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Immortalized feeders for the scale-up of human embryonic stem cells in feeder and feeder-free conditions

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Abstract

Human embryonic stem cells (hESC) are pluripotent cells that proliferate indefinitely in culture, whilst retaining their capacity for differentiation into different cell types. However, hESC cultures require culture in direct contact with feeder cells or conditioned medium (CM) from feeder cells. The most common source of feeders has been primary mouse embryonic fibroblast (MEF). In this study, we immortalized a primary MEF line with the E6 and E7 genes from HPV16. The immortal line, ΔE-MEF, was able to proliferate beyond 7–9 passages and has an extended lifespan beyond 70 passages. When tested for its ability to support hESC growth, it was found that hESC continue to maintain the undifferentiated morphology for >40 passages both in co-culture with ΔE-MEF and in feeder-free cultures supplemented with CM from ΔE-MEF. The cultures also continue to express the pluripotent markers, Oct-4, SSEA-4, Tra-1-60, Tra-1-81, alkaline phosphatase and maintain a normal karyotype. In addition, these hESC formed teratomas when injected into SCID mice. Lastly, we demonstrated the feasibility of scaling-up significant quantities of undifferentiated hESC (>10^8 cells) using ΔE-MEF in cell factories. The results from this study suggest that immortalized feeders can provide a consistent and reproducible source of feeders for hESC expansion and research.

Keywords: Human embryonic stem cells; Immortalized feeders; Undifferentiated

1. Introduction

Since the successful isolation of human embryonic stem cells (hESC) from the inner cell mass of pre-implantation embryos, it is now possible to culture undifferentiated hESC indefinitely in vitro and thereby potentially provide a starting source of material that can be differentiated into cells from the three embryonic germ layers to be used in regenerative therapy (Thomson et al., 1998). Conventionally, hESC are maintained directly on feeder layers as co-cultures and these feeders have been derived either from mouse or human sources (Amit et al., 2003; Choo et al., 2004; Hovatta et al., 2003; Reubinoff et al., 2000; Richards et al., 2002, 2003). More recently, hESC have been successful cultured under feeder-free conditions. Here,

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the cells are grown on extracellular matrices, such as matrigel and supplemented with conditioned medium (CM) from feeder layers (Carpenter et al., 2004; Xu et al., 2001). Regardless of the source, feeders are derived from primary tissues and hence have a limited lifespan in culture. For example, primary feeders can only be cultured for approximately seven to nine passages before the cells senesce. Hence, fresh batches of feeders have to be prepared on a routine basis, which may result in batch to batch variation. Furthermore, there are also concerns that pathogens may be transmitted from the feeders to the hESC.

One possible solution to obtaining a sustainable, validated and consistent source of feeders for the culture of hESC, especially for scale-up, is the immortalization of primary feeders. Several methods have been previously used for immortalization and these include the transduction of normal cells with genes from DNA tumor viruses such as simian virus 40 (SV40), Epstein–Barr virus (EBV) and human papillomavirus (HPV) (Bryan and Reddel, 1994; Gudjonsson et al., 2002; Jha et al., 1998; Sugimoto et al., 1999; Yamamoto et al., 2003). More recently, groups have also reported that the over-expression of the human telomerase reverse transcriptase, hTERT was also able to extend the lifespan of somatic or differentiated cells (Bodnar et al., 1998; Xu et al., 2004).

In this study, we investigated whether we could extend the proliferative capacity of primary MEF after infection with retrovirus vectors encoding for the E6 and E7 genes from HPV16 beyond its normal lifespan in vitro. The over-expression of the E6 protein causes the degradation of the p53 tumor suppressor protein, as well as the up-regulation of c-myc expression and the activation of telomerase (Klingelhutz et al., 1996). On the other hand, expressed E7 protein binds to the retinoblastoma protein, pRb resulting in the degradation of pRb (Boyer et al., 1996). It has previously been shown that the expression of both E6 and E7 can efficiently immortalize normal human fibroblast, mammary epithelial cells and foreskin keratinocytes (Gudjonsson et al., 2002; Halbert et al., 1991; Shiga et al., 1997). Here, we successfully immortalized primary MEF with the over-expression of the E6 and E7 antigens. The immortalized MEF, ΔE-MEF, continued to proliferate in vitro beyond 70 passages and this did not result in the cells gaining any tumorigenic phenotype. In addition, three hESC lines previously grown on primary MEF either on feeders or feeder-free conditions readily adapted to ΔE-MEF. Morphologically, the hESC remained undifferentiated and continued to express both intracellular and extracellular markers characteristic of pluripotency. The hESC cultures also retained normal karyotype and formed teratomas in SCID mouse models.

2. Materials and methods

2.1. Cell culture

Human embryonic stem cell lines, HES-2 (46 X,X), HES-3 (46 X,X) and HES-4 (46 X,Y) were obtained from ES Cell International. The cells were cultured at 37 °C/5% CO_2 either on mitomycin-C inactivated feeders ($\sim 4 \times 10^4$ cells cm^{-2}) in gelatin-coated organ culture dishes (co-cultures) or on matrigel-coated organ culture dishes supplemented with CM from feeders (feeder-free cultures). Medium used for culturing hESC was KNOCKOUT (KO) medium, which contained 85% KO–DMEM supplemented with 15% KO serum replacer, 1 mM L-glutamine, 1% non-essential amino acids and 0.1 mM 2-mercaptoethanol and 4–8 ng ml^{-1} of basic fibroblast growth factor (Invitrogen). Medium was changed daily and the cultures were passaged weekly following enzymatic treatment as previously described (Choo et al., 2004). Culture dishes for feeder-free cultures were incubated at 4 °C overnight with matrigel (Becton Dickinson) diluted in cold KO–DMEM (1:30 dilution).

2.2. Preparation of MEF and MEF conditioned medium

Primary MEF were isolated from the fetuses of 129X1/SvJ mice (day 13.5 post-coitum) using the methods described by Robertson (1987). Monolayers of primary MEF (passage 4) were cultured to confluency and treated with 10 μg ml^{-1} mitomycin-C for 2.5–3 h. Following treatment, cells were detached with 0.25% trypsin–EDTA and seeded onto organ culture dishes as described above in F-DMEM medium. This medium consists of 90% DMEM high glucose, 10% FBS, 2 mM L-glutamine, 25 U ml^{-1} penicillin and 25 μg ml^{-1} streptomycin (Invitrogen). Culture medium was changed to KO medium 24 h after