REVIEW

Embryonic stem cells

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Abstract. Embryonic stem cells have huge potential in the field of tissue engineering and regenerative medicine as they hold the capacity to produce every type of cell and tissue in the body. In theory, the treatment of human disease could be revolutionized by the ability to generate any cell, tissue, or even organ, 'on demand' in the laboratory. This work reviews the history of murine and human ES cell lines, including practical and ethical aspects of ES cell isolation from pre-implantation embryos, maintenance of undifferentiated ES cell lines in the cell culture environment, and differentiation of ES cells *in vitro* and *in vivo* into mature somatic cell types. Finally, we discuss advances towards the clinical application of ES cell technology, and some of the obstacles which must be overcome before large scale clinical trials can be considered.

INTRODUCTION

Murine embryonic stem (ES) cells were first described over 20 years ago, when they were isolated from the inner cell mass of the developing blastocyst and grown *in vitro* (Evans & Kaufman 1981; Martin 1981). ES cells have since been shown to contribute to all cell lineages, including the germ line, when incorporated into chimeras with intact mouse embryos (Bradley *et al.* 1984; Nagy *et al.* 1990). *In vitro*, murine ES cells can be propagated indefinitely in the undifferentiated state, but retain the capacity to differentiate to all mature somatic phenotypes when induced by the appropriate signals. The initial isolation of murine ES cell lines in 1981 heralded a major breakthrough for developmental biology as it provided a simple model system to study the basic processes of early embryonic development and cellular differentiation. However, it also promised something else. If ES cells could be derived from human blastocysts, their capacity for multilineage differentiation might be exploited for cell-based therapies in which virtually any tissue or cell type could be produced 'to order' in the laboratory. This would provide a radical new approach to the treatment of a wide variety of diseases where organ damage or dysfunction exceeds the body's capability for natural repair. Human ES cells were eventually derived in 1998 (Thomson *et al.* 1998; Reubinoff *et al.* 2000), finally making regenerative

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medicine and tissue engineering a real possibility for the future treatment of human disease. This article reviews the current status of ES cell research, with particular emphasis on progress towards the development of therapies for human disease.

WHAT IS A STEM CELL?

Two properties are generally considered to define a stem cell, the capacity for long-term self-renewal without senescence and pluripotency, the ability to differentiate into one or more specialized cell types. These cells could therefore provide a theoretically inexhaustible supply of cells for transplantation. Totipotent stem cells, which have an ability to generate all tissue types, play a critical role in human development, providing the raw material for the development of all tissues and organs in the embryo and all the extra-embryonic tissues. However, tissue-specific stem cells are also deposited in various niches throughout the body, such as bone marrow, brain, liver and skin, as a mechanism for tissue maintenance, growth and repair in later life (Lavker & Sun 2000; Uchida *et al.* 2000; Vessey & de la Hall 2001; Wagers *et al.* 2002). These 'adult' stem cells were originally thought to be committed to regenerating only a very restricted set of cell lineages, however, it is becoming increasingly evident that they can show considerably more plasticity (Blau *et al.* 2001; Morrison 2001; Prockop *et al.* 2003). In theory, these cells could be harvested from a patient, differentiated in the laboratory and transplanted back into the same individual for tissue repair, thus bypassing the need for immunosuppression. However, for some stem cell types, low frequency, difficulties in accessing the niche and isolating the cells, restricted lineage potential and poor growth in cell culture may render their use impractical for tissue engineering purposes (Vogel 2001). In these cases, ES cells are likely to provide a more appropriate cell source.

EMBRYONIC STEM CELLS

The first pluripotent cell lines to be established were embryonic carcinoma (EC) cell lines, derived from the undifferentiated compartment of murine and human germ cell tumours (Finch & Ephrussi 1967; Andrews 2002). These cells could be expanded continuously in culture and could also be induced to differentiate into derivatives of all three embryonic germ layers; endoderm, ectoderm and mesoderm (Kleinsmith & Cochran 1964). Murine EC cells can also contribute extensively to all the normal tissues of chimaeric mice generated by the injection of EC cells into mouse blastocysts (Illmensee & Mintz 1976). However, being cancer-derived and usually aneuploid, EC cells are not suitable for clinical application, although they have proven to be a very useful model system in the laboratory. Murine ES cells were first isolated in 1981, using culture techniques based on experience with the culture of EC cells (Evans & Kaufman 1981; Martin 1981). ES cells are derived from the pre-implantation blastocyst, a hollow sphere of cells containing an outer layer of trophoblast cells which give rise to the placenta and the inner cell mass (ICM), from which ES cells are derived. Cells of the ICM ultimately go on to form the embryo proper and therefore have the capacity to form all the tissues in the body. Although these truly pluripotent cells are relatively short-lived in the embryo *in vivo*, they can be propagated indefinitely in culture in an undifferentiated state, by growth in the presence of leukaemia inhibitory factor (LIF) and/or on a feeder layer of murine embryonic fibroblasts (MEF) (Smith

et al. 1988; Williams *et al*. 1988). Murine ES cells have had an enormous impact on many fields of research over the last 20 years. In particular, ES cells can be used to reconstitute early mouse embryos (Bradley *et al*. 1984), and this has formed the basis of genome manipulation technology that has produced hundreds of 'knock out' and 'knock in' transgenic animals for the investigation of gene expression and regulation *in vivo* (Thomas & Capecchi 1987). ES cells also allow studies of the initial stages of mammalian development *in vitro*, without the need to harvest peri-implantation embryos, and continue to be used to dissect the basic mechanisms underlying pluripotency and cell lineage specification. Not surprisingly, significant efforts have been made to isolate ES cells from other species and, to date, ES cell lines are available from rodents (Evans & Kaufman 1981; Martin 1981; Doetschman *et al*. 1988; Iannaconne *et al*. 1994), rabbits (Graves & Moreadith 1993), pigs (Li *et al*. 2003), primates (Thomson *et al*. 1995, 1996) and, significantly, an ever-increasing number from humans (Thomson *et al*. 1998; Amit *et al*. 2000; Reubinoff *et al*. 2000; Richards *et al*. 2002; Hovatta *et al*. 2003; Mitalipova *et al*. 2003), (summarized in Orive *et al*. 2003).

The generation of human ES cell lines has sparked a great deal of controversy in the media, with particularly strong objections being raised to the use of human embryos in scientific research by certain religious communities (Annas *et al*. 1999; De Wert & Mummery 2003; Orive *et al*. 2003). Legislation governing the use of human embryos to produce ES cell lines varies between countries and, at the time of writing, is still under debate in many cases. The initial human ES cell lines were derived from 'spare' embryos produced by *in vitro* fertilization and donated with the informed consent of the parents (Anonymous 2002). This source is widely held to be the most acceptable source of embryos for research, the embryos being originally created for reproductive, not scientific, purposes. Many people consider it to be ethically superior to use these embryos for medical research from which the human population as a whole is likely to benefit, than to simply destroy them or store them indefinitely. An alternative method of deriving human ES cells is somatic nuclear transfer, or cloning, which bypasses the need to destroy an embryo produced 'naturally' by the fusion of sperm and egg, which could otherwise develop to term if implanted. This procedure was first described in sheep, where a somatic cell nucleus was transferred into an enucleated oocyte leading to apparently normal embryonic development in a proportion of cases (Campbell *et al*. 1996). Cloned embryos have since been generated in a number of species, using a variety of somatic cell types (McGrath & Solter 1983; Wilmut *et al*. 2002). The clinical development of regenerative medicine could be markedly expedited by the use of autologous cloned human embryos as it would circumvent any potential problems with the rejection of foreign cells, which remains the bane of transplantation medicine. However, the creation of a cloned human embryo specifically for the purposes of research is even more ethically loaded than the use of 'spare' embryos from IVF treatment. In all cases where animal embryos have been successfully cloned using this technique, a small proportion of embryos have survived and developed to become live young after implantation (Wilmut *et al*. 1997, 2002). The even remote possibility of generating a live human clone has been sufficient to ban the technique of somatic nuclear transfer from being applied to human cells in most western countries (Andorno 2002; Bosch 2002). Although there has been a single scientific report of the cloning of a human embryo (Cibelli *et al*. 2001), these results have met widespread scepticism as the embryos were only allowed to develop to the 6-cell stage, before nuclear DNA begins to regulate embryonic development (Stix 2001). However, a report of the cloning of non-human primate embryos suggests that cloning by nuclear transfer is technically possible in humans (Mitalipov *et al*. 2002), and ES cells have been recently developed by somatic nuclear transfer of human nuclei into rabbit oocytes (Chen *et al*. 2003). Although this latter work was carried out to avoid the use of human oocytes, which are in short supply for clinical use and difficult to obtain for

scientific research, the creation of a 'human-rabbit hybrid' added fuel to the fire of an already acrimonious debate. Nevertheless, the universally extreme inefficiency of somatic nuclear transfer in generating viable embryos (less than 1% development to blastocysts of cloned rhesus money embryos) is likely to render it impractical for human use, even if the cloning of human embryos were to become less morally dubious (Colman & Kind 2000).

Human ES cells show several important differences from murine ES cells in culture. They grow more slowly, tend to form flat rather than spherical colonies and are more easily dissociated into single cells than their mouse counterparts (Laslett *et al.* 2003). Human ES cells are also unresponsive to LIF and require culture on MEF feeder layers in the presence of basic fibroblast growth factor (Thomson *et al.* 1998; Amit *et al.* 2000; Reubinoff *et al.* 2000; Laslett *et al.* 2003), or on matrigel or laminin in MEF-conditioned medium (Xu *et al.* 2001). The two sources also differ in some antigenic phenotypes, for example, undifferentiated murine ES cells express the embryonic antigen SSEA-1A but not SSEA-3 and 4, but undifferentiated human ES cells have precisely the opposite phenotype (Thomson *et al.* 1998; Reubinoff *et al.* 2000). The demonstration of pluripotency for murine ES cells usually involves reconstitution of embryos and the generation of chimaeric mice, however, for human cells this test cannot be applied for obvious reasons. Instead, for human ES cells, the 'gold standard' test for pluripotency which has evolved is the demonstration that cells can differentiate into all three germ layers *in vivo* as teratomas when implanted into immunodeficient mice (Thomson *et al.* 1998; Amit *et al.* 2000; Reubinoff *et al.* 2000). A growing number of human ES cell lines are now available from several research groups and commercial companies, including some that have been clonally derived (summarized in Annas *et al.* 1999). For therapeutic purposes, it is unlikely that human ES cells grown on murine feeder layers, or in cell culture medium containing animal-derived products, will be acceptable due to the risk of transferring animal pathogens into the human population. For this reason, human ES cells are now beginning to be derived on human feeder cells, or without feeder layers entirely, under completely animal-free conditions (Richards *et al.* 2002; Amit *et al.* 2003; Hovatta *et al.* 2003; Richards *et al.* 2003).

DIFFERERENTIATION OF EMBRYONIC STEM CELLS *IN VITRO*

One of the most intriguing and important aspects of ES cell lines is their ability to differentiate into multiple mature somatic cell types in cell culture, presumably via precursor cells, when the appropriate stimuli are applied. The most common method for initiating differentiation in culture is the formation of 3-D spherical structures in suspension culture, termed embryoid bodies (EBs) (Fig. 1a) due to their similarity to post-implantation embryonic tissue *in vivo*. These aggregated structures contain derivatives of all three embryonic germ layers (Fig. 1b) (Abe *et al.* 1996; Leahy *et al.* 1999; Itskovitz-Eldor *et al.* 2000), and their formation is a prerequisite for the generation of most mature somatic cell lineages as ES cells differentiated in monolayer culture alone form large quantities of an endodermal-like cell which is identified by ES cell colonies flattening and developing a 'rough' appearance. It should be noted, however, that EB differentiation does not reconstitute the full array of embryonic development, having no form of polarity or 'body plan'. As such, ES cells cannot form viable human embryos. EB formation can be achieved in a number of ways following the withdrawal of LIF and/or the feeder layer (Keller 1995). Cells can be transferred to suspension culture at high density (Doetschman *et al.* 1995) or in methylcellulose-containing medium (Wiles & Keller 1991), to form EBs of a range of sizes and shapes; alternatively 'hanging drop' cultures provide a more controlled
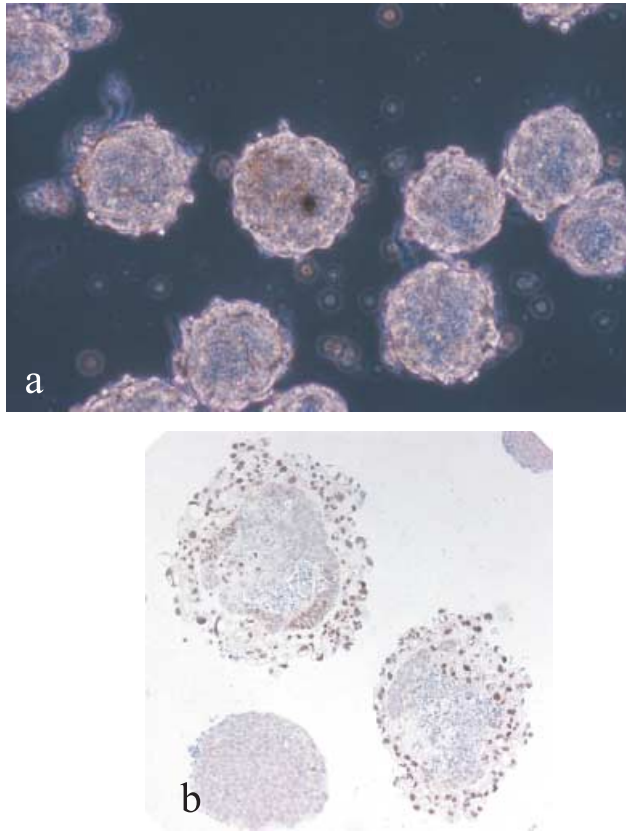
Figure 1. (a) Murine embryoid bodies, derived spontaneously from differentiating embryonic stem cells, floating in culture. **(b) Sections of murine embryoid bodies** after 8 days of differentiation immunostained for the transcription marker hepatocyte nuclear factor 3β, demonstrating the formation of endoderm on the outside of the bodies (ABC method).

method of generating single EBs from a defined cell number in individual droplets of culture medium (Wobus *et al*. 1991; Boheler *et al*. 2002). The length of EB culture time is dependent upon the ultimate target cell type and EB differentiation appears to correlate well temporally with the post-implantation development of embryos (Keller 1995). Mesodermal and ectodermal precursors form within a few days, whereas some endodermal cell types may benefit from more extended culture time (up to 10 days) to a stage where most EBs have cavitated and become cystic (Abe *et al*. 1996; Leahy *et al*. 1999).

Following the formation of EBs, they are returned to adherent culture conditions upon which specialized cells develop in the areas of outgrowth. Not surprisingly, progress in directing and characterizing the *in vitro* differentiation of murine ES cells is significantly more advanced than for human ES cells. One of the most visually impressive phenotypes of differentiated ES cells is the appearance of primitive cardiomyocytes in the culture, which can initially be observed as areas of 'twitching' in EBs after several days in suspension culture, and then later as large patches of synchronously contracting cells in adherent culture. Murine ES cell-derived cardiomyocytes have been shown to express tissue-specific markers, including structural cardiac proteins, cardiac receptors and cardiac transcription factors (summarized in Wobus 2001 and

Boheler *et al*. 2002). Within the population of contractile cells, proportions representing a variety of specialized functions of the heart have also been identified by electrophysiology, including atrial-like, ventricular-like, purkinje-like and nodal-like (Wobus 2001; Boheler *et al*. 2002). Preliminary data on engraftment of mouse ES cell-derived cardiomyocytes into mouse models has also indicated that these cells are capable of integrating into endogenous heart tissue, vascularizing, and continuing to differentiate *in vivo* (Klug *et al*. 1996; Johkura *et al*. 2003). Towards the production of cardiomyocytes for tissue engineering, work is now proceeding on techniques to scale-up the differentiation process (Zandstra *et al*. 2003), strategies to select out the appropriate cell type from the differentiated population (Muller *et al*. 2000), and cardiomyocytes are now being generated from human ES cells based on protocols optimized for murine ES cells (Kehat *et al*. 2001; He *et al*. 2003; Mummery *et al*. 2003). Tissue engineering of the heart is undoubtedly the aspect of regenerative medicine closest to a clinical application; however, there is also a word of warning. As well as issues surrounding immunocompatibility which will affect any cell transplantation technology, questions have also been raised about the differentiation state of ES cell-derived cardiomyocytes, which tend to recapitulate the phenotype of the fetal heart rather than adult cells (Doevendans *et al*. 2000; Fijnvandraat *et al*. 2003a). It has been suggested that implanting spontaneously beating cells may, in fact, cause serious cardiac arrhythmias which could not be corrected once the cells had engrafted into the endogenous heart tissue (Fijnvandraat *et al*. 2003b). ES cell-based therapies still have many hurdles to overcome before they can be transferred from the laboratory to the clinic, and it will be critical to validate all types of differentiated progeny of ES cells for the ability to integrate fully and functionally with host tissue.

Many other somatic cell types have also been derived from both murine and human ES cells. As well as cardiac differentiation, other mesodermal cell types that have been obtained from ES cells include chondrocytes (Kramer *et al*. 2000), adipocytes (Dani *et al*. 1997) and endothelial cells (Risau *et al*. 1988; Wang *et al*. 1994; Yamashita *et al*. 2000). In our own laboratory, a robust system has been developed for osteoblast differentiation, which involves dispersal of murine EBs into a culture medium designed for the maintenance and growth of explanted osteoblasts (Buttery *et al*. 2001). Differentiation is further augmented sevenfold by the addition of dexamethasone 14 days after EB dispersal, illustrating that, not only the stimulus itself, but also the time at which it is administered, can be significant. Osteoblasts have now also been derived at high yield from human ES cells using a similar protocol (Sottile *et al*. 2003).

Neuroectodermal differentiation is also relatively easy to achieve from murine ES cells and high yields of neuron-, astrocyte- and oligodendrocyte-like cells have been achieved with several, very different protocols (Stavridis & Smith 2003). These protocols have since been further simplified to remove the EB stage and differentiate murine and human ES cells into neuroectodermal lineages in monolayer, the only somatic cell type for which this is known to be possible (Reubinoff *et al*. 2001; Pachernik *et al*. 2002; Ying *et al*. 2003). Preliminary studies of the implantation of ES cell-derived neurons into mouse models have also indicated that these cells are functionally active (i.e. form synapses and can generate action potentials), integrate into the brain and, in at least one case, have corrected the phenotype of a neurodegenerative disease (Barberi *et al*. 2003; Benninger *et al*. 2003; Chiba *et al*. 2003).

As well as deriving osteoblasts, our laboratory has also focused on the differentiation of ES cells to alveolar epithelium. Lung tissue is formed from the definitive endoderm during embryogenesis, and the picture gradually emerging from ES cell research is that endodermal cell lineages are substantially more difficult to derive in significant yields than either ectoderm or mesodermal cell types. Using a similar approach to that which was used for osteoblast differentiation, we demonstrated that differentiated murine ES cell cultures could be enriched for type II alveolar epithelial-like cells by the application of SAGM (small airway growth medium), a
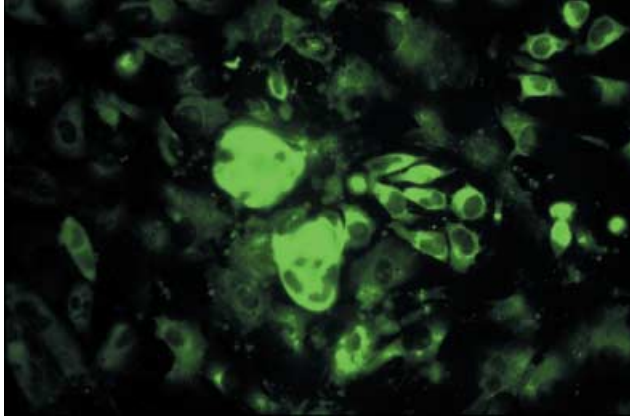
Figure 2. Clusters of cells derived from murine embryonic stem cells immunostained for surfactant protein C, a specific marker for type II pneumocytes (indirect immunofluorescence).

medium designed for the maintenance of primary distal lung epithelium in culture (Ali *et al*. 2002). Type II cells were identified by their expression of a cell-specific gene product, surfactant protein C (Fig. 2), and by the presence of a cell-specific organelle, lamellar bodies. However, despite the enrichment step, these cells were present only at very low frequency and work is ongoing to increase the yield of the target cell type by modulating the composition of the culture medium. Other endodermal cell types that have been obtained from ES cells include hepatocytes (Chinzei *et al*. 2002; Kuai *et al*. 2003; Rambhatla *et al*. 2003) and pancreatic islet cells (Lumelsky *et al*. 2001; Kim *et al*. 2003). However, progress in deriving endodermal lineages from ES cells is lagging significantly behind that of the other two germ layers at the present time.

The majority of the reports of ES cell differentiation *in vitro* have so far focused on differentiation of cells in adherent culture following EB formation. However, for many tissue engineering applications, the incorporation of differentiated cells into higher-order structures will be essential for implants to be functional, and the acquisition of an appropriate 3-D structure may also further direct the maturation and specialization of differentiated cell types. In general, differentiated progeny of ES cells have been difficult to sustain in 3-D culture due to problems in controlling cell proliferation and survival, and the presence of a highly mixed population of cell types. Recently, human ES cells have been differentiated on a polymeric scaffold designed for the support of complex tissue structures rather than via EB formation, and these were observed to form structures with the characteristics of neural tissues, cartilage and liver, as well as a network of blood vessel-like tubules (Levenberg *et al*. 2003). Organization of the structures could be enhanced by conditioning the scaffold with specific growth factors and by implantation into severe combined immunodeficient (SCID) mice, in which the constructs were observed to be viable for at least 2 weeks. Most intriguingly, in implanted constructs, the vessel-like structures were shown to contain intraluminal red blood cells, suggesting that they had anastamosed with the host vascular system. ES cells might therefore provide a means to generate histologically complete tissue constructs, which would not rely entirely on full vascularization by the host circulatory system alone for survival. In addition to the potential for transplantation, in the shorter term, such constructs could also provide extremely useful in vitro models to recapitulate the physiological state sufficiently for the evaluation of new drugs and identification of new therapeutic targets in tissue culture.

EMBRYONIC STEM CELLS FOR REGENERATIVE MEDICINE

Although the potential of ES cells in transplantation medicine is vast, before any clinical application of ES cells can succeed there are a number of obstacles that must be overcome. Firstly, no approach to the differentiation of ES cells has yet yielded a 100% pure population of mature progeny. It will be essential to avoid implanting undifferentiated ES cells or inappropriate cell lineages because of the risk of teratoma formation or further perturbation of tissue function, therefore there must be an efficient means to purify the required population. Methods such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) allow such purification but are dependent on the cell type of interest expressing a surface marker that can be recognized by a fluorescent or magnetic microbead-tagged antibody and, to be fully effective, the marker needs to be absolutely cell-type specific. In many cases, such a marker is not presently available, and sorting methods then rely on genetic modification of the ES cells with a marker gene under the control of a lineage-specific promoter. Alternatively, cells could be transduced with a drug-resistance gene instead of a marker, to allow for preferential selection of subpopulations.

Finally, as with all transplants, there is a risk that allogeneic ES cell-derived implants could be rejected by the host. Although the immunogenicity of the transplant can be contained through the lifelong use of immunosuppressive drugs, they are associated with many unpleasant side-effects and render the patient extremely susceptible to infection. As discussed, the production of autologous ES cells by somatic nuclear transfer is unlikely to provide a satisfactory solution, however, the amenability of ES cells to genetic modification provides a means to reduce their immunogenicity. This could be achieved by the insertion of immunosuppressive molecules such as Fas ligand, or by deleting immunoreactive molecules such B7 antigens (Walker *et al*. 1997; Harlan & Kirk 1999). More ambitiously, foreign major histocompatability complex (MHC) genes could be replaced by the recipient's MHC genes, increasing the immunocompatability of the cells.

CONCLUSIONS

The potential of human ES cells to generate any cell, tissue or organ 'on demand' for tissue repair or replacement promises to revolutionize the treatment of human disease. In the shorter term, 3-D ES cell-derived 'organ' cultures could be used for pharmacological testing, streamlining the drug-testing process and reducing the number of animals used, and also for the detailed mechanistic investigation of differentiation itself. However, although it is over 20 years since murine ES cells were first isolated, human ES cell technology is still in its infancy and many technical and ethical hurdles must be cleared before clinical trials can begin.

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