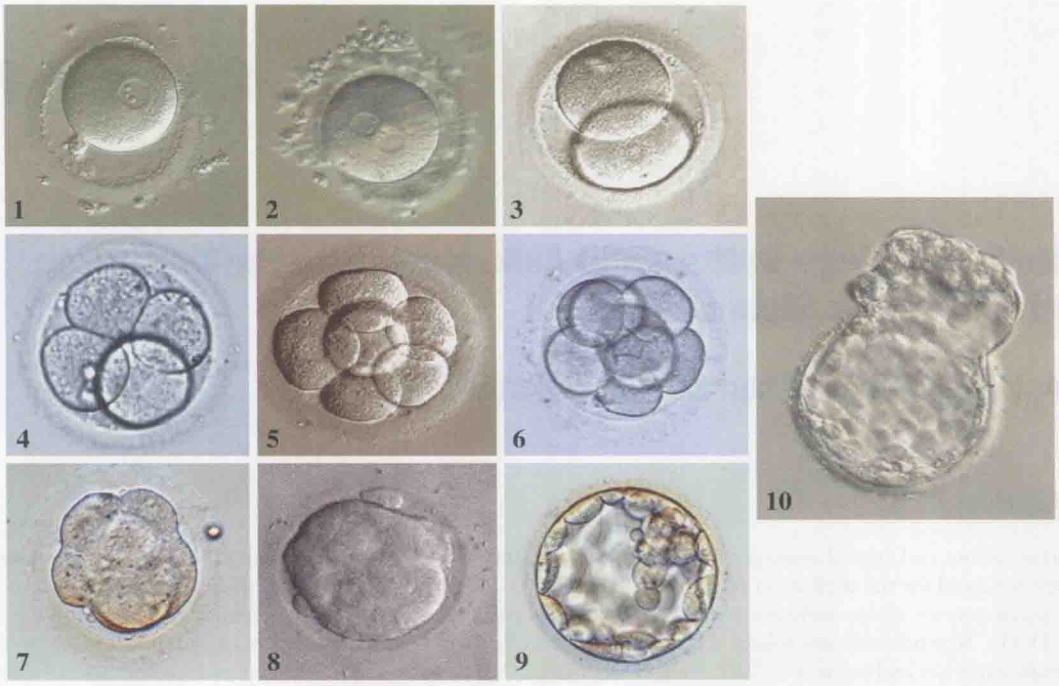

Fig. 1.1. Normal whole embryos – 1-cell to blastocyst (Phase contrast). 1. Activated oocyte; 2. Fertilized ovum (2PN); 3. 2-cell; 4. 4-cell; 5. 6-cell; 6. 8-cell; 7. Compaction; 8. Morula; 9. Blastocyst; 10. Hatching Blastocyst (9).

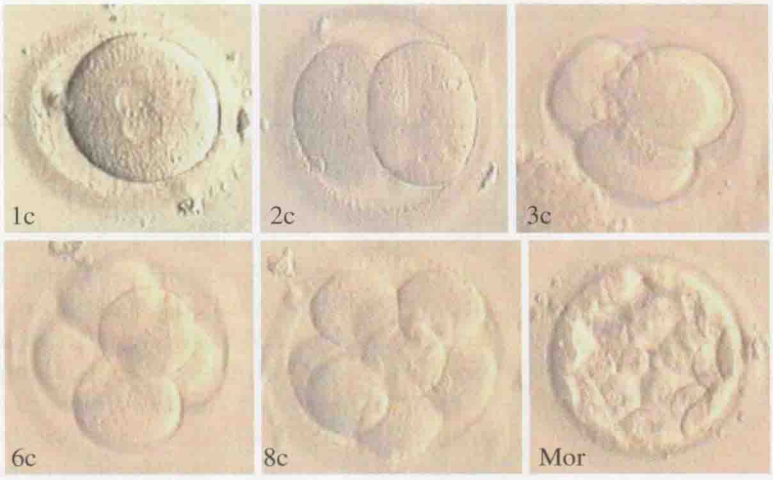

Fig. 1.2. Normal human embryos – 1-cell to morula (phase-contrast). The cleavage embryos have equal blastomeres and minimal cytoplasmic fragmentation, except the 3-cell embryo ×400 (20).

The preferred stage used to derive ESC is the blastocyst on day 5, where the ICM (embryoblast) has already differentiated from a peripheral trophoblast, which becomes the placenta (**Fig. 1.2**). The ICMs of blastocysts have been successfully used in the

derivation of ESC lines, which is now a routine procedure (5–7). These cells are pluripotent and can differentiate into all 3 germ layers: endoderm, ectoderm, and mesoderm, from which all tissues of the human body are formed (8). A time table of embryo development in week 1 is shown in **Table 1.1**.

Table 1.1
Normal embryonic growth from day 2 to 7

Day	Embryo	Appearance/hours
D1	Fertilized ovum	2 PN (12 h) and Syngamy (18–24 h)
D2	Cleaving embryo	2–6 cells: rounded blastomeres
D3	Cleaving embryo Compacting embryo	8–10 cells or rounded blastomeres Blastomeres show evidence of adhesion
D4	Compacted morula Early cavitating	Blastomeres show increased adhesion Beginning of blastocoele formation
D5	Early blastocyst Mid-blastocyst Expanding blastocyst	Blastocoele formed ICM, trophoblast, and blastocyst clearly seen Trophoblast expanding, Zona thinning out. Embryo growing, blastocoele much increased
D6/7	Late blastocyst Hatching blastocyst Hatched blastocyst	Expanded ~150–200 cells; diameter ~215 mm Trophoblast hatching out of zona Trophoblast and ICM hatched out of empty zona

An embryo that develops to this time table is likely to be more viable than the one which shows delayed growth. Modified from (9)

This chapter deals with the culture and assessment of viable preimplantation embryos from pronuclear ova to blastocysts (**Figs. 1.1** and **1.2**), since it is important to select the most normal and vigorous embryos for ESC culture. The assessment is morphological and noninvasive using an inverted phase microscope with advanced Hoffman optics and images recorded on video with a digital camera. If you have already an established IVF laboratory, culturing blastocysts for ESC is not too difficult.

2. Materials

2.1. Culturing Embryos (see* Notes 1–9*)

1. 4-well nunclon dishes (Becton Dickinson, Lane Cove, NSW, Australia)
2. 1-, 5-, and 10-ml falcon sterilogical pipettes (Becton Dickinson)

3. Art pipette tips (In vitro Technologies, Noble Park, Vic, Australia)
4. Glass Pasteur Pipettes (Pacific Lab Products, Blackburn, Vic, Australia) (*see* **Note 5**)
5. SIVF Cleaveage and Blastocyst media and Paraffin oil (COOK Medical, Eight Mile Planes, Qld, Australia). All culture media are stored at 2–8°C (*see* **Note 2**)
6. MINC incubators used for embryo culture (COOK Medical, Eight Mile Planes, Qld, Australia) (*see* **Note 4**)
7. Sanyo Trigas incubators (Quantum Scientific, Australia) used for culture media equilibration
8. Nitrogen, CO_2, and Special Mix (6% CO_2, 5% O_2, 89% N_2) cylinders (BOC, Preston, Vic, Australia)

3. Methods

Some of the embryos cultured and assessed noninvasively in the laboratory were generated at Monash IVF, Melbourne, a premier assisted reproductive technology (ART) center for the treatment of infertility, with over 20,000 successful pregnancies (take-home babies): website www.monashivf.com (*see* **Section 3.1**). Others were developed in well-established IVF hospitals in Singapore and Bangalore. Both in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) procedures were used to generate these embryos in our laboratories here and overseas (9–13).

3.1. Culturing Human Embryos In Vitro Involves

3.1.1. Fertilization Assessment

Oocytes are viewed for fertilization 14–18 h post sperm insemination. Oocytes exhibiting two pronuclei are deemed normally fertilized zygotes (monospermic) and are suitable for culture (**Figs. 1.3–1.6**). More than two pronuclei, usually 3, means that abnormal fertilization has taken place and the embryos are not suitable for culture (e.g., dispermy or digyny). These embryos might grow quite successfully but have an abnormal (triploid) complement of chromosomes. Oocytes with no pronuclei are indicative of failure to fertilize and those with one pronucleus are either activated or show abnormal fertilization – asynchrony of pronuclear formation or silent fertilization, when sperm heads do not expand to form male pronuclei. These oocytes are not suitable for culture (*see* **Note 6**).

3.1.2. Cleavage Stage Culture

On the day of insemination (day 0), cleavage stage culture dishes are prepared in a clean laminar flow (**Figs. 1.7–1.15**):

1. Using a displacement pipette 2 × 10 µl of cleavage media is dispensed into each well of a labeled 4-well nunclon dish.

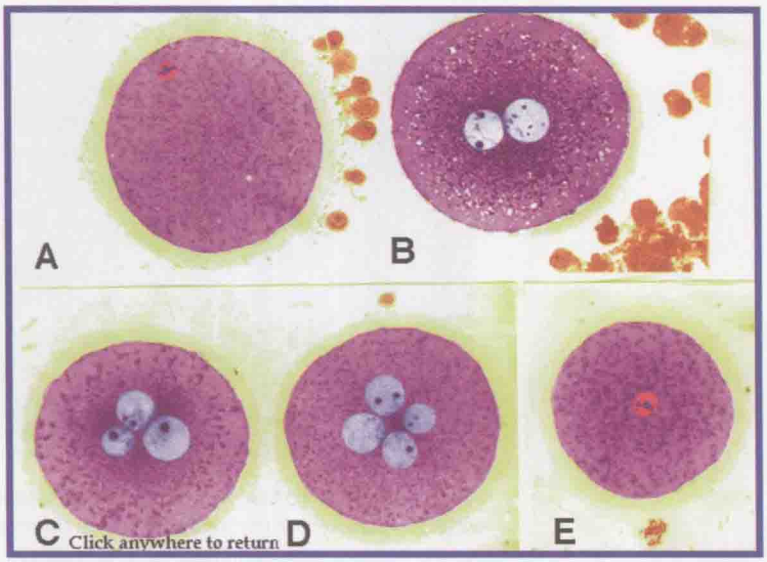

Fig. 1.3. Fertilization: oocyte to pronuclear ova and syngamy – sections (LM). **a**. Mature oocyte (Metaphase II spindle). **b**. Bipronuclear ovum. **c**. Tripronuclear ovum (Dispermy). **d**. Tetrapronuclear ovum. **e**. Syngamy – first mitotic spindle – ×400 (22).

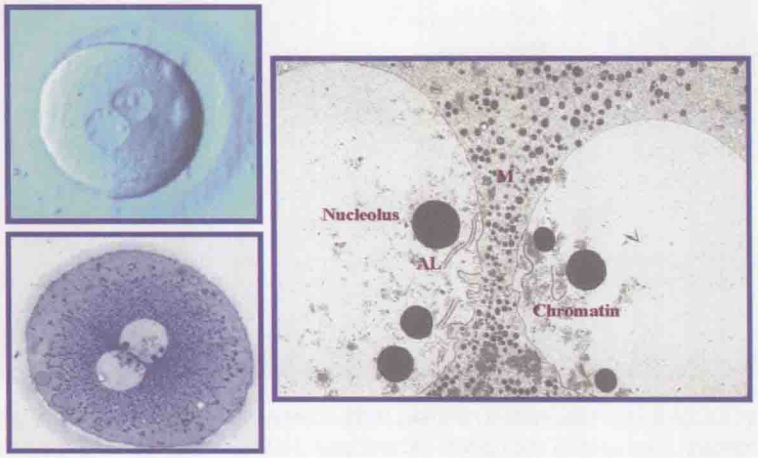

Fig. 1.4. Normal bipronuclear ova (Phase, LM, and TEM). These bipronuclear, after monospermic fertilization, seem normal. Note alignment of nucleoli adjacent to apposing pronuclear membranes. What is more significant is the alignment of chromatin, associated with nucleoli, which would condense to form the male and female chromosomes at syngamy ×400, ×35,700 (22).

2. Paraffin oil is over-laid, approximately 0.5 ml with either a 1-ml displacement pipette or using a 1- or 5-ml serological pipette and an electric pipette aid.
3. Up to 8 zygotes can be accommodated per dish so enough dishes should be made for the number of zygotes being cultured for individuals.

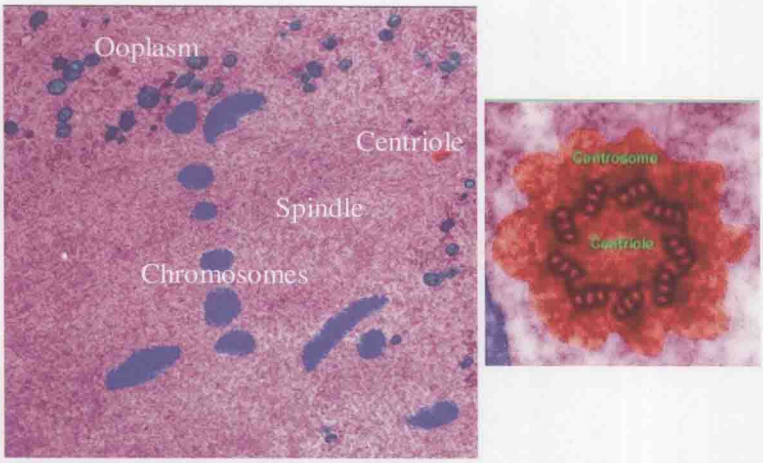

Fig. 1.5. Syngamy: the first mitotic spindle at metaphase TEM. Syngamy is the culmination of fertilization when maternal and paternal chromosomes come together on a bipolar spindle. Note displaced chromosomes outside spindle. The centriole is a descendant of the sperm centrosome in a 8-cell embryo (22).

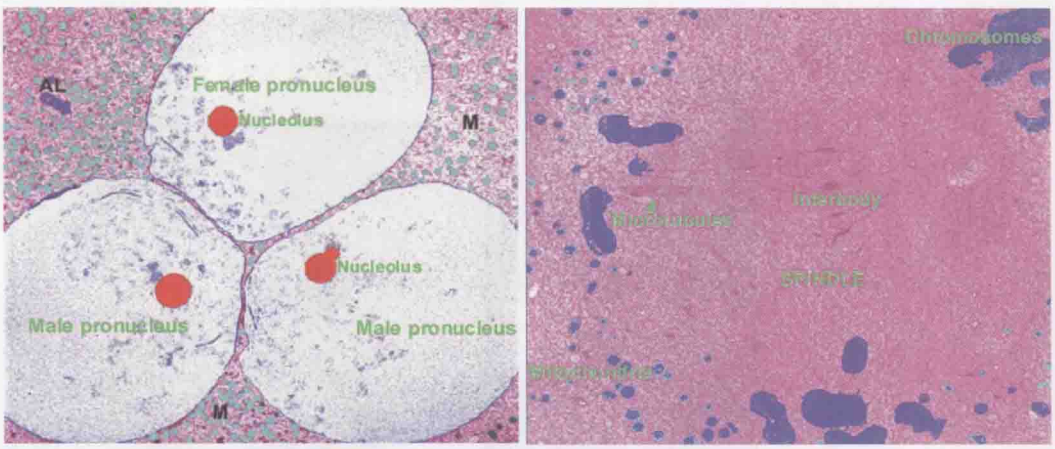

Fig. 1.6. Dispermic tripronuclear (3PN) ova (TEM). Classical images of 3PN ova at the pronuclear stage and syngamy. Note chromatin (blue specks) and nucleoli located toward adjacent membranes of the pronuclear envelopes. The spindle is tripolar enabling the ovum to divide into 3 cells, instead of 2 cells. ×5000, ×8000 (22).

4. Once dishes are complete, they are placed into a CO_2 incubator (*see* **Note 3**). Dishes are equilibrated for a minimum of 4 h in a 6% CO_2 environment (may vary depending on culture media being used) and 5% O_2 in nitrogen and 37°C. Having a 5% O_2 environment is not vital for dish equilibration. However, it is important for culturing embryos.
5. After fertilization assessment (day 1), individual zygotes are moved from the fertilization dish to the cleavage culture dish (day 1–3 culture dish) using a glass flame pulled Pasteur