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# Cytotoxic antibody fragments for eliminating undifferentiated human embryonic stem cells

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ABSTRACT

Human embryonic stem cells (hESC) possess great potential for applications in regenerative medicine due to their ability to differentiate into any cell type in the body. However, it is crucial to remove residual undifferentiated hESC from the differentiated population to avoid teratoma formation *in vivo*. The monoclonal antibody, mAb 84, has been shown to bind and kill undifferentiated hESC and is very useful for the elimination of contaminating undifferentiated hESC prior to transplantation. As mAb 84 is an IgM, its large size may impede penetration into embryoid bodies (EB) or cell clumps. To improve penetration, four antibody fragment formats of mAb 84 were engineered and expressed in *Escherichia coli*: Fab 84, scFv 84, scFv 84-diabody and scFv 84-HTH. All 4 fragments bound specifically to hESC, but only scFv 84-HTH, a single chain variable fragment with a dimerizing helix–turn–helix motif, could recapitulate the cytotoxicity of mAb 84 on multiple hESC lines. The results suggest that multivalency and flexibility between the antigen-binding sites may be essential features required for killing of hESC by mAb 84 and its derivatives. Imaging of EB treated with scFv 84-HTH or mAb 84 showed an even distribution of scFv 84-HTH throughout the EB whereas mAb 84 was localized more to the periphery.



## 1. Introduction

The use of human embryonic stem cells (hESC) as a starting source to derive differentiated cells holds enormous potential for applications in cell therapy and toxicology screening (Arenas, 2010; Teo and Vallier, 2010; Urbaniak et al., 2010). Following differentiation of hESC to a specific cell lineage, the removal of residual undifferentiated stem cells from the differentiated population is essential as these undifferentiated cells may form teratomas after *in vivo* transplantation (Hentze et al., 2007).

The use of magnetic separation technology relying on hESC-specific surface antigens to separate undifferentiated stem cells from the differentiated population has been reported with hESC retention efficacies around 81–83% (Fong et al., 2009; Hill et al., 2010). Hentze et al. demonstrated that as few as 2 hESC colonies (approximately 30,000 hESC/colony) implanted into SCID (severe combined immunodeficiency) mice resulted in teratoma formation. When the clumps were trypsinized to single cell suspensions before injection, 245 cells were sufficient to form teratomas after 10 weeks (Hentze et al., 2009). Hence, magnetic separation alone may not be sufficient to achieve the level of cell removal required.

The cytotoxic monoclonal antibody, mAb 84, was previously demonstrated to effectively eliminate teratoma formation by undifferentiated hESC in a SCID mouse model (Choo et al., 2008). Single cell suspensions of hESC pre-treated with mAb 84 *in vitro* before transplantation into mice did not form tumors even after 18 weeks. In contrast, transplantation of untreated hESC resulted in palpable tumor formation by 7 weeks (Choo et al., 2008). Hence, a combination of cell surface antigen-based magnetic cell separation followed by mAb 84 treatment could potentially be part of a purification scheme to eliminate residual undifferentiated stem cells from single cell suspensions.

However, the derivation of certain cell lineages requires differentiation from embryoid bodies or cell clumps rather than single cells, for example, cardiomyocytes (Graichen et al., 2008) and neural progenitors (Banin et al., 2006). In these cases, the elimination of residual undifferentiated stem cells within the cell clumps will be challenging since accessibility of large molecules may be limited. As mAb 84 is an IgM (MW ~ 960 kDa), the large size of the intact antibody may impede penetration into the differentiated cell clump. This is similar to situations where solid tumors were resistant to treatment with intact antibodies because of poor penetration (Goel et al., 2001; Thurber et al., 2008).

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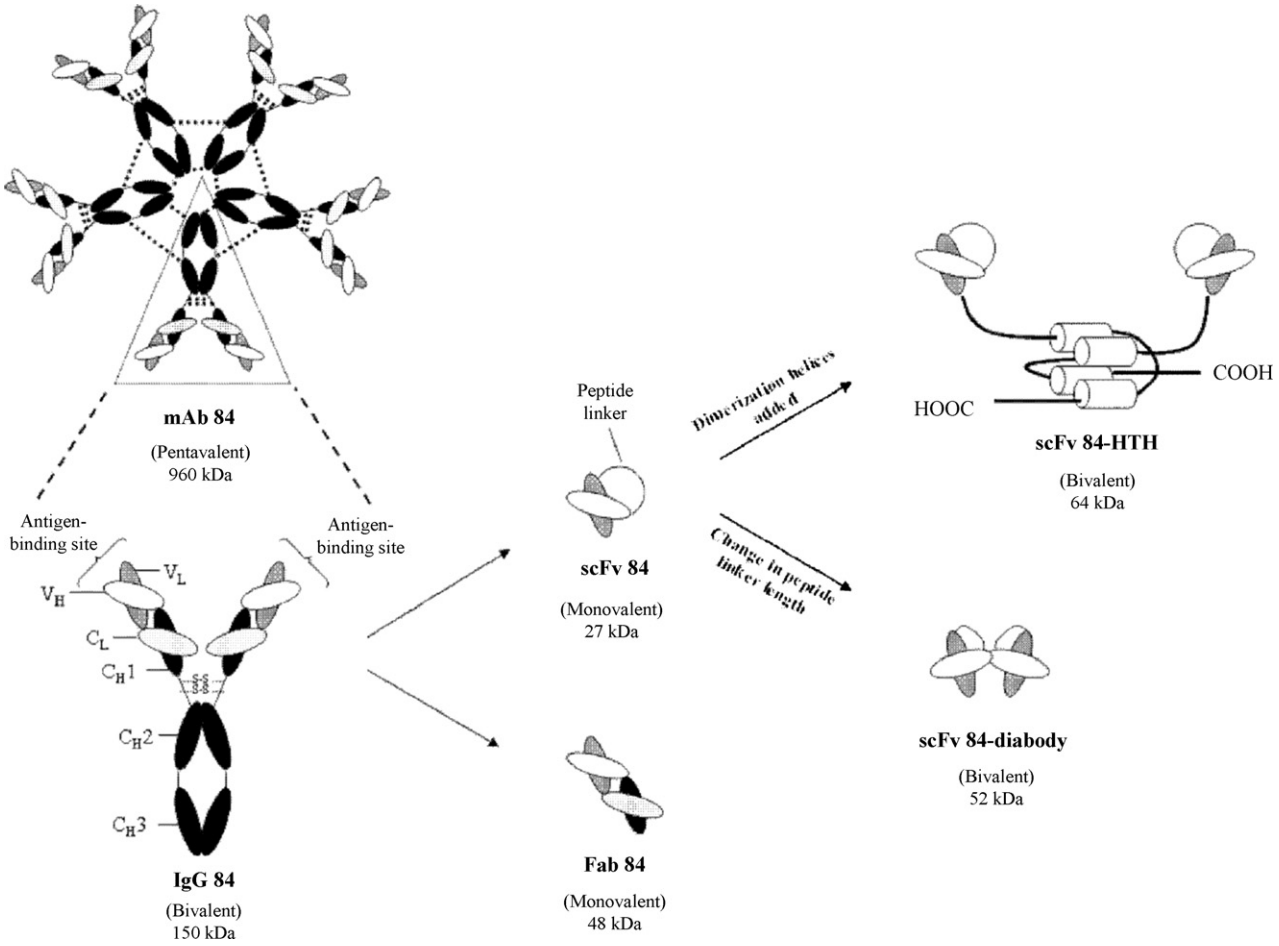


**Fig. 1.** Antibody fragment formats generated for this study.

A strategy that has been successful in overcoming the limited tumor penetration of intact antibodies is to engineer recombinant antibody fragments. In a number of studies, improved tumor uptake was observed for the smaller size fragments compared to whole antibodies (Goel et al., 2001; Wu and Senter, 2005). A spectrum of antibody fragment formats, including multivalent or bispecific antibodies, have been successfully developed for therapeutic and imaging applications (Enever et al., 2009; Holliger and Hudson, 2005). In an attempt to improve penetration into embryoid bodies and cells clumps, 4 antibody fragment variants of mAb 84 were engineered in this study (Fig. 1). Besides the monovalent binding fragments, Fab 84 (fragment antigen-binding) and scFv 84 (single chain variable fragment), two bivalent fragments were also constructed. scFv 84-diabody has a short 5 amino acid linker between the heavy and light variable chain compared to the 15 amino acid linker in scFv 84. The shorter linker limits intra-molecular association between the heavy and light chains within the molecule and forces inter-molecular association to form dimeric structures with the antigen-binding sites on the 2 ends (Holliger et al., 1993). In contrast, scFv 84-HTH dimerizes via a helix–turn–helix motif at the C-terminus of the scFv. In addition, a flexible linker composed of the upper hinge region of a mouse IgG3 connects the scFv to the helix–turn–helix motif. This antibody format has been termed "miniantibody" (Pluckthun and Pack, 1997; Willuda et al., 2001). We show that though all 4 antibody fragments retained specific binding to hESC, only scFv 84-HTH was cytotoxic to multiple hESC lines without affecting differentiated cells, thus recapitulating the unique characteristic of the parental IgM, but with a significantly reduced size.

## 2. Materials and methods

### 2.1. Cell culture and media

#### 2.1.1. Human embryonic stem cells

Human embryonic stem cell lines, HES-2 (46, XX) and HES-3 (46, XX) were obtained from ES Cell International (ESI, Singapore). Cells were cultured at 37 °C/5% $CO_2$ on Matrigel-coated culture dishes supplemented with condition media (CM) from immortalized mouse feeders, ΔE-MEF (Choo et al., 2006). Medium used for culturing hESC was KnockOut (KO) medium containing 85% KO-DMEM (DMEM, Dulbecco's modified Eagle's medium) supplemented with 15% KO serum replacer, 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM 2-mercaptoethanol and 4 ng/mL fibroblast growth factor-2 (Invitrogen, Carlsbad, CA).

#### 2.1.2. IMR90 fibroblasts

IMR90 fibroblasts (ATCC No: CCL-186) was cultured in medium consisting of 90% DMEM high glucose, 10% FBS, 2 mM L-glutamine, 25 U/ml penicillin and 25 μg/ml streptomycin (Invitrogen). Upon confluency, cells were washed in 1× phosphate-buffered saline (PBS) (Final conc: 137 mM NaCl, 2.7 mM KCl, 10 mM Phosphate Buffer) (1st Base, Singapore), detached with 0.25% trypsin–ethylene-diamine tetraacetic acid (EDTA), and re-plated at a ratio of 1:4.