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ARTICLE

The fine structure of human germ layers *in vivo*: clues to the early differentiation of embryonic stem cells *in vitro*

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Abstract The fine structure of the three germ layers in human ectopic embryos (stage 7) have been documented by digital light and electron microscopy. The formation of ectoderm, endoderm and mesoderm and notochordal cells, and also the extraembryonic membranes, amnion and yolk sac, are imaged. The germ layers give rise to all the cells and tissues of the human body. Possible clues to the early differentiation of embryonic stem cells (ESC) *in vitro* were obtained, since these events are more or less mimicked in cultures of ESC derived from the inner cell mass of human blastocysts. The findings are discussed with reference to previous studies on the fine structure of ESC using the same technique.

KEYWORDS: ectoderm, ectopic embryo, endoderm, mesoderm, primitive streak, TEM

Introduction

The ectopic embryo is a useful model to study early development *in vivo* and understand early cell differentiation in embryos, embryonic stem cells (ESC) grown in colonies and embryoid bodies (EB) derived from ESC. About 95% of ectopic pregnancies are located in the oviduct and reported to be normal in microstructure (Pereda and Motta, 1999) with gestational sacs that could be easily recovered intact by laparoscopic salpingectomy (Sathananthan and Selvaraj, 2007).

There are numerous reports of early human embryos *in vivo*, especially from the Carnegie collection in Washington DC, edited by R Gasser (http://virtualhumanembryo.lsuhsc.edu; Moore, 1982; O'Rahilly, 1973). Most of these images were documented by routine light microscopy of paraffin sections at lower resolution. The germ layer cells are the stem cell primordia from which all other stem cells originate to form the major tissues and organs of the human body. This is a fine structural study of fresh ectopic embryos using Araldite thin sectioning and advanced digital

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microscopy in order to find possible clues to the spontaneous differentiation of ESC in cultures (Sathananthan, 2007, 2010).

Materials and methods

Six embryo sacs (38—40 days old) were recovered from oviduct ampullae, fixed and dissected, enabling the recovery of two embryos (week 3) at the primitive streak stage, corresponding to stage 7 of the Carnegie collection (~19 days old). The other embryos were at more advanced stages (stages 8—9) and appeared normal in structure, although somewhat lagging in development. Cell differentiation in these later stages will be reported in a further communication. The primitive streak embryos were sectioned serially for digital light microscopy and routine transmission electron microscopy (TEM). The overall aim of this study was to produce an image library in the future to compare with embryonic and adult stem cells grown in culture (see www.sathembryoart.com).

The TEM procedure is outlined in detail by Sathananthan and Nottola (2007). Briefly the embryo sacs were fixed in 3% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.2) in hospital and sent to Melbourne. The sacs were dissected to retrieve the embryos, postfixed in 1% osmium tetroxide and embedded in Araldite. Thick survey sections (1 μm) were cut serially, stained with 1% toluidine blue in borax, and corresponding thin sections (~70 nm) were cut with a diamond knife and stained with alcoholic uranyl acetate followed by Reynold's lead citrate. Thin sections were examined with Joel and Philips TEM. Light micrographs of the thick sections were taken with a Leitz Q Win digital microscope. The ease by which one could produce thin (1 μm) epoxy resin sections for digital light microscopy is highlighted by Sathananthan and Nottola (2007) and one need not do TEM if facilities and staff are unavailable. Details of the TEM procedure for embryos and stem cells are covered in this review. Since all the images were computer generated, only original magnifications at microscopy are indicated in the figure legends.

Results

The oval, discoidal embryos were trilaminar showing the formation of the three primary germ layers — ectoderm, endoderm and mesoderm — in thin sections. Diagrams of trilaminar embryos are illustrated in textbooks of embryology, such as Moore (1982). Also evident were the extraembryonic fetal membranes — amnion, chorion and yolk sac (**Figures 1 and 2**). The layer of surface epiblast was composed of a stratified epithelium that will eventually differentiate into ectoderm, mesoderm and some cells of the endoderm (**Figures 3—5**). It varied in thickness (rostral to caudal) and showed a medial primitive streak through which surface cells were migrating inwards and laterally to form mesoderm. This migration or involution of cells is the most important event in week 3 of development, completing the process of gastrulation, which began with endoderm formation in the late blastocyst on day 6 by a process of delamination (Sathananthan et al., 2003). As the primitive streak

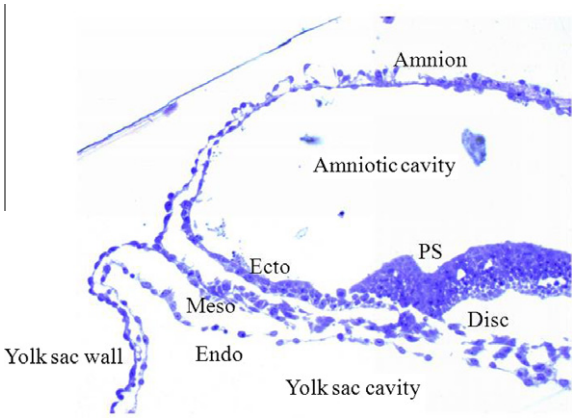

Figure 2 Embryonic disc with the extraembryonic fetal membranes: amnion (top) and yolk sac (bottom). The embryo is protected by the amnion and yolk sac. Note primitive streak (PS) and the three-layered embryonic disc showing ectoderm, mesoderm and endoderm. Light microscopy, ×200.

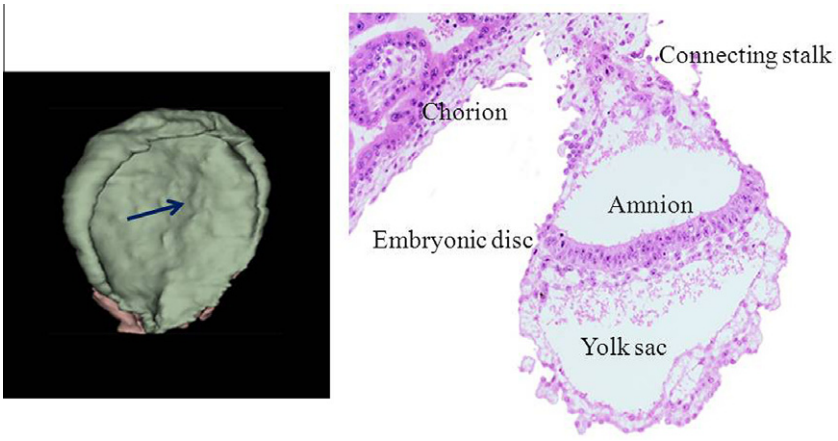

Figure 1 Carnegie embryo stage 7 (day 19). Computer-generated, pear-shaped embryonic disc with medial primitive streak (arrow) (left panel). Wax section through the primitive streak embryo showing the fetal membranes — amnion, yolk sac and chorion (right panel). Reproduced with permission, http://virtualhumanembryo.lsuhsc.edu.

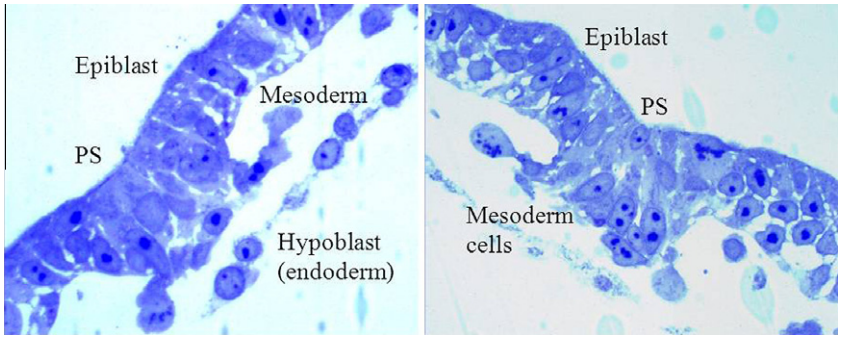

Figure 3 Primitive streak (PS) and germ layers (sectioned at two levels). Migration of mesodermal cells from the surface epiblast (future ectoderm and mesoderm) are shown at the PS. The three germ layers are now being established — a fundamental process in early embryogenesis. Light microscopy, left panel ×200, right panel ×1000.

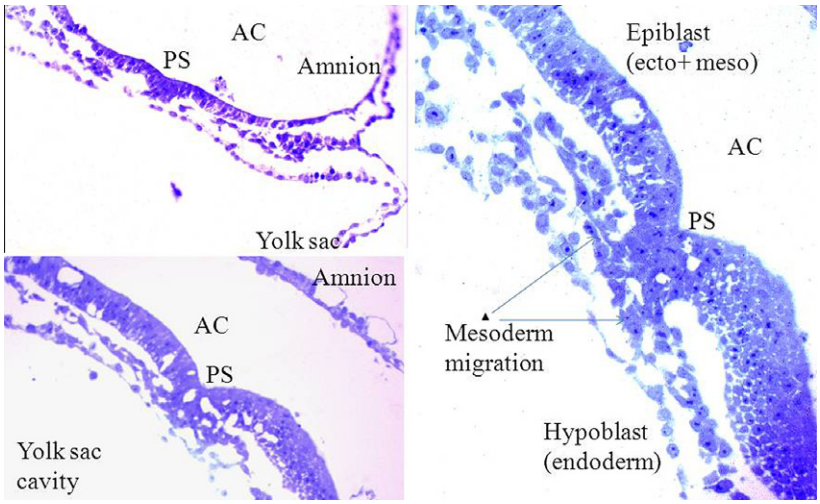

Figure 4 Variation of primitive streak thickness: caudal (left); rostral (right). Serial sections of embryonic disc showing involution of mesodermal cells through the streak, establishing the three germ layers — ectoderm, mesoderm and endoderm. AC = amniotic cavity, PS = primitive streak. Light microscopy, ×200, ×1000.

deepens rostrally, the epiblast thickens on either side of the streak due to a piling up of migrating cells medially. Endoderm that had delaminated earlier now forms the roof of the primitive gut and lining of the yolk sac. The cells were, as usual, phagocytic with vacuoles. The amnion and chorion had ectoderm and mesoderm, whilst the yolk sac had endoderm and mesoderm, but had no blood islands. The surrounding chorion of the embryo sac had numerous chorionic villi, which eventually form a haemochorial placenta (**Figures 1** and **2**).

Serial sections of the embryonic disc showed progressive migration of mesodermal cells through the streak and laterally between the surface epiblast and endoderm, thereby establishing the three germ layers (**Figures 2** and **3**). The mesoderm cells were flask-shaped as they migrated inwards from the epiblast, becoming amoeboidal as they migrated laterally (**Figures 4** and **5**), best visualized by TEM (**Figures 6** and **7**). Mitotic cells were evident below the streak, while microvilli were abundant on the surface of the epiblast. Mesoderm cells were most abundant in the early embryo, located between the ectoderm and endoderm (**Figures 4**

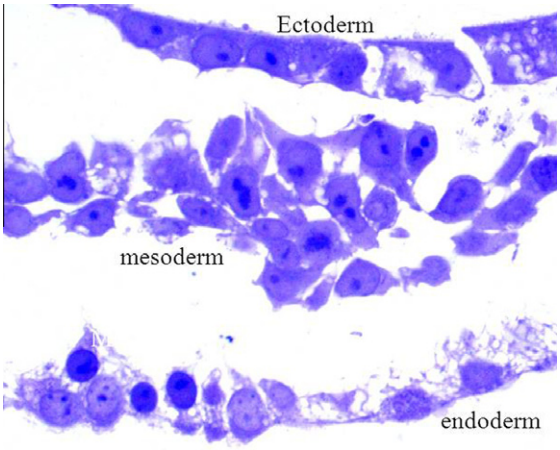

Figure 5 Trilaminar embryo with the three germ layers: germ layers are formed during week 3 of development. Mesoderm cells have migrated laterally beneath the surface ectoderm (epiblast). Endoderm represents the hypoblast. Light microscopy, ×1000.

and 7). The cells were amoeboidal and seemed to be migrating in every direction — laterally, rostrally and caudally — and represent primitive mesenchyme, which would differentiate into most of the tissues and organs of the human body. The lateral plate mesoderm formed a clump of cells before it split to line the amnion above and yolk sac below and form somatic and splanchnic mesoderm (**Figure 8**). The primitive knot, or Henson's node, situated anterior to the primitive streak, showed ingression of primitive notochordal cells which attach to the endoderm — the beginning of the rostral notochordal process (**Figure 9**).

Regarding the extraembryonic membranes, the primitive amnion formed on day 9 consisted of two layers, mesoderm outside and ectoderm inside, and enclosed a clear amniotic cavity above the embryonic disc (**Figures 10** and **11**). The yolk sac showed intraembryonic endoderm, which forms the roof of the gut, and extraembryonic endoderm, forming the primitive yolk sac lined by endoderm inside and mesoderm outside (**Figure 12**). There were no blood islands in the mesoderm. Endoderm is characterized by vacuolated phagocytic cells as demonstrated in day-6 blastocysts (Sathananthan et al., 2003). The chorion enclosed the embryo with all its membranes. It had numerous chorionic villi projecting from the surface of the embryo sac (**Figure 1**) and was lined by ectoderm on the outside and mesoderm inside. This forms the fetal segment of a haemochorial placenta. Chorionic villi were lined by a cellular cytotrophoblast, surrounded by a multinucleated syncytiotrophoblast.

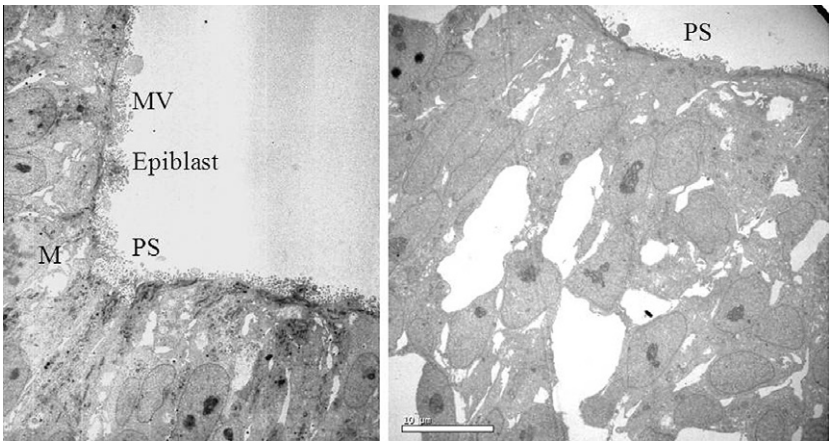

Figure 6 Primitive streak (PS) and involution of mesoderm cells. Cells are flask-shaped and become amoeboidal as they migrate inwards from the epiblast. A mitotic cell (M) is evident below the streak. Note microvilli (MV) on the surface of the epiblast. Transmission electron microscopy, ×3000.

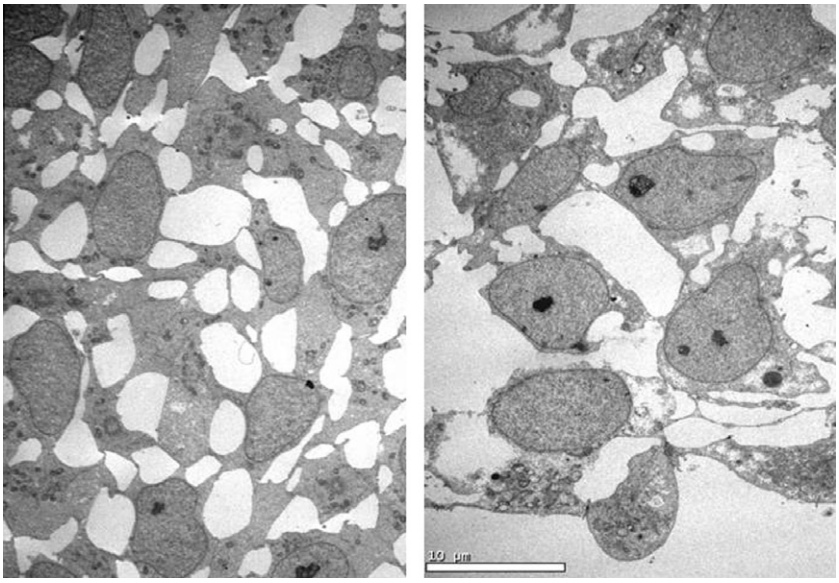

Figure 7 Lateral plate mesoderm. Mesoderm cells are the most abundant in the early embryo, located between ectoderm and endoderm. The cells are amoeboidal and seem to be migrating in every direction and represent primitive mesenchyme. They differentiate into most of the tissues and organs of the human body, such as connective, skeletal, muscle, heart, blood. Transmission electron microscopy, ×2000 (left), ×3000 (right).

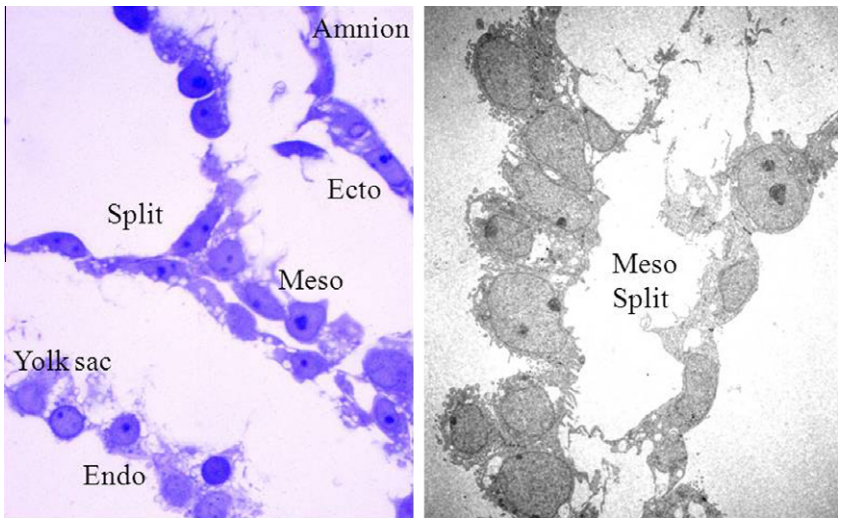

Figure 8 Lateral mesoderm split. The extraembryonic mesoderm splits into two and contributes to the amnion dorsally and yolk sac ventrally to establish these fetal membranes. Light microscopy, ×1000 (left); transmission electron microscopy, ×3000 (right).

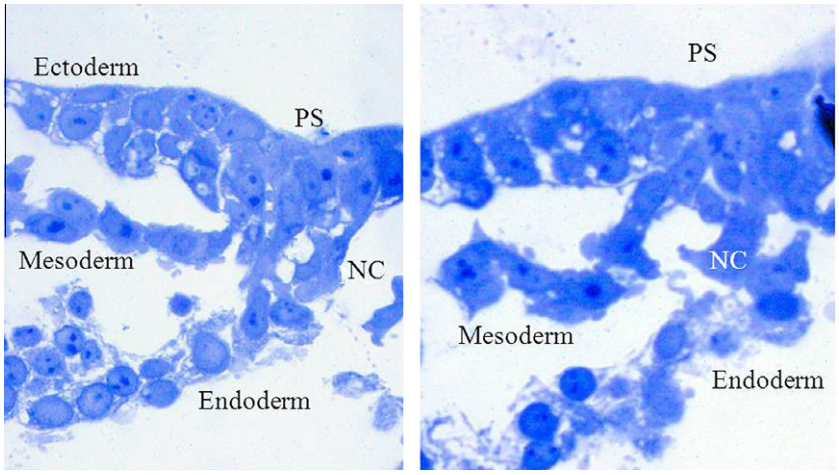

Figure 9 Primitive knot and ingression of notochordal cells (two levels). This is the transition from primitive streak (PS) to the anterior knot, where primitive notochord cells (NC) are formed and then attach to endoderm beneath, the beginning of the rostral notochordal process (axial skeleton of the human embryo). Light microscopy, ×1000.

Discussion

The three primary germ layers formed during week 3 of development establishes a trilaminar embryo, a fundamental process in early embryogenesis and important for stem cell studies. Germ layers are derived from the inner cell mass of blastocysts, as are ESC, which originate around day 9 of development (Sathananthan, 2007, 2010). These cells are the precursors from which all other cell types of the human body are derived at the next level of cell differentiation in weeks 3 and 4 of embryogenesis. Further studies of more advanced ectopic embryos, already retrieved at stages 8—10, will further elucidate the evolution of the microstructure of these cell types *in vivo*. The predominant stem cells at the next level of organization are neuroepithelial (ectodermal), mesenchymal (mesoderm) and endodermal derivatives, which are well documented in the literature (Moore, 1982).

Such cells are also seen in ESC and EB and even in neurospheres at about weeks 2 or 3 of cell differentiation in culture (Sathananthan, 2007, 2010, in press; Sathananthan and Trounson, 2005, 2007; Sathananthan et al., 2002). However, these primordial stem cells are not organized or structured in time and space, as they are in the developing embryo. Spontaneously differentiated cells of almost all tissues ranging from epithelial to neural stem cells were found in groups at the next level of cell differentiation and could be identified by their microstructure. Neural rosettes and neural stem cells could be traced to surface epithelia in EB and neurospheres (Peh, 2007; Sathananthan, 2010, in press) There is also evidence of trophectoderm (Thomson et al., 1998). The mesenchymal stem cells eventually differentiate

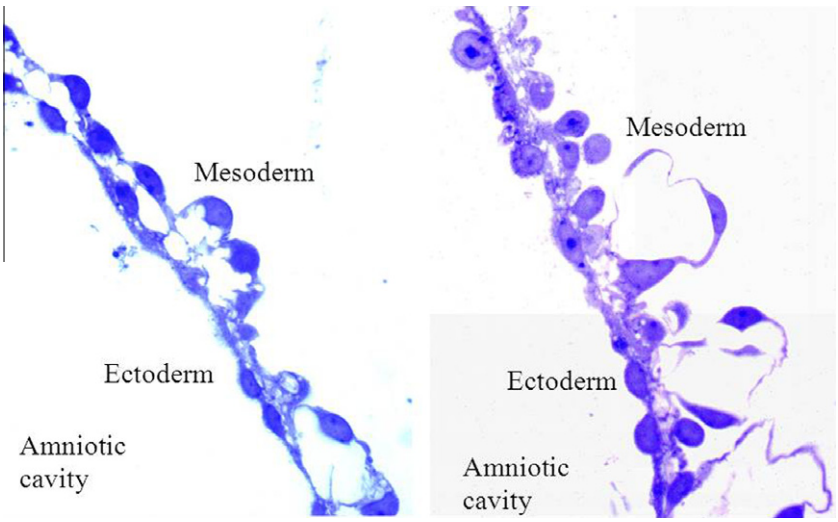

Figure 10 The amnion or water bag from two embryos. Amnion consists of two layers, mesoderm outside and ectoderm inside and a cavity. Note blebs of mesoderm in amnion. Amniotic cells are now used in stem cell research. Light microscopy, ×400 (left), ×1000 (right).

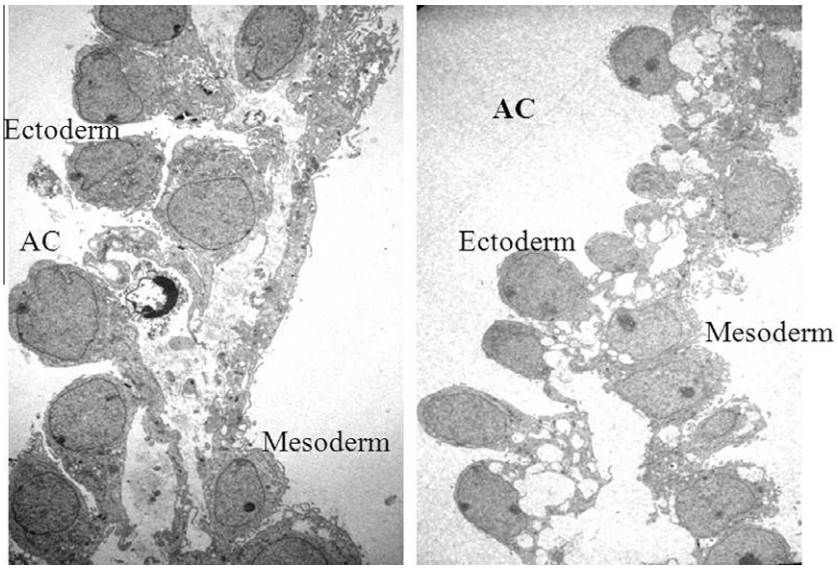

Figure 11 Amnion: mid and caudal regions. The primitive amnion has two layers: ectoderm inside and mesoderm outside and encloses an amniotic cavity (AC) above the embryonic disc. Transmission electron microscopy, ×3000 (left), ×2000 (right).

into connective, muscle, heart and haematopoietic tissue (Trounson, 2006). The first cell types to appear among ESC and EB are fibroblasts and branched cardiac muscle fibres. In addition, there were capillary-like endothelial formations and germ cells. Endodermal derivatives included goblet cells, lung cells resembling those in alveoli and clumps of cells, perhaps of hepatic and pancreatic origins. These cell types have been documented in previous reports (Sathananthan, 2010; Sathananthan and Trounson, 2005, 2007).

In conclusion, this study's aim was to compare embryo structure with early differentiation of cells in ESC, EB and neurospheres. Ectopic embryos will no doubt prove to be a valuable source of stem cell precursors *in vivo* and should provide hitherto unavailable material for stem cell research. This study realized that cell differentiation in culture somewhat recapitulated the processes documented in embryos, Characterizing the fine structure of these cell types must now be confirmed by current stem cell techniques, like fluorescent microscopy, using specific markers. This can be done using ectopic embryos fixed in formaldehyde and frozen sectioning. We need to confirm stem cells in germ layers using cytokeratin 8 for potential ectoderm; alpha-1-fetoprotein for potential endoderm; and vimentin for potential mesoderm, as reported in advanced bovine embryos (Maddox-Hyttel et al., 2003). These authors used paraformaldehyde for fixation and paraffin sections. Another alternative is to perform electron cytochemistry using specific gold-labelled antibodies. Such

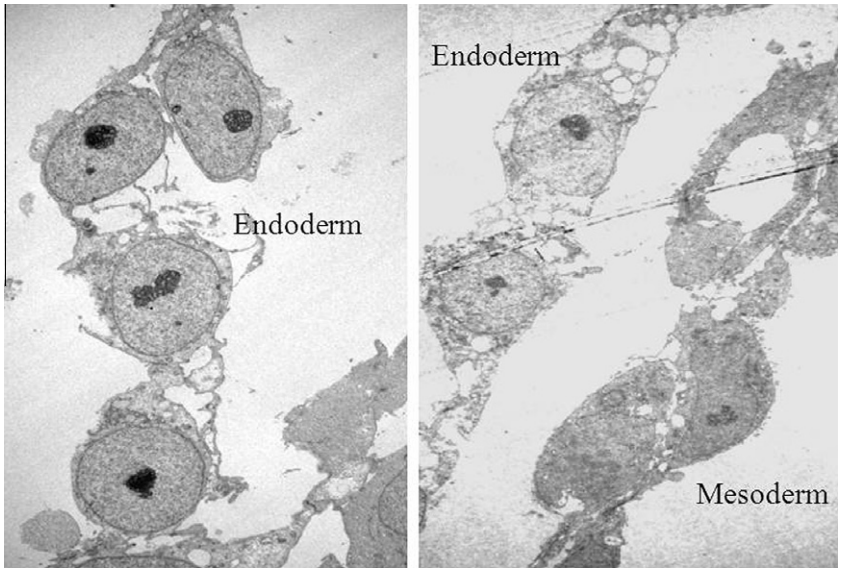

Figure 12 Endoderm (left) and yolk sac (right), showing intraembryonic endoderm (roof of the gut) and extraembryonic endoderm forming the primitive yolk sac (endoderm + mesoderm). Endoderm is characterized by vacuolated phagocytic cells, often found in ESC colonies. Transmission electron microscopy, ×3000.

studies can be extended to blastocysts to see the progressive evolution of stem cells during embryogenesis and will set the gold standard for research in the characterization of stem cells for clinical therapy (Sathananthan and Trounson, 2007).

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References

Maddox-Hyttel, P., Alexopoulos, N.I., Vajta, G., Lewis, I., Rogers, P., Cann, L., Callesen, H., 2003. Immunohistochemical and ultrastructural characterization of the initial post-hatching development of bovine embryos. Reproduction 125, 607—623.

Moore, K.L., 1982. The Developing Human: Clinically Oriented Embryology, third ed. Saunders, Philadelphia, p. 479.

O'Rahilly, R., 1973. Developmental Stages in Human Embryology, Embryos of the First 3 Weeks (Stages 1—9). Carnegie Institution, Washington, DC.

Peh, G.S.L., 2007. Characterization of neural progenitors derived from human embryonic stem cells. Ph.D. Thesis, Monash University, Melbourne, Australia.

Pereda, J.T., Motta, P.M., 1999. New advances in human embryology: morphofunctional relationship between the embryo and the yolk sac. Med. Electron. Microsc. 2, 67—78.

Sathananthan, H., Pera, M., Trounson, A., 2002. The fine structure of human embryonic stem cells. Reprod. Biomed. Online 4, 56—61.

Sathananthan, A.H., Gunasheela, S., Menezes, J., 2003. Critical evaluation of human blastocysts for assisted reproductive techniques and embryonic stem cell biotechnology. Reprod. Biomed. Online 7, 219—227.

Sathananthan, A.H., Trounson, A., 2005. Human embryonic stem cells and their spontaneous differentiation. Ital. J. Anat. Embryol. 110, 151—157.

Sathananthan, A.H., 2007. The derivation of embryonic stem cells for therapy: new technologies. Reprod. Biomed. Online 14, 635—639.

Sathananthan, A.H., Nottola, S.A., 2007. Digital imaging of stem cells by electron microscopy. In: Vermuri, M.C. (Ed.), Stem Cell Assays. Humana Press, pp. 21—41.

Sathananthan, A.H., Selvaraj, K., 2007. Human ectopic embryos: clues to the origins of stem cells. In: World Congress on Fallopian Tubes, Calcutta, India.

Sathananthan, A.H., Trounson, A., 2007. In vitro differentiation of human embryonic stem cells. In: Sullivan, S., Cowan, C., Eggan, K. (Eds.), Human Embryonic Stem Cells: A Practical Handbook. Wiley Press, pp. 149—168.

Sathananthan, A.H., 2010. Spontaneous differentiation of tissue cell types in ESC, EB and NS cultured from ICM of blastocysts *in vitro*. In: Poster/DVD Fifth Warm World Congress, Moscow, Russia, October 2010.

Sathananthan, A.H., in press. Neural stem cells in neurospheres, embryoid bodies and CNS of human embryos. Microsc. Microanal.

Thomson, J.A., Itskovitz-Eldor, J., Sander, S., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145—1147.

Trounson, A., 2006. The production and directed differentiation of human embryonic stem cells. Endocr. Rev. 27, 208—219.

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