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From teratocarcinomas to embryonic stem cells

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The recent derivation of human embryonic stem (ES) cell lines, together with results suggesting an unexpected degree of plasticity in later, seemingly more restricted, stem cells (so-called adult stem cells), have combined to focus attention on new opportunities for regenerative medicine, as well as for understanding basic aspects of embryonic development and diseases such as cancer. Many of the ideas that are now discussed have a long history and much has been underpinned by the earlier studies of teratocarcinomas, and their embryonal carcinoma (EC) stem cells, which present a malignant surrogate for the normal stem cells of the early embryo. Nevertheless, although the potential of EC and ES cells to differentiate into a wide range of tissues is now well attested, little is understood of the key regulatory mechanisms that control their differentiation. Apart from the intrinsic biological interest in elucidating these mechanisms, a clear understanding of the molecular process involved will be essential if the clinical potential of these cells is to be realized. The recent observations of stem-cell plasticity suggest that perhaps our current concepts about the operation of cell regulatory pathways are inadequate, and that new approaches for analysing complex regulatory networks will be essential.

Keywords: embryonic stem cells; teratocarcinoma; embryonal carcinoma; human; differentiation; plasticity

1. INTRODUCTION

The present flurry of interest in stem cells and their potential for 'regenerative' medicine has arisen from the confluence of several streams of biological thought and investigation. The first is the notion of stem cells themselves, which evolved among biologists considering the regeneration of tissues that are necessarily replaced throughout adult life, most obviously the blood, the lining of the gut and the skin. It was proposed that the replacement of these complex tissues, comprising many different cell types, depends upon the existence of small populations of undifferentiated cells, 'stem cells', that not only proliferate to replace themselves indefinitely ('self-renewal'), but also differentiate into daughter cells with a limited proliferative capacity. These so-called 'transit amplifying' cells are committed to differentiate further to a restricted range of terminal cell types responsible for the principal functions of such tissues (Lajtha 1979; Potten & Lajtha 1982). Such terminally differentiated cells typically lose their capacity for further proliferation.

This type of tissue architecture may very well have a selective advantage in minimizing the susceptibility of an organism to cancer, which is almost certainly the result of the accumulation of mutations that affect both cell proliferation and differentiation. It was pointed out by Pierce (1974) that many cancers seem to be caricatures of their tissue of origin, with a malignant stem cell similar to the stem cell of the healthy tissue, as well as a variety of cells at progressive stages of differentiation. He proposed that malignancy is a consequence of a failure to regulate the balance between proliferation and differentiation, resulting in the accumulation of cells at earlier stages of the differentiation pathways. Where the issue has been analysed in detail, notably in the haematological malignancies, possibly only few cells within a tumour mass, the so-called 'clonogenic cells', are capable of transferring a malignancy to a new host (Greaves 1982). Cairns (1975) pointed out that it is mutations occurring in the stem-cell population that are most likely to become 'fixed' in a tissue and cause long-term damage because mutations in transit amplifying and terminally differentiated cells would normally be lost as the tissue turned over. He suggested that there would then be a selective advantage for an organism to maintain only small stem populations to minimize the target for mutagenesis, and hence carcinogenesis.

Many tissues that generally appear quiescent also turn over slowly (e.g. bone), or may proliferate or regenerate in response to physiological cues (mammary tissue in pregnancy) or injury (liver). Presumably these, too, retain stem cells, or cells that are capable of conversion into stem cells. But it has now become evident that tissues not previously thought capable of regeneration, notably the nervous system, also harbour stem-cell populations (Temple 2001). However, the results that have caused general surprise come from a series of experiments, by different groups working on different organ systems, suggesting that stem cells from one tissue, hitherto thought committed to the lineages of that tissue, can give rise to distinct cell types of embryologically unrelated tissues under some circumstances. Thus, evidence has been presented for neural stem cells giving rise to blood cells (Bjornson *et al.* 1999) and to muscle (Galli *et al.* 2000), haematopoietic stem cells giving rise to neurons (Brazelton *et al.* 2000; Mezey *et al.* 2000), muscle (Goodell *et al.* 2001) and liver (Alison

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et al. 2000; Peterson *et al.* 1999; Lagasse *et al.* 2000), muscle stem cells giving rise to blood cells (Jackson *et al.* 1999; Seale *et al.* 2001), and mesenchymal stem cells from bone giving rise to a wide variety of tissues (Pittenger *et al.* 1999). Perhaps the most striking observation was that neural stem cells transferred to a blastocyst could contribute to tissues belonging to all three germ layers (Clarke *et al.* 2000).

Many questions still remain about these observations of apparent stem-cell plasticity; only in a few cases have the experiments been conducted with clonal, well-characterized stem cells—a necessity if one is to conclude that a particular stem cell is able to give rise to several distinct cell types. In any case, if we take the results at face value, are we observing a normal physiological function of stem cells, or are we seeing the consequences of rare switches in their differentiative capacity or potency? In the latter case, is this because of the exposure of the cells to environments that they do not normally see—an issue of the difference between what cells 'can do'—'prospective potency'—and what they normally do when undisturbed—'prospective fate' (Weiss 1939). Or are the changes in their patterns of differentiation due to rare events involving intrinsic changes to the patterns of gene activity that regulate the determined state of a cell? Regardless of the answers to these questions, the observations have nevertheless raised the prospect of clinically using stem cells from adult tissues in cell replacement therapies designed to repair or replace tissues damaged by accident or disease.

The second stream of work contributing to the current debate is one that began with a long-standing fascination with a peculiar type of tumour, the teratomas, and has culminated in the derivation of stable, pluripotent human embryonic stem (ES) cell lines in culture. Teratomas present a haphazard array of cell differentiation that appears to recapitulate many of the events that occur during early embryonic development but in a disorganized manner (Damjanov 1993; Dixon & Moore 1952; Mostofi & Price 1973). They have intrigued clinicians and biologists for many hundreds of years; excellent summaries of the history of this interest are provided by Damjanov & Solter (1974) and Wheeler (1983). The experimental study of teratomas began with the discovery by Stevens & Little (1954) that they occur spontaneously in the testes of about 1% of male mice of the 129 strain, or that they can be induced by transplanting the genital ridges of embryos from strain 129 mice, and a few other strains, to the testes of adult mice (Stevens 1964, 1970*a*,*b*). Some of these tumours were evidently malignant and could be re-transplanted to successive hosts. Such 'teratocarcinomas', as the re-transplantable, malignant tumours were known, contained a relatively undifferentiated cell type known as an 'embryonal carcinoma' (EC) cell, long suspected as the stem cell of the tumour. This stem-cell character of EC cells was confirmed by Kleinsmith & Pierce (1964), who showed that single EC cells transferred to a new host could reform a complex teratocarcinoma that could again be re-transplanted to another host. A long series of experiments through the 1960s and 1970s, seeking to characterize these EC cells and their relationship to embryonic cells, culminated with the isolation, in 1981, of pluripotent cells from very early mouse embryos (Evans & Kaufman 1981; Martin 1981) and eventually, in 1998, from human embryos (Thomson *et al.* 1998). These 'normal' embryo-derived cells became known as ES cells. In fact, the term 'stem cell' was rarely used previously by embryologists (e.g. Wilson 1896) and it then generally referred to precursor cells within the developing embryo. Such cells usually exist only transiently, and their self-renewal is normally limited, unlike 'stem cells' in the adult. Although ES cell lines in culture do meet the criteria used in the context of 'adult stem cells' of indefinite self-renewal together with a capacity for differentiation, the embryonic cells of the late inner cell mass (ICM), to which they correspond, generally disappear from the developing embryo by the time of gastrulation. However, it is intriguing to speculate that a number of childhood cancers may arise because of the abnormal persistence of 'stem cells' present during embryogenesis.

The third stream of relevant research encompasses the work of those who sought first to determine whether terminal differentiation of cells during embryogenesis results from the loss of genes, or whether altered regulation without a significant change in the complement of genes present in a cell could be responsible. Following earlier studies of Briggs & King (1952), Gurdon (1962) showed that, in *Xenopus laevis*, nuclear transfer from differentiated somatic cells to enucleated oocytes supported embryonic development, indicating that the nuclei of differentiated cells in amphibians contain a full genetic complement. These experiments incidentally demonstrated an approach for producing 'clones' of genetically identical individuals. For many years it seemed that this might not be possible in the case in mammals (McGrath & Solter 1984), and definitive evidence that mammalian 'cloning' by nuclear transfer from somatic cells is possible was not acquired until experiments with sheep embryos led to the birth of 'Dolly', born following the transfer of a nucleus from a mammary cell of an adult sheep to an enucleated oocyte (Campbell *et al.* 1996; ilmut *et al.* 1997). That observation was quickly followed by finding that 'cloning' is also possible in mice (Wakayama *et al.* 1998), cows (Cibelli *et al.* 1998) and pigs (Onishi *et al.* 2000). It is a short step to imagine that this would also be possible in humans, if anyone chose to take this step.

The idea of using such nuclear transfer techniques for so-called 'reproductive cloning' in humans is an anathema to most scientists, not least because the process is both inefficient and subject to serious errors so that most animals developing by this route have proved to be defective. However, it rapidly occurred to several people that embryo cloning by this route, coupled with the ability to derive ES cells from very early embryos at the blastocyst stage, could allow the development of almost any differentiated cell type that would be genetically identical to a prospective patient, who could then receive such cells in tissue replacement therapies (essentially autografts), the so-called 'therapeutic cloning' approach. As a proof of concept, Munsie *et al.* (2000) have isolated murine ES cells from blastocysts derived by somatic nuclear transplantation. The derivation of human embryos following somatic nuclear transfer to an enucleated oocyte has also been reported, though subsequent development arrested prior to blastocyst formation at the six-cell stage (Cibelli *et al.* 2001). So far, no one has described the derivation

of human ES cells from such embryos. Whether it will ever prove a practicable proposition to derive cells tailored for specific patients, instead of finding other ways for defeating the immune system to permit allografts from established, non-autologous ES lines, is a moot point.

Thus, the current excitement about the potential of stem-cell biology for regenerative medicine arises in part from the identification and culture of various types of stem cells, whether from embryos or adults, and in part from results indicating that adult stem-cell plasticity or embryo cloning by somatic cell nuclear transplantation may be realistic approaches for providing autologous tissues for grafting to evade problems of immunological incompatibility. Nevertheless, it is arguable that, despite considerable progress in the biology of stem cells from various adult tissues, ES cells from the early embryo are perhaps the best understood. Clear identification of the various adult stem cells has often been elusive; some, notably haematopoietic stem cells, cannot be easily cultured and expanded *in vitro*, while others require continual re-isolation as indefinite culture *in vitro* does not seem possible. By contrast, ES cells from mice and humans have been cloned (Amit *et al.* 2000), in the sense that it has been shown that single identifiable cells can give rise to a variety of distinct cell types by differentiation, they can be cultured and expanded *in vitro*, apparently indefinitely, without loss of potency, and they could, in principle, be used to generate all tissues of the body. These features mean that it should be possible establish an inexhaustible supply of well-characterized cells for clinical use—an important issue for ensuring safe protocols. It is also important to recognize that ES cells, and their malignant EC cell counterparts, provide invaluable tools for analysing cell differentiation throughout embryogenesis, which also has implications for understanding diseases such as cancer. In turn, ES cells may also provide keys for understanding the biology of 'adult stem cells'.

2. THE BIOLOGY OF TERATOCARCINOMAS

Teratomas are generally benign tumours that occur most commonly in the ovary, where they are also known as benign ovarian cysts (figure 1*a*). These arise from oocytes that have undergone parthenogenetic activation, begun development and then become disorganized to form a mass of embryonic tissue (Stevens & Varnum 1974). Teratomas are also found, though more rarely, in other sites, including the base of the spine in newborn infants.

Similar tumours occur in the testis, but in this case they are generally highly malignant and consequently known as teratocarcinomas (Dixon & Moore 1952; Mostofi & Price 1973). These form a subgroup of the germ-cell tumours (GCTs) that account for almost all testicular cancers. Testicular GCTs, which appear to arise from abnormal gonocytes within the seminiferous tubules (figure 1*b*) (Skakkebaek 1972) are rare, but have a peak incidence in young post-pubertal men making them the most common malignancy in this age group (Møller 1993). Furthermore, their incidence has increased dramatically over the past 50 years. The uncommonly young age of GCT patients contributes to their medical significance but, fortunately, GCT are amongst the most treatable cancers since the advent of *cis*-platinum-based therapy in the 1970s (Einhorn 1987; Stoter 1987).

GCTs are typically divided into seminomas and non-seminomas (Damjanov 1990, 1993). Seminomas consist of cells that resemble primordial germ cells; they do not occur in mice. In contrast, non-seminomas are histologically heterogeneous and frequently contain somatic tissues such as nerve, bone, muscle, etc.; sometimes they contain structures, embryoid bodies, in which these cells are organized to resemble closely an early embryo (figure 1*c*,*d*). These tumours also contain histologically undifferentiated elements composed of EC cells, the key malignant pluripotent stem cell of these tumours. The term teratocarcinoma is generally used for tumours containing both EC and teratoma components.

In 1954, Stevens & Little reported that males of the 129 strain develop spontaneous testicular teratomas and teratocarcinomas that can be observed as incipient tumours, forming structures described as embryoid bodies within the seminiferous tubules of the developing gonad, as early as 13 days of embryonic development (Stevens 1964). Stevens also showed that these tumours can be induced experimentally in strain 129 mice and a limited range of other strains by explanting genital ridges of foetuses, between 11 and 13.5 days of development, to ectopic sites (Stevens & Hummel 1957; Stevens 1967*a*, 1970*a*). These results suggested that the origins of teratomas were from primordial germ cells as, in the mouse embryo, these migrate into the genital ridge at 11 days (Bendel-Stenzel *et al.* 1998). The upper limit of 13 days implies some further changes in the germ cells, which could be associated with their entering mitotic arrest soon after their arrival in the genital ridge. Confirmation of the germ-cell origin of the spontaneous and experimental testicular teratomas came from studies of mice homozygous for the *Steel* (*Sl*) mutation (Stevens 1967*b*). Viable *Sl*/*Sl* homozygotes are infertile as the primordial germ cells do not survive migration. Stevens found that the genital ridges from homozygous *Sl*/*Sl* 129 mice did not yield teratomas.

Although, testicular teratomas can only be induced in a limited number of strains, teratomas can also be formed from many strains of mice by the transplantation of rather earlier embryos, at the egg cylinder stage (about 7 days of development) to ectopic sites (Solter *et al.* 1970, 1979, 1981). As in the spontaneous ovarian tumours derived from parthenogenotes, these embryos become disorganized and form teratomas or teratocarcinomas, depending upon the host strain into which the embryo is transplanted and, interestingly, not upon the genotype of the embryo itself. Curiously, the range of mouse strains from which teratomas and teratocarcinomas can be derived by this route, and so from which EC cell lines can be established, is considerably greater than the range of strains from which ES cells have been derived by blastocyst culture, or teratocarcinomas from genital ridges, whether spontaneously or by transplantation. These results raise the question of what is the cell of origin of the EC cells derived from egg cylinder explants—presumably they are neither ICM/epiblast cells nor primordial germ cells. A further curiosity is that, apart from the spontaneous human tumours, it has not proved possible to derive teratocarcinomas routinely by such techniques in other species, though in the rat the yolk sac can give rise

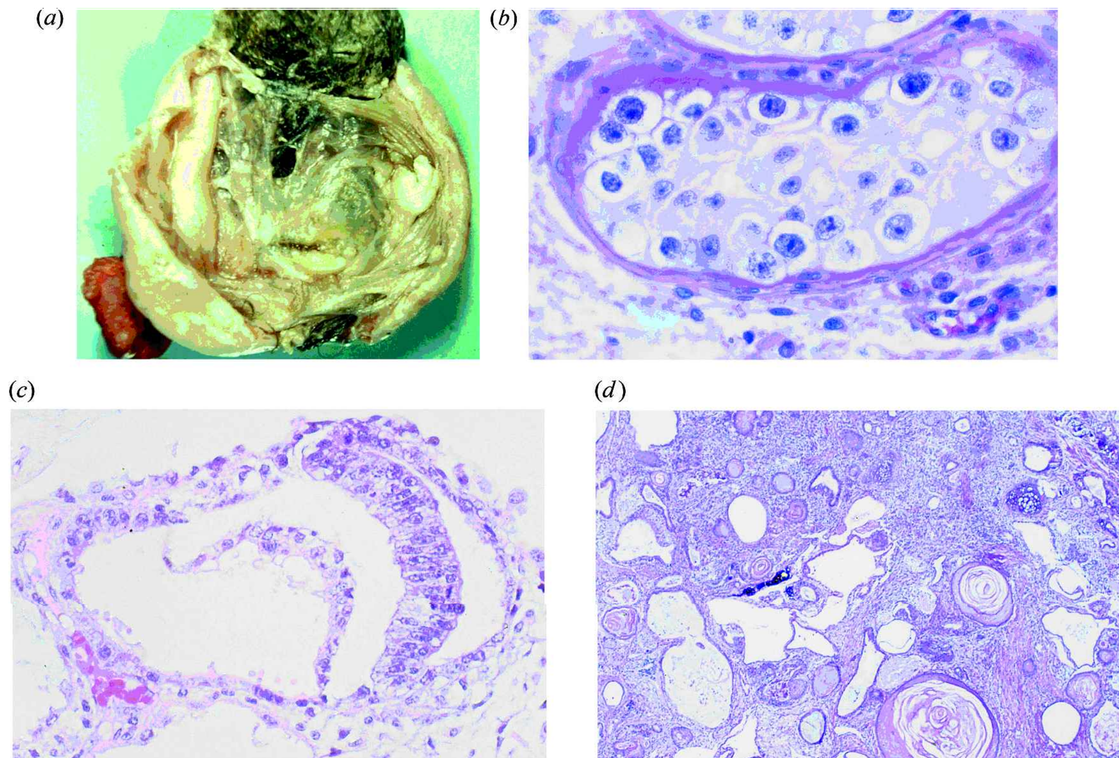

Figure 1. Examples of ovarian and testicular teratocarcinomas. (*a*) A mature human teratoma, or dermoid cyst of the ovary, showing hair and a tooth. (*b*) Carcinoma *in situ*: the pre-invasive phase of human testicular teratocarcinomas in which abnormal gonocytes fill the seminiferous tubules of the testis. (*c*) An embryoid body within a human testicular teratocarcinoma. (*d*) A well-differentiated mouse teratocarcinoma. Note extensive differentiation including keratin pearls and cartilage.

to a teratoma after the fetus is surgically removed (Sobis *et al.* 1993). These rat tumours are not re-transplantable and the isolation of a rat EC, or indeed ES, cell line has proved elusive.

3. EMBRYONAL CARCINOMA CELL LINES

Further study of the biology of mouse EC cells awaited the derivation of EC cell lines established in culture, which was first reported by Finch & Ephrussi (1967) and Kahn & Ephrussi (1970). Subsequently, several groups established murine EC cell lines throughout the 1970s (Bernstine *et al.* 1973; Jakob *et al.* 1973; Evans 1972; Martin & Evans 1974, 1975; Nicolas *et al.* 1975). Many of these lines remained pluripotent and, although they could be maintained as undifferentiated EC cells under certain culture conditions, they retained the ability to form teratocarcinomas when transplanted back into an appropriate mouse host. Many of the pluripotent lines would also differentiate in culture, but the circumstances varied between lines: some EC cell lines remained undifferentiated if kept proliferating in subconfluent cultures, but differentiated spontaneously to yield a variety of cell types, including nerve and muscle, if maintained in confluent cultures for several days (Nicolas *et al.* 1975). Other pluripotent EC lines required culture on feeder layers of transformed mouse fibroblasts to prevent differentiation (Martin & Evans 1974, 1975). In this case, differentiation could be induced by removing the EC cells from the feeder cells, and particularly if the cells were grown in suspension when they aggregated to form structures known as embryoid bodies with an inner core of EC cells surrounded by a layer of cells resembling the visceral endoderm of an early mouse conceptus. Gradually these embryoid bodies became histologically complex and a wide variety of differentiated cells would grow out when they plated on a substrate that permitted attachment.

The uncontrolled nature of spontaneous differentiation of EC cells, although dramatic, made study of the underlying processes difficult. The discovery by Strickland & Mahadavi (1978), that an apparently nullipotent EC cell line, F9, could be induced to differentiate by exposure to retinoic acid, proved a significant advance. After exposure to both retinoic acid and cAMP these F9 EC cells generated cells that closely resemble parietal endoderm (Strickland *et al.* 1980). Subsequently, it was found that if F9 cells are allowed to form clusters in suspension in the presence of retinoic acid, they form embryoid bodies in which the outer layer of cells resembles visceral endoderm, while the inner cells retained an EC phenotype (Hogan *et al.* 1983). Thus, apparently F9 cells can be switched between differentiating into visceral or parietal endoderm depending upon external cues. It was later shown that retinoic acid, as well as other agents, notably dimethylsulphoxide (DMSO) and hexamethylene bisacetamide (HMBA), could also induce the differentiation of a number of mouse EC cell lines (Jakob *et al.* 1978; McBurney *et al.* 1982). In particular, at least in the case of the P19 EC cell line, retinoic acid tended to induce neurectodermal differentiation, whereas DMSO induced mesoderm differentiation and the appearance of cardiac muscle (Jones-Villeneuve *et al.* 1982).

4. RELATIONSHIP TO THE EARLY EMBRYO

During the 1970s, it was found that murine EC cells express various features that are typical of cells of the ICM and primitive ectoderm of the early conceptus (Artzt *et al.* 1973; Jacob 1978). Following the line of reasoning that EC cells are a malignant equivalent of those embryonic cell types, EC cells from tumours were transplanted into blastocysts that were then re-implanted into the uterus of pseudo-pregnant female mice (Brinster 1974; Papaioannou *et al.* 1975; Mintz & Illmensee 1975). Under these conditions, the EC cells, which would typically form teratomas if transplanted to ectopic sites of an adult mouse, often appear to become normalized under the influence of the blastocyst and to participate in embryonic development giving rise to a range of normal tissues in the chimeric mice that subsequently develop.

In rare cases, it was reported that the germ cells of such chimeric mice also derived from the transplanted EC cells, but these observations have not been confirmed. Despite early indications to the contrary, it appears that many EC cells when transplanted into blastocysts do give rise to teratomas in the developing mice, so that their tumour characteristics are not completely suppressed by the host embryo. The latter result is, perhaps, not surprising as EC cells have generally adapted over many generations to growth either in culture or as a tumour in a host mouse. Because their differentiated derivatives usually have a limited lifespan and are non-malignant, one can easily imagine that the accumulation of mutations that inhibit differentiation would provide the EC cells with a selective advantage. When mouse EC cells are fused with somatic cells, notably thymocytes from adult mice, the resulting hybrid cells often exhibit the general features of EC cells (Miller & Ruddle 1976; McBurney 1977; Rousset *et al.* 1980; Gmür *et al.* 1980). However, in several cases, it has been noted that these hybrid EC cells show a greater capacity for differentiation than their EC parents (Andrews & Goodfellow 1980; Rousset *et al.* 1983). The simplest explanation for these observations is that the normal genome of the somatic cell introduces genes that are capable of complementing accumulated EC cell mutations that have tended to inhibit their ability to differentiate.

The recognition that EC cells are the malignant counterparts of embryonic ICM cells eventually resulted in the experiments of Evans & Kaufman (1981) and Martin (1981), who showed that it is possible to derive permanent lines of cells directly from mouse blastocysts, which closely resemble the EC cells derived from teratomas. They termed these cells ES cells. The normal cells to which these lines are thought to be equivalent, namely the cells of the late ICM, do not normally persist for any great length of time. The apparent ability of ES cells to grow indefinitely and exhibit an immortal characteristic, i.e. to present classical 'stem-cell features', seems to be a consequence of their removal from the embryo and maintenance in tissue culture. A further development, stimulated by the origins of testicular teratocarcinomas from primordial germ cells, was the finding that when such primordial germ cells are cultured *in vitro* they convert to cells, called embryonic germ (EG) cells, that closely resemble EC and ES cells (Matsui *et al.* 1992; Resnick *et al.* 1992).

5. HUMAN EC AND ES CELL LINES

Human teratocarcinoma cell lines were first isolated in the 1950s as xenografts in hamster cheek pouches (Pierce *et al.* 1957). Several lines were subsequently established *in vitro* during the 1970s, notably TERA1, TERA2 (Fogh & Trempe 1975) and SuSa (Hogan *et al.* 1977). Many of these human lines showed little capacity for differentiation, but they provided the basis for the identification of a number of characteristic features of human EC cells. Eventually, several human EC cell lines capable of differentiation were obtained, including: GCT27 (Pera *et al.* 1989; Roach *et al.* 1993, 1994; Pera & Herszfeld 1998), NCCIT (Teshima *et al.* 1988; Damjanov *et al.* 1993) and NCG.R3 (Hata *et al.* 1989; Umezawa *et al.* 1996) and, ironically, TERA2 (Andrews *et al.* 1984*b*), one of the oldest extant human teratocarcinoma cell lines. The pluripotent character of TERA2, and indeed its identity as an EC cell line, was overlooked for some time (e.g. see Andrews *et al.* 1980) because the specific culture conditions required to maintain pluripotent, undifferentiated human EC cells were not fully appreciated. Thus, in contrast to many mouse EC lines, it is necessary to maintain human EC cells at high cell densities, and it is generally best to passage TERA2 by scraping rather than by using trypsin to harvest the cells—retaining the cells in small clumps, which would be disrupted by trypsinization, seems to inhibit spontaneous differentiation. The widely used clonal subline of TERA2, NTERA2 cl.D1 (NT2/D1), was re-isolated after passage of a well-differentiated culture of TERA2 as a xenograft tumour in a nude mouse, which appeared to 'rescue' persisting EC cells within the culture (Andrews *et al.* 1984*b*).

Human and mouse EC cells differ significantly from one another, although they share some common features. For example, their morphology and growth patterns are similar, as both tend to grow in clusters of tightly packed cells with relatively little cytoplasm and prominent nucleoli. Also, both express high levels of alkaline phosphatase (Bernstine *et al.* 1973; Benham *et al.* 1981). However, the differences include a distinct pattern of surface antigen expression, as well as a propensity for human but not murine EC cells to differentiate into trophectoderm (Andrews *et al.* 1980, 1982, 1984*b*, 1996; Damjanov & Andrews 1983). The specific features of murine EC cells that differ from human EC cells, notably their expression of the lactoseries glycolipid antigen, stage-specific embryonic antigen 1 (SSEA1) (Kannagi *et al.* 1982; Gooi *et al.* 1981; Solter & Knowles 1978), their lack of expression of two globoseries glycolipid antigens, SSEA3 (Shevinsky *et al.* 1982) and SSEA4 (Kannagi *et al.* 1983), and their lack of ability to differentiate into trophectoderm, are all shared with murine ES cells, consistent with the idea that they resemble embryonic cells of the ICM and primitive ectoderm: although SSEA3 and SSEA4 are expressed by cleavage stage mouse embryos, the ICM lacks these antigens, but expresses SSEA1. Further, the ICM cells soon lose the capacity for trophectodermal differentiation. Thus, without the existence of human ES lines and without direct information from human embryos, the differences between human and mouse EC cells made the relationship of human EC cells to the early human embryo uncertain.

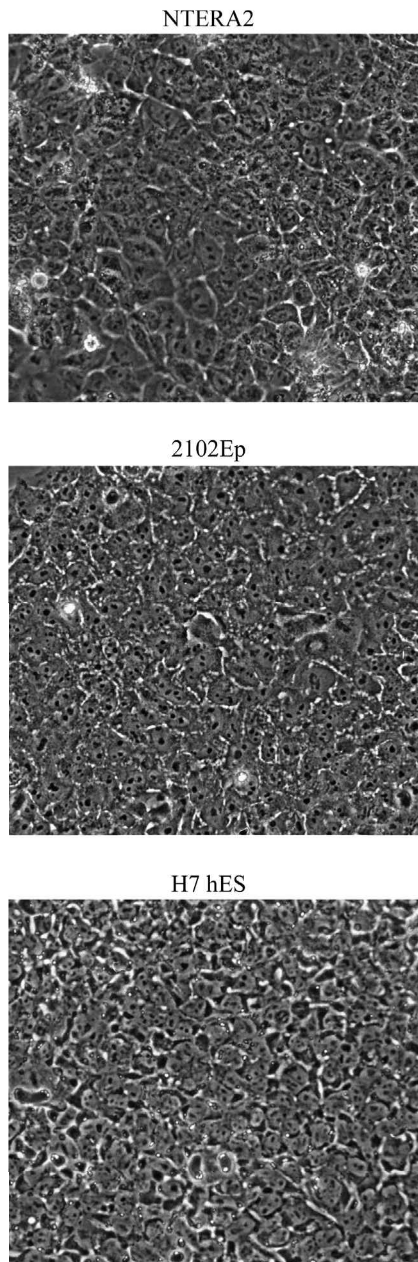

Figure 2. A comparison of the morphology of a human ES cell line, H7, provided by Dr James Thomson, and two human EC cell lines: NTERA2, capable of extensive differentiation (Andrews *et al.* 1984*b*), and 2102Ep, capable of only limited differentiation (Andrews *et al.* 1982). Note the tightly packed cells with little cytoplasm, pale nuclei and prominent nucleoli.

Logistical problems, as well as legal and ethical ones, delayed the derivation of ES cell lines from human embryos. However, rhesus monkey ES cell lines were first reported in 1995 (Thomson *et al.* 1995) and marmoset ES lines in 1996 (Thomson *et al.* 1996). These primate ES cells more closely resemble human EC cells than murine EC and ES cells. Eventually the derivation of human ES cell lines was reported by Thomson *et al.* (1998) and Reubinoff *et al.* (2000). These human ES cells also resemble the rhesus monkey ES cells and human EC cells (figure 2) rather than the murine cells—they are able to differentiate into trophoblast and are SSEA3(+), SSEA4(+), SSEA1(−). Also like human EC cells, but not those of the mouse, they express class 1 MHC antigens and Thy1 (Draper *et al.* 2002). Conversely, human ES cells do resemble the mouse cells in their expression of alkaline phosphatase and the stem-cell-specific transcription factor, *Oct4* (Yeom *et al.* 1996). Recently, we have confirmed that indeed the ICM of human blastocysts resembles human EC and ES cells in several of these features (Henderson *et al.* 2002), notably the expression of SSEA3 and SSEA4, the lack of SSEA1 and also the expression of two human cell-surface, keratan sulphate-related antigens, TRA-1-60 and TRA-1-81, which were previously defined on human EC cells (Andrews *et al.* 1984*a*; Badcock *et al.* 1999). Thus, there do appear to be significant differences in the biology of early embryonic cells of human and mouse embryos.

The precise significance of these differences remains to be resolved, especially as the function of the surface antigen markers, mostly involving carbohydrate epitopes, is unknown. Nevertheless, the regulation of these molecules is subject to tight control during differentiation; SSEA1 has been suggested to mediate compaction in mouse embryos (Bird & Kimber 1984; Fenderson *et al.* 1984), and a role for SSEA3 and SSEA4 in embryo pathology has been suggested (Tippet *et al.* 1986). SSEA3 and SSEA4 belong to the P-blood-group system and are expressed on red cells, except in individuals with the pp or p^k phenotypes. Women with these latter phenotypes are reported to be susceptible to recurrent early spontaneous abortions (Race & Sanger 1975); the expression of SSEA3 and SSEA4 by early embryonic cells suggests that these could be the target for a proposed immune response to the early embryos in such women. However, regardless of the functional significance of these particular differences between mouse and human embryos, the results show that lessons from the mouse cannot necessarily be extrapolated to human embryogenesis. They also suggest that human EC cells remain a useful complement of human ES cells for exploring the mechanisms that regulate human development; while ES cells are 'genetically normal' and differentiate into a wide range of cell types, EC cells are easier to grow and exhibit a simplified pattern that can be analysed more easily.

Human EG cells have also been described but, although pluripotent, they seem to differ from human EC and ES cells with respect to various surface antigen markers (Shamblott *et al.* 1998). However, the significance of these differences remains to be resolved.

6. DIFFERENTIATION OF HUMAN EC AND ES CELLS

Although EC cells are the stem cells of teratocarcinomas, a striking feature of many human EC cell lines is their lack of ability to differentiate into well-recognizable cell types. This might, in part, reflect their origins in tumours, as the acquisition of an inability to differentiate could provide a strong selective advantage. Human EC cells are highly aneuploid and it is easy to envisage that genetic changes might occur to inhibit their differentiation. Indeed, cell-hybrid studies with both mouse and human EC cells support this idea and suggest that a loss of pluripotency results from the loss of key gene functions

(Andrews & Goodfellow 1980; Duran *et al.* 2001). Nevertheless, several human EC cell lines can differentiate well and have been studied extensively. One line, GCT27, requires maintenance on feeder layers and differentiates into a wide range of cell types when removed from the feeders (Pera *et al.* 1989). Another human EC cell line, TERA2 and its NTERA2 sublines, does not require feeders and differentiates extensively, but in distinct directions, in response to retinoic acid (Andrews 1984), HMBA (Andrews *et al.* 1990) and the bone morphogenetic proteins (BMPs) (Andrews *et al.* 1994).

Apart from forming well-differentiated teratomas when grown as xenografts in nude mice, TERA2 and NTERA2 cells rapidly lose their EC phenotype and differentiate into a wide array of cell types, including neurons, after exposure to 10^{-5} or 10^{-6} M retinoic acid (Andrews 1984). This differentiation is marked by a switch in glycolipid synthesis from globoseries to lactoseries and ganglioseries structures (Fenderson *et al.* 1987), and can conveniently be followed by the loss of antigens, such as SSEA3 and SSEA4, as well as TRA-1-60 and TRA-1-81, and the acquisition of antigens, such as SSEA1, A2B5 (ganglioside GT3) and ME311 (ganglioside 9-*O*-acetyl GD3). There are also substantial changes in gene activity, most notably activation of the *HOX* genes in a retinoic acid concentration-dependent manner (Simeone *et al.* 1990), and the appearance of susceptibility to infection and replication of human cytomegalovirus (Gönczöl *et al.* 1984) and human immunodeficiency virus (Hirka *et al.* 1991), neither of which will grow in the undifferentiated EC cells.

As differentiation progresses, neural markers become evident, and neurons that express neurofilament proteins and a typically neuronal morphology appear (figure 3*a*) after one to two weeks (Andrews 1984; Lee & Andrews 1986). The pattern of expression of genes related to the early stages of neural differentiation during embryonic development—nestin in mitotic neural precursors and NeuroD in post-mitotic neuroblasts—seems to be followed by differentiating NTERA2 cells (Przyborski *et al.* 2000; Pleasure *et al.* 1992; Pleasure & Lee 1993). Furthermore, the terminal neurons are functional and express tetrodotoxin-sensitive sodium channels (Rendt *et al.* 1989), glutamate receptors and voltage-gated calcium channels (Squires *et al.* 1996). Several studies have recently been conducted of their potential for implanting into the central nervous system, initially in experimental rats in which NTERA2-derived neurons will apparently integrate functionally to correct neural defects, such as those resulting from stroke (Borlongan *et al.* 1998; Hurlebert *et al.* 1999; Kleppner *et al.* 1995; Philips *et al.* 1999). Experiments to implant these EC-cell-derived neurons into human stroke patients have also been reported (Kondziolka *et al.* 2000). The tumour origins of these neurons provokes some disquiet about safety, and whether their implantation will provide benefits to patients remains to be seen. Nevertheless, the experiments pave the way for future studies using neurons derived from genetically normal ES cells.

When differentiation of NTERA2 EC cells is induced by HMBA or BMP7, distinct differences from retinoic-acid-induced differentiation are seen (Andrews *et al.* 1990, 1994). Thus, ganglioside antigens are only expressed late, on a small proportion of cells, and few neurons are evident. The identity of many of the cells induced by HMBA and BMP has not been clearly defined, although smooth muscle actin was particularly noted after BMP induction. One idea we have considered is that NTERA2 differentiates predominantly in an ectodermal direction but that, depending upon the conditions, this can take a more 'dorsal' (predominantly neural) or 'ventral' (epidermal) character. By this notion, retinoic acid may favour a more dorsal pattern of differentiation, whereas HMBA and BMP may promote a more ventral pattern.

Not surprisingly, the embryo-derived human ES cells exhibit a considerably greater potential for differentiation. When grown in immunodeficient mice, these cells form well-differentiated teratomas with well-organized tissues representing all three germ layers (Thomson *et al.* 1998). When grown *in vitro*, as for mouse ES cells, the human ES cells require maintenance on feeder layers to prevent differentiation, but the cytokine LIF, which is able to prevent the differentiation of mouse ES cells in the absence of feeders, is apparently not able to do so for human ES cells. Conversely, it has been reported that conditioned medium from fibroblasts will inhibit differentiation of cells grown on Matrigel (Xu *et al.* 2001).

Many studies of differentiation in human ES cell cultures have focused upon allowing the cells first to form 'embryoid bodies' by culture in suspension; after a period of suspension culture, a variety of differentiated cells can be detected in the resulting cell clusters, which will grow out when allowed to attach. Under these conditions, neurons, glia, skeletal and cardiac muscle, liver and even insulin-secreting islet cells have been reported (Assady *et al.* 2001; Kaufman *et al.* 2001; Kehat *et al.* 2001; Itskovitz-Eldor *et al.* 2000; Odorico *et al.* 2001; Pera 2001; Schuldiner *et al.* 2000).

Differentiation also occurs in attached cultures, either spontaneously, or by induction with agents such as retinoic acid. Even when maintained on feeders, colonies of clearly non-ES cells can often be seen, and gene expression indicative of extra-embryonic cell types (e.g. chorionic-gonadotropin-indicating trophoblast and α-fetoprotein-indicating yolk sac) can be detected in the SSEA3(−) presumptive non-ES cells isolated by 'fluorescence-activated cell sorting' from such cultures (Henderson *et al.* 2002). These spontaneous differentiated derivatives can present a problem to continued maintenance of the ES lines if they are allowed proliferate, and culture techniques need to be adapted to remove them during passaging.

Retinoic acid, HMBA and DMSO also induce differentiation of attached cultures, whether in the presence or absence of feeders (Draper *et al.* 2002). Many of the changes seen during the differentiation of human EC cells induced by these agents are also seen in the human ES cultures. For example, all the typical EC/ES marker antigens, SSEA3, SSEA4, TRA-1–60, TRA-1-81, etc. are downregulated. Also, neural differentiation is common (figure 3*b*). However, although the induction of some of the antigens that appear during EC differentiation is seen, there are many differences in the patterns of antigens observed, probably because the ES cells are capable of a much wider range of differentiation.

Although it is clear that human ES cells are capable of extensive differentiation, as in the mouse, and although

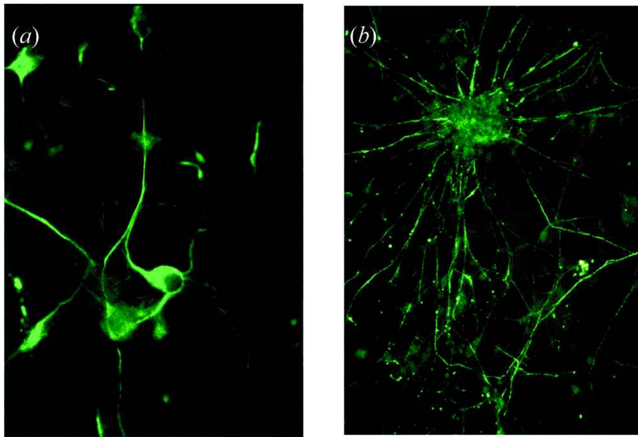

Figure 3. (*a*) Neurons differentiating from NTERA2 human EC cells induced with retinoic acid, and (*b*) a low-power view of an extended network of neurons in a culture of human ES cells (H7), also induced with retinoic acid. Both are stained with an antibody to the 200 kDa neurofilament protein.

various protocols are being developed to favour particular lineages of differentiation, we currently know very little of the mechanisms that regulate their decision to differentiate in the first place, or that regulate which lineages are followed once differentiation is initiated. Answers to these fundamental questions could provide valuable insights into a wide range of issues pertinent to human health, from the causes of abnormal embryonic development and the formation of birth defects, to understanding the relationships between cell proliferation and differentiation that underlie the development of cancer. Such answers are also crucial if ES cells are to realize their promise of providing tissues for transplantation and 'regenerative' medicine. Thus, efficient maintenance of ES cells suitable for use in human patients will demand culture conditions that minimize not only spontaneous, unwanted differentiation, but also the use of animal products, whether feeder cells, serum or growth supplements, which could introduce adventitious pathogens. Equally, although a wide range of differentiation is easy to obtain, particular cell types often only constitute a small proportion of a differentiated culture. Efficiency of production, apart from the need to avoid contamination with unwanted cell types that may be deleterious, demands that culture techniques are developed to promote differentiation in only specific directions.

Although murine EC and ES cells were originally derived with approaches to addressing such questions in mind, most of the use of mouse ES cell technology over the past 20 years has been directed towards production of transgenic mice, and not for answering questions of fundamental cell biology pertinent to ES cells *per se*. Nevertheless, some studies of the mechanisms that regulate mouse ES cell differentiation have been carried out. Most notable are studies that have highlighted the crucial role of specific discrete levels of *Oct4* expression for the maintenance of an undifferentiated phenotype (Niwa *et al.* 2000). Thus, if the level of *Oct4* expression in mouse ES cells is reduced, differentiation into trophectoderm ensues, whereas if levels are raised, differentiation to extra-embryonic endoderm is promoted. Furthermore, the maintenance of an undifferentiated phenotype depends upon activation of the STAT3 signalling pathway and inhibition of the MAPK/ERK pathway, following interaction of LIF with its receptor (Niwa *et al.* 1998; Burdon *et al.* 1999; Boeuf *et al.* 1997). However, as LIF does not appear to influence the behaviour of human ES cells (Thomson *et al.* 1998; Reubinoff *et al.* 2000), it remains uncertain to what extent these lessons will apply to human ES cells. It is possible that the STAT3 and MAPK pathways could be regulated by other receptor ligand systems; alternatively, other signalling pathways, yet to be identified, might perform similar functions in humans.

7. CONCLUDING THOUGHTS

A common notion that has prevailed in developmental biology for many years is one of cell differentiation during embryogenesis proceeding through a series of successive binary decisions by which cells adopt alternative phenotypes. Thus, embryogenesis is commonly seen in terms of cells following branching pathways of differentiation. Waddington (1956, 1966) has described such a process as 'canalization', envisaging cells moving 'downhill' through a series of valleys making up the 'epigenetic landscape' (figure 4*a*). A further notion that is often linked to this concept, although by no means essential, is that the process of differentiation is unidirectional and, in normal circumstances, irreversible. The branching pathways are seen as representing successive commitment of cells progressively to restricted options of eventual cell fate.

While the idea of 'de-differentiation', implying reversal of specific steps of differentiation, has often been discussed, particularly in the context of tumour biology, it has not found general favour. In the case of cancers that appear, for example, to express so-called 'oncofetal' proteins, it may be more appropriate to consider that it is the stem cells of specific tissues that are the target for carcinogenesis, and that it is their overgrowth that gives the appearance of reversion, or 'de-differentiation' (Pierce 1974). However, the recent reports of stem cells from different adult tissue, displaying quite unexpected plasticity and apparent lack of specific commitment, suggests that perhaps the concepts of unidirectional, irreversible differentiation along distinct cell lineages should be revised. Indeed, 'trans-differentiation', as in metaplasia, is well known to pathologists, while 'trans-determination' of imaginal disc cells in *Drosophila* was described many years ago by Hadorn (1968).

When considering the factors that regulate cell behaviour, whether commitment and determination, or differentiation, attention commonly focuses on individual signalling pathways by which cells respond to external cues, e.g. growth factors, the extracellular matrix, or interactions with other cells. To keep the analysis simple, such signalling pathways within a cell are often considered in isolation, and are also considered as simple switches—either 'on' or 'off'. However, any molecules within a signalling pathway will obey the normal chemical laws affecting reaction rates and equilibria. The activity of particular regulatory molecules will be influenced by the overall state of all the other regulatory and metabolic reactions taking place within the cell. Thus, the various signalling and

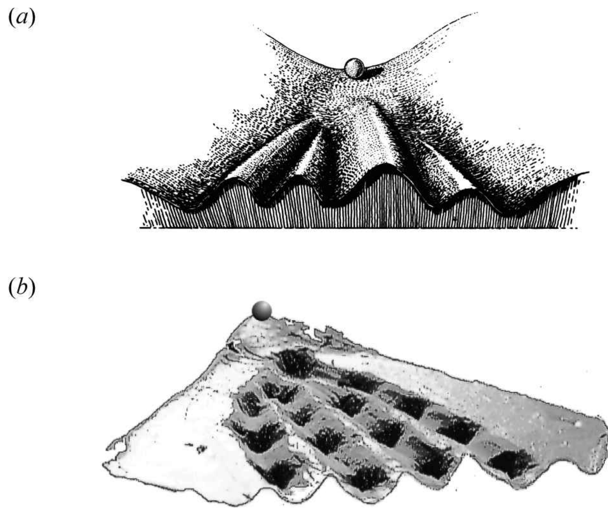

Figure 4. The 'epigenetic landscape': (*a*) a diagram reproduced from a drawing by C. H. Waddington (Waddington 1966) to illustrate his concept of 'canalization'. The image of a ball rolling downhill and selecting between different valleys represents the selection of different lineages by embryonic cells as they differentiate; if something were to push the ball up the side of a valley, it would nevertheless tend to return to the valley bottom, effectively a representation of regulation within the developing embryo. (*b*) A modification of the idea of Waddington: in this image the 'landscape' includes a series of depressions or hollows, which may nevertheless be arranged in valleys. The hollows would represent lowest free energy states, i.e. stable states, of the regulatory/metabolic network that makes up a cell. Movement of a cell from one state to another (differentiation) would tend to be from a stable state of higher free energy to one of lower free energy: the likelihood of such a transition would depend upon the height of the surrounding terrain—low barriers, say between successive hollows in a given valley, would permit a high frequency, or probability, of transition, whereas high barriers, say between neighbouring valleys, would permit transitions only infrequently. Thus, the height of the ground represents something akin to activation energy.

regulatory pathways, as well as the metabolic pathways of a cell, make up a complex, dynamic network in which various 'stable states' or equilibria would probably exist. These stable states, which would correspond to free energy minima for the network, would represent the various differentiated states that a cell could adopt. Under this view, it may be appropriate to modify the 'valleys' envisaged in Waddington's epigenetic landscape to a series of depressions (figure 4*b*), possibly still linked in 'valley systems', that would represent the commonly observed differentiation pathways. The height of the terrain would represent the free energy of the regulatory/metabolic network that constitutes the cells. Therefore the probability of a cell moving from one state to another would depend upon the heights of the hills surrounding the hollows and valleys. Cells finding themselves on the 'uplands' of 'unstable' or 'transition' states would tend to move to the nearest 'stable' state. In such a model, normally observed lineages represent transitions for which there is the highest probability of movement, hence the appearance of irreversibility. However, reversion, or de-differentiation, is entirely possible, although the probability may be low, as would movements from one set of hollows within one valley system to another valley system—trans-differentiation or trans-determination.

Waddington (1962) has indeed discussed the concept of stable states for cells and referred to mathematical models developed by Goodwin (1961). Slack (1991) has also discussed a similar idea. Perhaps a substantial challenge from the recent observations of stem-cell plasticity will be to develop a more detailed understanding of the chemistry underlying the complex network of signalling and metabolic pathways within a cell, to provide a more solid basis for such a concept of 'stable states' as opposed to fixed cell lineages. Certainly, considerable work lies ahead to understand the molecular mechanisms that regulate stem-cell differentiation, before their full potential for regenerative medicine can be realized.

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REFERENCES

Alison, M. R., Poulsom, R., Jeffery, R., Dhillon, A. P., Quaglia, A., Jacob, J., Novelli, M., Prentice, G., Williamson, J. & Wright, N. A. 2000 Hepatocytes from non-hepatic adult stem cells. *Nature* **406**, 257.

Amit, M., Carpenter, M. K., Inokuma, M. S., Chiu, C. P., Harris, C. P., Waknitz, M. A., Itskovitz-Eldor, J. & Thomson, J. A. 2000 Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* **227**, 271–278.

Andrews, P. W. 1984 Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. *Dev. Biol.* **103**, 285–293.

Andrews, P. W. & Goodfellow, P. N. 1980 Antigen expression by somatic cell hybrids of a murine embryonal carcinoma cell with thymocytes and L cells. *Somat. Cell Genet.* **6**, 271–284.

Andrews, P. W., Bronson, D. L., Benham, F., Strickland, S. & Knowles, B. B. 1980 A comparative study of eight cell lines derived from human testicular teratocarcinoma. *Int. J. Cancer* **26**, 269–280.

Andrews, P. W., Goodfellow, P. N., Shevinsky, L., Bronson, D. L. & Knowles, B. B. 1982 Cell surface antigens of a clonal human embryonal carcinoma cell line: morphological and antigenic differentiation in culture. *Int. J. Cancer* **29**, 523–531.

Andrews, P. W., Banting, G. S., Damjanov, I., Arnaud, D. & Avner, P. 1984*a* Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. *Hybridoma* **3**, 347–361.

Andrews, P. W., Damjanov, I., Simon, D., Banting, G., Carlin, C., Dracopoli, N. C. & Fogh, J. 1984*b* Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2: differentiation *in vivo* and *in vitro*. *Lab. Invest.* **50**, 147–162.

Andrews, P. W., Nudelman, E., Hakomori, S.-i. & Fenderson, B. A. 1990 Different patterns of glycolipid antigens are expressed following differentiation of TERA-2 human embryonal carcinoma cells induced by retinoic acid, hexa-

methylene bisacetamide (HMBA) or bromodeoxyuridine (BUdR). *Differentiation* **43**, 131–138.
Andrews, P. W., Damjanov, I., Berends, J., Kumpf, S., Zappavingna, V., Mavilio, F. & Sampath, K. 1994 Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by osteogenic protein-1 (or bone morphogenetic protein-7). *Lab. Invest.* **71**, 243–251.
Andrews, P. W. (and 15 others) 1996 Comparative analysis of cell surface antigens expressed by cell lines derived from human germ cell tumors. *Int. J. Cancer* **66**, 806–816.
Artzt, K., Dubois, P., Bennett, D., Condamine, H., Babinet, C. & Jacob, F. 1973 Surface antigens common to mouse cleavage embryos and primitive teratocarcinoma cells in culture. *Proc. Natl Acad. Sci. USA* **70**, 2988–2992.
Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K. L. & Tzukerman, M. 2001 Insulin production by human embryonic stem cells. *Diabetes* **50**, 1691–1697.
Badcock, G., Pigott, C., Goepel, J. & Andrews, P. W. 1999 The human embryonal carcinoma marker antigen TRA-1-60 is a sialylated keratan sulphate proteoglycan. *Cancer Res.* **59**, 4715–4719.
Bendel-Stenzel, M., Anderson, R., Heasman, J. & Wylie, C. 1998 The origin and migration of primordial germ cells in the mouse. *Semin. Cell Dev. Biol.* **9**, 393–400.
Benham, F. J., Andrews, P. W., Bronson, D. L., Knowles, B. B. & Harris, H. 1981 Alkaline phosphatase isozymes as possible markers of differentiation in human teratocarcinoma cell lines. *Dev. Biol.* **88**, 279–287.
Bernstine, E. G., Hooper, M. L., Grandchamp, S. & Ephrussi, B. 1973 Alkaline phosphatase activity in mouse teratoma. *Proc. Natl Acad. Sci. USA* **70**, 3899–3903.
Bird, J. M. & Kimber, S. J. 1984 Oligosaccharides containing fucose linked α(→) and α(→) to *N*-acetylglucosamine cause decompaction of mouse morale. *Dev. Biol.* **104**, 449–460.
Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C. & Vescovi, A. L. 1999 Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo*. *Science* **283**, 534–537.
Boeuf, H., Hauss, C., De Graeve, F., Baran, N. & Kedinger, C. 1997 Leukemia inhibitory factor-dependent transcriptional activation in embryonic stem cells. *J. Cell Biol.* **138**, 1207–1217.
Borlongan, C. V., Tajima, Y., Trojanowski, J. Q., Lee, V. M. & Sanberg, P. R. 1998 Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. *Exp. Neurol.* **149**, 310–321.
Brazelton, T. R., Rossi, F. M. V., Kekshet, G. I. & Blau, H. M. 2000 From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290**, 1175–1179.
Briggs, R. & King, T. J. 1952 Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc. Natl Acad. Sci USA* **38**, 455–463.
Brinster, R. L. 1974 The effect of cells transferred into the mouse blastocyst on subsequent development. *J. Exp. Med.* **140**, 1049–1056.
Burdon, T., Stracey, C., Chambers, I., Nichols, J. & Smith, A. 1999 Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Dev. Biol.* **210**, 30–43.
Cairns, J. 1975 Mutation selection and the natural history of cancer. *Nature* **255**, 197–200.
Campbell, K. H., McWhir, J., Richie, W. A. & Wilmut, I. 1996 Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**, 64–66.
Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon, F. A. & Ro, J. M. 1998 Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–1258.
Cibelli, J. B., Kiessling, A. A., Cunniff, K., Richards, C., Lanza, R. P. & West, M. D. 2001 Somatic cell nuclear transfer in humans: pronuclear and early embryonic development. *J. Regenerative Med.* **2**, 25–31.
Clarke, D. L., Johansson, C. B., Wilbertz, J., Veress, B., Nilsson, E., Karlström, H., Lendahl, U. & Frisén, J. 2000 Generalised potential of adult neural stem cells. *Science* **288**, 1660–1663.
Damjanov, I. 1990 Teratocarcinoma stem cells. *Cancer Surv.* **9**, 303–319.
Damjanov, I. 1993 Pathogenesis of testicular germ cell tumors. *Eur. Urol.* **23**, 2–7.
Damjanov, I. & Andrews, P. W. 1983 Ultrastructural differentiation of a clonal human embryonal carcinoma cell line *in vitro*. *Cancer Res.* **43**, 2190–2198.
Damjanov, I. & Solter, D. 1974 Experimental teratoma. *Curr. Top. Pathol.* **59**, 69–130.
Damjanov, I., Horvat, B. & Gibas, Z. 1993 Retinoic acid-induced differentiation of the developmentally pluripotent human germ cell tumor-derived cell line, NCCIT. *Lab. Invest.* **68**, 202–232.
Dixon, F. S. & Moore, R. A. 1952 Tumors of the male sex organs. In *Atlas of Tumor Pathology,* vol. 8, fasicles 31b & 32. Washington, DC, USA: Armed Forces Institute of Pathology.
Draper, J. S., Pigott, G., Thomson, J. A. & Andrews, P. W. 2002 Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J. Anat.* (In the press.)
Duran, C., Talley, P. J., Walsh, J., Pigott, C., Morton, I. & Andrews, P. W. 2001 Hybrids of pluripotent and nullipotent human embryonal carcinoma cells: partial retention of a pluripotent phenotype. *Int. J. Cancer* **93**, 324–332.
Einhorn, L. H. 1987 Treatment strategies of testicular cancer in the United States. *Int. J. Androl.* **10**, 399–405.
Evans, M. J. 1972 The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratoma cells. *J. Embryol. Exp. Morphol.* **28**, 163–176.
Evans, M. J. & Kaufman, M. H. 1981 Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.
Fenderson, B. A., Zehavi, U. & Hakomori, S. 1984 A multivalent lacto-*n*-fucopentaose III-lysylysine conjugate decompacts pre-implantation-stage mouse embryos, while the free-oligosaccharide is ineffective. *J. Exp. Med.* **160**, 1591–1596.
Fenderson, B. A., Andrews, P. W., Nudelman, E., Clausen, H. & Hakomori, S.-i. 1987 Glycolipid core structure switching from globo- to lacto- and ganglio-series during retinoic acid-induced differentiation of TERA-2-derived human embryonal carcinoma cells. *Dev. Biol.* **122**, 21–34.
Finch, B. W. & Ephrussi, B. 1967 Retention of multiple developmental potentialities by cells of a mouse testicular teratocarcinoma during prolonged culture *in vitro* and their extinction upon hybridisation with cells of permanent lines. *Proc. Natl Acad. Sci. USA* **57**, 615–621.
Fogh, J. & Trempe, G. 1975 New human tumor cell lines. In *Human tumor cells* in vitro (eds. J. Fogh). pp. 115–159. New York: Plenum.
Galli, R., Borello, U., Gritti, A., Minasi, M. G., Bjornson, C., Coletta, M., De Angelis, M. G., Fiocco, R., Cossu, G. & Vescovi, A. L. 2000 Skeletal myogenic potential of human and mouse neural stem cells. *Nature Neurosci.* **3**, 986–991.
Gmür, R., Solter, D. & Knowles, B. B. 1980 Independent regulation of H-2K and H-2D gene expression in murine teratocarcinomas somatic cell hybrids. *J. Exp. Med.* **151**, 1349–1358.
Gönczöl, E., Andrews, P. W. & Plotkin, S. A. 1984 Cyto-

megalovirus replicates in differentiated but not undifferentiated human embryonal carcinoma cells. *Science* **224**, 159–161.

Goodell, M. A. (and 10 others) 2001 Stem cell plasticity in muscle and bone marrow. *Ann. N.Y. Acad. Sci.* **938**, 208–218.

Goodwin, B. C. 1961 Studies in the general theory of development and evolution. PhD thesis, Edinburgh University.

Gooi, H. C., Feizi, T., Kapadia, A., Knowles, B. B., Solter, D. & Evans, M. J. 1981 Stage specific embryonic antigen involves α→ fucosylated type 2 blood group chains. *Nature* **292**, 156–158.

Greaves, M. F. 1982 'Target' cells, cellular phenotypes, and lineage fidelity in human leukaemia. *J. Cell. Physiol.* (*Suppl.*) **1**, 113–125.

Gurdon, J. B. 1962 The developmental capacity of nuclei taken from intestinal epithelial cells of feeding tadpoles. *J. Embryol. Exp. Morphol.* **10**, 622–640.

Hadorn, E. 1968 Transdetermination in cells. *Sci. Am.* **219**, 110–114.

Hata, J., Fujita, H., Ikeda, E., Matsubayashi, Y., Kokai, Y. & Fujimoto, J. 1989 Differentiation of human germ cell tumor cells. *Hum. Cell.* **2**, 382–387.

Henderson, J., Draper, J. S., Haillie, H. S., Fishel, S., Thomson, J. A., Moore, H. & Andrews, P. W. 2002 Preimplantation human embryos and embryonic stem cells show comparable expression of stage specific embryonic antigens. (Submitted.)

Hirka, G., Prakesh, K., Kawashima, H., Plotkin, S. A., Andrews, P. W. & Gönczöl, E. 1991 Differentiation of human embryonal carcinoma cells induces human immunodeficiency virus permissiveness which is stimulated by human cytomegalovirus coinfection. *J. Virol.* **65**, 2732–2735.

Hogan, B., Fellows, M., Avner, P. & Jacob, F. 1977 Isolation of a human teratoma cell line which expresses F9 antigen. *Nature* **270**, 515–518.

Hogan, B. L. M., Barlow, D. P. & Tilly, R. 1983 F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surv.* **2**, 115–140.

Hurlebert, M. S., Gianani, R. I., Hutt, C., Freed, C. R. & Kaddis, F. G. 1999 Neural transplantation of hNT neurons for Huntington's disease. *Cell Transplant.* **8**, 143–151.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H. & Benvenisty, N. 2000 Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. *Mol. Med.* **6**, 88–95.

Jackson, K. A., Mi, T. & Goodell, M. A. 1999 Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc. Natl Acad. Sci. USA* **96**, 14 193–14 195.

Jacob, F. 1978 Mouse teratocarcinoma and mouse embryo. *Proc. R. Soc. Lond.* B **201**, 249–270.

Jakob, H., Boon, T., Gaillard, J., Nicolas, J.-F. & Jacob, F. 1973 Tératocarcinoma de la souris: isolement, culture, et proprieties de cellules à potentialities multiples. *Ann. Microbiol. Inst. Pasteur* B **124**, 269–282.

Jakob, H., Dubois, P., Eisen, H. & Jacob, F. 1978 Effets de l'hexaméthylènebisacétamide sur la differenciation de cellules de carcinome embryonnaire. *C. R. Acad. Sci. Paris* **286**, 109–111.

Jones-Villeneuve, E. M., McBurney, M. W., Rogers, K. A. & Kalnins, V. I. 1982 Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94**, 253–262.

Kahn, B. W. & Ephrussi, B. 1970 Developmental potentialities of clonal *in vitro* cultures of mouse testicular teratoma. *J. Natl Cancer Inst.* **44**, 1015–1029.

Kannagi, R., Nudelman, E., Levery, S. B. & Hakomori, S. 1982 A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen, SSEA-1. *J. Biol. Chem.* **257**, 14 865–14 874.

Kannagi, R., Cochran, N. A., Ishigami, F., Hakomori, S.-i., Andrews, P. W., Knowles, B. B. & Solter, D. 1983 Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J.* **2**, 2355–2361.

Kaufman, D. S., Hanson, E. T., Lewis, R. L., Auerbach, R. & Thomson, J. R. 2001 Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc. Natl Acad. Sci. USA* **98**, 10 716–10 721.

Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J. & Gepstein, L. 2001 Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* **108**, 407–414.

Kleinsmith, L. J. & Pierce, G. B. 1964 Multipotentiality of single embryonal carcinoma cells. *Cancer Res.* **24**, 1544–1552.

Kleppner, S. R., Robinson, K. A., Trojanowski, J. Q. & Lee, V. M. 1995 Transplanted human neurons derived from a teratocarcinoma cell line (NTERA2) mature, integrate and survive for over 1 year in the nude mouse brain. *J. Comp. Neurol.* **357**, 618–632.

Kondziolka, D. (and 11 others) 2000 Transplantation of cultured human neuronal cells for patients with stroke. *Neurology* **55**, 565–569.

Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I. L. & Grompe, M. 2000 Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. *Nature Med.* **6**, 1229–1234.

Lajtha, L. G. 1979 Stem cell concepts. *Differentiation* **14**, 23–34.

Lee, V. M.-Y. & Andrews, P. W. 1986 Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. *J. Neurosci.* **6**, 514–521.

McBurney, M. W. 1977 Hemoglobin synthesis in cell hybrids formed between teratocarcinomas and Friend erythroleukemia cells. *Cell* **12**, 653–662.

McBurney, M. W., Jones-Villeneuve, E. M., Edwards, M. K. & Anderson, P. J. 1982 Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* **299**, 165–167.

McGrath, J. & Solter, D. 1984 Inability of mouse blastomere nuclei transfered to enucleated zygotes to support development *in vitro*. *Science* **226**, 1317–1319.

Martin, G. R. 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl Acad. Sci. USA* **78**, 7634–7636.

Martin, G. R. & Evans, M. J. 1974 The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture. *Cell* **2**, 163–172.

Martin, G. R. & Evans, M. J. 1975 Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. *Proc. Natl Acad. Sci. USA* **72**, 1441–1445.

Matsui, Y., Zsebo, K. & Hogan, B. L. 1992 Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**, 841–847.

Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. & McKercher, S. R. 2000 Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* **290**, 1779–1782.

Miller, R. A. & Ruddle, F. H. 1976 Pluripotent teratocarcinoma–thymus somatic cell hybrids. *Cell* **9**, 45–55.

Mintz, B. & Illmensee, K. 1975 Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc. Natl Acad. Sci. USA* **72**, 3585–3589.

Møller, H. 1993 Clues to the aetiology of testicular germ cell tumors from descriptive epidemiology. *Eur. Urol.* **23**, 8–15.

Mostofi, F. K. & Price, E. B. 1973 Tumours of the male genital system. In *Atlas of tumour pathology*, 2nd series, fasicle 8. Washington, DC, USA: Armed Forces Institute of Pathology.

Munsie, M. J., Michalska, A. E., O'Brien, C. M., Trounson, A. O., Pera, M. F. & Mountford, P. S. 2000 Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. *Curr. Biol.* **10**, 989–992.

Nicolas, J.-F., Dubois, P., Jakob, H., Gaillard, J. & Jacob, F. 1975 Tératocarcinome de la souris: différenciation en culture d'une lignée de cellules primitives à potentialities multiples. *Ann. Microbiol. Inst. Pasteur* A **126**, 3–22.

Niwa, H., Burdon, T., Chambers, I. & Smith, A. G. 1998 Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.* **12**, 2048–2060.

Niwa, H., Miyazaki, J. & Smith, A. G. 2000 Quantitiative expression of *Oct-3/4* defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genet.* **24**, 372–376.

Odorico, J. S., Kaufman, D. S. & Thomson, J. A. 2001 Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* **19**, 193–204.

Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. & Perry, A. C. 2000 Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289**, 1188–1190.

Papaioannou, V. E., McBurney, M. W., Gardner, R. L. & Evans, M. J. 1975 Fate of teratocarcinoma cells injected into early mouse embryos. *Nature* **258**, 70–73.

Pera, M. F. 2001 Human pluripotent stem cells: a progress report. *Curr. Opin. Genet. Dev.* **11**, 595–599.

Pera, M. F. & Herszfeld, D. 1998 Differentiation of human pluripotent teratocarcinomas stem cells induced by bone morphogenetic protein-2. *Reprod. Fertil. Dev.* **10**, 551–555.

Pera, M. F., Cooper, S., Mills, J. & Parrington, J. M. 1989 Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. *Differentiation* **42**, 10–23.

Peterson, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S. & Goff, J. P. 1999 Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168–1170.

Philips, M. F., Muir, J. K., Saatman, K. E., Raghupathi, R., Lee, V. M. Y., Trojanowski, J. Q. & McIntosh, T. K. 1999 Survival and integration of transplanted postmitotic human neurons following experimental brain injury in immunocompetent rats. *J. Neurosurg.* **90**, 116–124.

Pierce, G. B. 1974 Neoplasms, differentiations and mutations. *Am. J. Pathol.* **77**, 103–118.

Pierce, G. B., Verney, E. L. & Dixon, F. J. 1957 The biology of testicular cancer. I. Behaviour after transplantation. *Cancer Res.* **17**, 134–138.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M., Simonetti, D. W., Craig, S. & Marshak, D. R. 1999 Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.

Pleasure, S. J. & Lee, V. M. Y. 1993 NTERA-2 cells a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. *J. Neurosci. Res.* **35**, 585–602.

Pleasure, S. J., Page, C. & Lee, V. M.-Y. 1992 Pure, postmitotic, polarized human neurons derived from Ntera2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci.* **12**, 1802–1815.

Potten, C. S. & Lajtha, L. G. 1982 Stem cells versus stem lines. *Ann. N.Y. Acad. Sci.* **397**, 47–60.

Przyborski, S. A., Morton, I. E., Wood, A. & Andrews, P. W. 2000 Developmental regulation of neurogenesis in the pluripotent human embryonal carcinoma cell line NTERA-2. *Eur. J. Neurosci.* **12**, 3521–3528.

Race, R. R. & Sanger, R. 1975 The P blood groups. In *Blood groups in man*, 6th edn, pp. 169–171 Oxford: Blackwell Scientific.

Rendt, J., Erulkar, S. & Andrews, P. W. 1989 Presumptive neurons derived by differentiation of a human embryonal carcinoma cell line exhibit tetrodotoxin-sensitive sodium currents and the capacity for regenerative responses. *Exp. Cell Res.* **180**, 580–584.

Resnick, J. L., Bixler, L. S., Cheng, L. & Donovan, P. J. 1992 Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359**, 550–551.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A. & Bongso, A. 2000 Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nature Biotechnol.* **18**, 399–404.

Roach, S., Cooper, S., Bennett, W. & Pera, M. F. 1993 Cultured cell lines from human teratomas: windows into tumour growth and differentiation and early human development. *Eur. Urol.* **23**, 82–88.

Roach, S., Schmid, W. & Pera, M. F. 1994 Hepatocytic transcription factor expression in human; embryonal carcinoma and yolk sac carcinoma cell lines: expression of HNF3α in models of early endodermal cell differentiation. *Exp. Cell Res.* **215**, 189–198.

Rousset, J.-P., Jami, J., Dubois, P., Avilès, D. & Ritz, E. 1980 Developmental potentialities and surface antigens of mouse teratocarcinomas × lymphoid cell hybrids. *Somat. Cell. Genet.* **6**, 419–433.

Rousset, J.-P., Bucchini, D. & Jami, J. 1983 Hybrids between F9 nullipotent teratocarcinomas and thymus cells produce multidifferentiated tumors in mice. *Dev. Biol.* **96**, 331–336.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A. & Benvenisty, N. 2000 Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. Natl Acad. Sci. USA* **97**, 11 307–11 312.

Seale, P., Asakura, A. & Rudnicki, M. A. 2001 The potential of muscle stem cells. *Dev. Cell* **1**, 333–342.

Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumethal, P. D., Huggins, G. R. & Gearhard, J. D. 1998 Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl Acad. Sci. USA* **95**, 13 726–13 731.

Shevinsky, L., Knowles, B. B., Damjanov, I. & Solter, D. 1982 Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. *Cell* **30**, 697–705.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P. W., Boncinelli, E. & Mavilio, F. 1990 Sequential activation of human *HOX2* homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature* **346**, 763–766.

Skakkebaek, N. E. 1972 Possible carcinoma-*in-situ* of the testis. *Lancet* **ii**, 516–517.

Slack, J. M. W. 1991 Theoretical embryology. In *From egg to embryo*, 2nd edn, pp. 34–43 Cambridge University Press.

Sobis, H., Verstuyf, A. & Vandeputte, M. 1993 Visceral yolk sac-derived tumors. *Int. J. Dev. Biol.* **37**, 155–168.

Solter, D. & Knowles, B. B. 1978 Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl Acad. Sci. USA* **75**, 5565–5569.

Solter, D., Škreb, N. & Damjanov, I. 1970 Extrauterine growth of mouse egg-cylinders results in malignant teratoma. *Nature* **227**, 503–504.

Solter, D., Dominis, M. & Damjanov, I. 1979 Embryo-derived

teratocarcinomas. I. The role of strain and gender in the control of teratocarcinogenesis. *Int. J. Cancer* **24**, 770–772.

Solter, D., Dominis, M. & Damjanov, I. 1981 Embyro-derived teratocarcinoma. III. Development of tumors from teratocarcinoma-permissive and non-permissive strain embryos transplanted to F1 hybrids. *Int. J. Cancer* **28**, 479–483.

Squires, P. E., Wakeman, J. A., Chapman, H., Kumpf, S., Fiddock, M. D., Andrews, P. W. & Dunne, M. J. 1996 Regulation of intracellular Ca^{2+} in response to muscarinic and glutamate receptor antagonists during the differentiation of NTERA2 human embryonal carcinoma cells into neurons. *Eur. J. Neurosci.* **8**, 783–793.

Stevens, L. C. 1964 Experimental production of testicular teratomas in mice. *Proc. Natl Acad. Sci. USA* **52**, 654–661.

Stevens, L. C. 1967*a* The biology of teratomas. *Adv. Morphol.* **6**, 1–31.

Stevens, L. C. 1967*b* Origin of testicular teratomas from primordial germ cells in mice. *J. Natl Cancer Inst.* **38**, 549–552.

Stevens, L. C. 1970*a* Experimental production of testicular teratomas in mice of strains 129, A/He and their F1 hybrids. *J. Natl Cancer Inst.* **44**, 929–932.

Stevens, L. C. 1970*b* The development of transplantable teratocarcinomas from intratesticular grafts of pre and post implantation mouse embryos. *Dev. Biol.* **21**, 364–382.

Stevens, L. C. & Hummel, K. P. 1957 A description of spontaneous congential testicular teratomas in strain 129 mice. *J. Natl Cancer Inst.* **18**, 719–747.

Stevens, L. C. & Little, C. C. 1954 Spontaneous testicular teratomas in an inbred strain of mice. *Proc. Natl Acad. Sci. USA* **40**, 1080–1087.

Stevens, L. C. & Varnum, D. S. 1974 The development of teratomas from parthenogenetically activated ovarian mouse eggs. *Dev. Biol.* **37**, 369–380.

Stoter, G. 1987 Treatment strategies of testicular cancer in Europe. *Int. J. Androl.* **10**, 407–415.

Strickland, S. & Mahdavi, V. 1978 The induction of differentiation in teratocarcinomas stem cells by retinoic acid. *Cell* **15**, 393–403.

Strickland, S., Smith, K. K. & Marotti, K. R. 1980 Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* **21**, 347–355.

Temple, S. 2001 The development of neural stem cells. *Nature* **414**, 112–117.

Teshima, S., Shimosato, Y., Hirohashi, S., Tome, Y., Hayashi, I., Kanazawa, H. & Kakizoe, T. 1988 Four new human germ cell tumor cell lines. *Lab. Invest.* **59**, 328–336.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A. & Hearn, J. P. 1995 Isolation of a primate embryonic stem cell line. *Proc. Natl Acad. Sci. USA* **92**, 7844–7848.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P. & Hearn, J. P. 1996 Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. *Biol. Reprod.* **55**, 688–690.

Thomson, J. A., Itskovitz-Eldor, J., 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.

Tippett, P., Andrews, P. W., Knowles, B. B., Solter, D. & Goodfellow, P. N. 1986 Red cell antigens P (globoside) and Luke: identification by monoclonal antibodies defining the murine stage-specific embryonic antigens -3 and -4 (SSEA-3 and -4). *Vox Sanguinis* **51**, 53–56.

Umezawa, A., Maruyama, T., Inazawa, J., Imai, S., Takano, T. & Hata, J. 1996 Induction of mcl1/EAT, Bcl-2 related gene, by retinoic acid or heat shock in the human embryonal carcinoma cells, NCR-G3. *Cell Struct. Funct.* **21**, 143–150.

Waddington, C. H. 1956 *Principles of embryology*. London: Allen & Unwin.

Waddington, C. H. 1962 *New patterns in genetics and development*. Columbia University Press.

Waddington, C. H. 1966 *Principles of development and differentiation*. New York: Macmillan Co.

Wakayama, T., Perry, A. C., Zuccotti, M., Johnson, K. R. & Yanagimachi, R. 1998 Full-term development of mice from enucleated oocytes injected with cumulus nuclei. *Nature* **394**, 369–374.

Weiss, P. 1939 *Principles of development*. New York: Henry Holt.

Wheeler, J. E. 1983 History of teratomas. In *The human teratomas: experimental and clinical biology* (ed. I. Damjanov, B. B. Knowles & D. Solter). pp. 1–22. Clifton, NJ, USA: Humana.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. 1997 Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813.

Wilson, E. B. 1896 *The cell in development and inheritance*. New York: Macmillan Co.

Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D. & Carpenter, M. K. 2001 Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnol.* **19**, 971–974.

Yeom, Y. II, Fuhrmann, G., Ovitt, C. E., Brehm, A., Ohbo, K., Gross, M., Hubner, K. & Scholer, H. R. 1996 Germline regulator element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* **122**, 881–894.