Materials and Methods

Mouse ES cell culture. R1 mouse ES cells were cultured according to previously described methods [1]. All cell culture reagents were obtained from Invitrogen, unless otherwise noted. Undifferentiated ES cells were maintained feeder-free on gelatin-coated dishes with Dulbecco's Modified Eagle Media (DMEM) media supplemented with 15% fetal bovine serum (FBS, Schenk), 2 mmol/L L-glutamine, 0.1 mmol/L non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.15 mmol/L monothioglycerol (MTG, Sigma) and 1000 U/ml leukemia inhibitory factor (LIF, Chemicon). The cells were passaged routinely at 60-70% confluence, and differentiation was induced by placing the cells in suspension at 7×10^5 cells/ml in 100 mm bacteriological Petri dishes in media lacking LIF and MTG, whereupon they form embryoid bodies (EBs). The EBs were re-fed every other day, and after 6-7 days in suspension culture, plated onto gelatin-coated substrates and EB outgrowths were cultured for up to several weeks thereafter.

At various time points during differentiation, cultures were pulsed with 5-bromo-2-deoxyuridine (BrdU, 10 μmol/L) for 2 hours and outgrowths were scraped, pelleted, and fixed with ice-cold methanol. Fixed samples were processed for paraffin embedding and sections of EB pellets were analyzed by immunohistochemical methods (described below) to detect BrdU+ cardiomyocytes.

Human ES cell culture. Human ES cells were cultured according to previously established techniques [2]. In brief, undifferentiated H1 or H7 human ESCs between passages 28 and 53 were cultured with mouse embryonic fibroblast (MEF)-conditioned media on substrates coated with Matrigel (Becton Dickinson) in the absence of feeder layers, re-fed daily and passaged

weekly at a 1:6 - 1:12 split. The media conditioned by the MEFs consists of 80% Knockout DMEM (KO-DMEM), 20% serum replacement, 1% non-essential amino acids, 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol (Sigma) and 4 ng/ml of basic fibroblast growth factor. Differentiation cultures were established by treating the adherent ES cells with 200 U/ml collagenase for 10 minutes at 37°C and detached cell clusters were maintained in suspension culture in non-adherent dishes to form EBs. The differentiation medium consists of 80% KO-DMEM, 1 mmol/L L-glutamine, 1% non-essential amino acids, and 20% FBS (Biowhitaker). EBs were plated onto gelatin-coated substrates after 4 days in suspension and adherent outgrowths were re-fed every other day thereafter with differentiation media. In some instances, human EB cultures were pulsed with BrdU, fixed, processed and analyzed by immunohistochemistry, similarly to the methods described for mouse EB cultures described above.

Percoll enrichment. In order to isolate an enriched fraction of cardiomyocytes, the differentiating EB outgrowths were gently dissociated with 0.56 U/ml of Blendzyme (Roche) in PBS and separated over a discontinuous Percoll gradient. The Percoll gradient was formed by pouring a 40.5% layer of Percoll on top of a 58.5% Percoll layer and the cells were fractionated by centrifugation at 1500 g for 30 minutes. Fractions containing an enriched population of cardiomyocytes (fraction IV – 58.5% Percoll) were re-plated and cultured. This procedure, based on previous methods used to purify cardiomyocytes from mouse [3] and human ES cells [4], typically yielded enriched populations of human cardiomyocytes with 10-30% purity based on immunostaining for sarcomeric actin or myosin heavy chain.

References

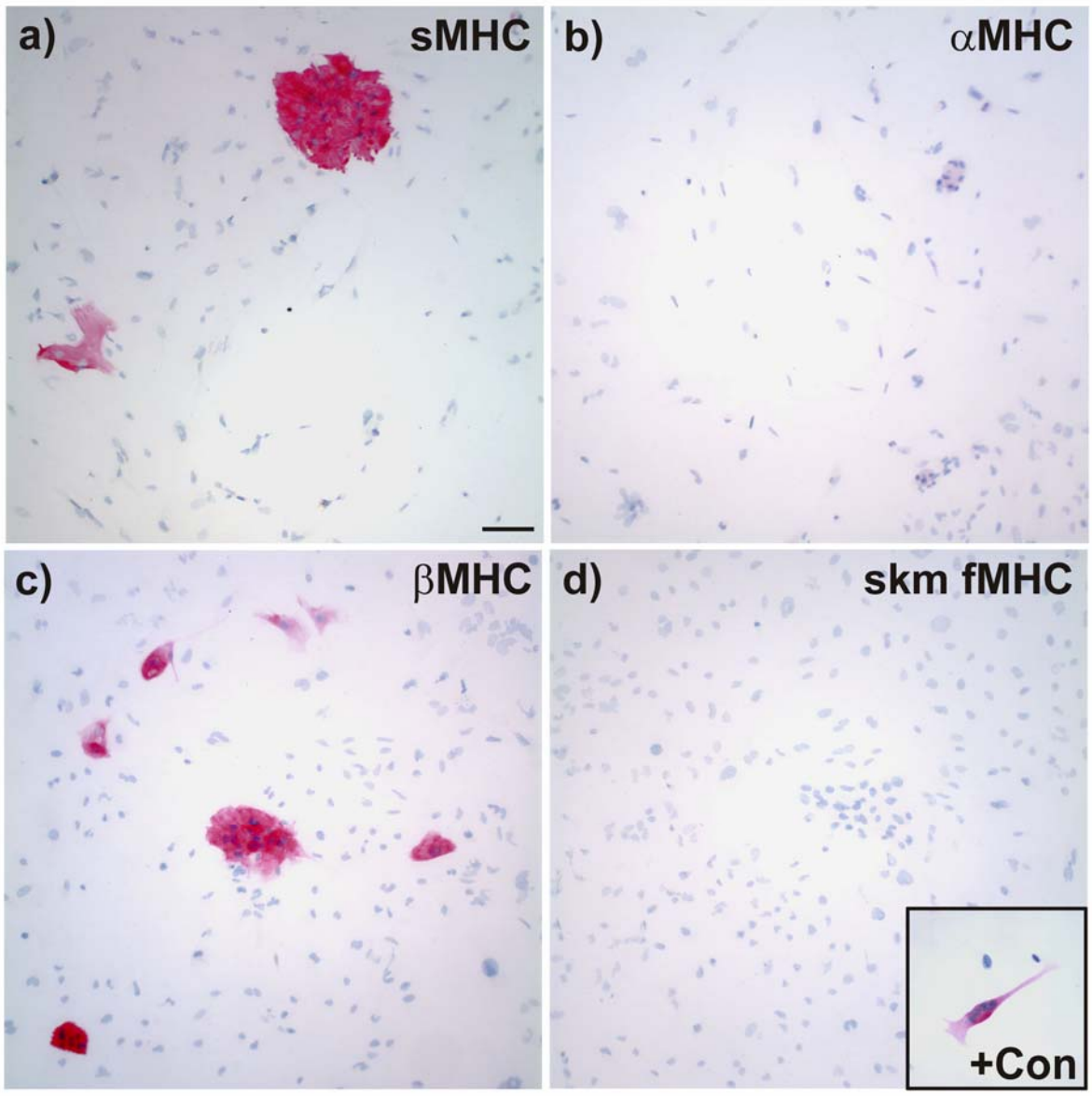
[1] Wobus AM. Potential of embryonic stem cells. Mol Aspects Med 2001; 22: 149-64.

[2] Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001; 19: 971-4.

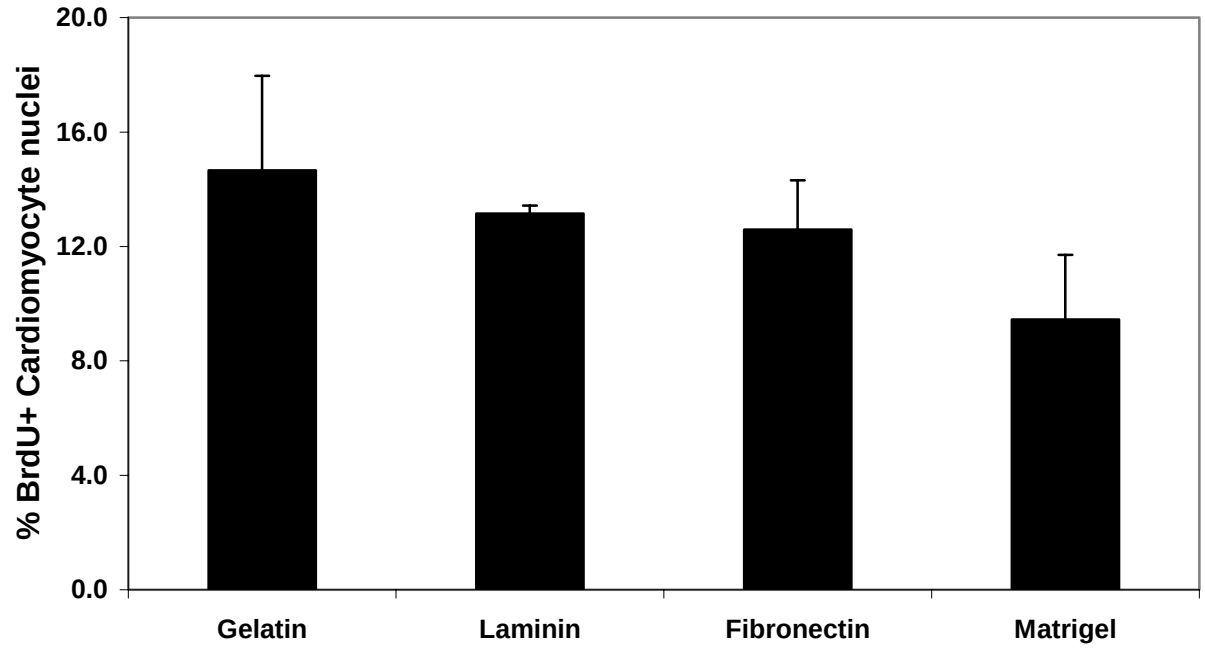
[3] Doevendans PA, Kubalak SW, An RH, Becker DK, Chien KR, Kass RS. Differentiation of cardiomyocytes in floating embryoid bodies is comparable to fetal cardiomyocytes. J Mol Cell Cardiol 2000; 32: 839-51.

[4] Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002; 91: 501-8.

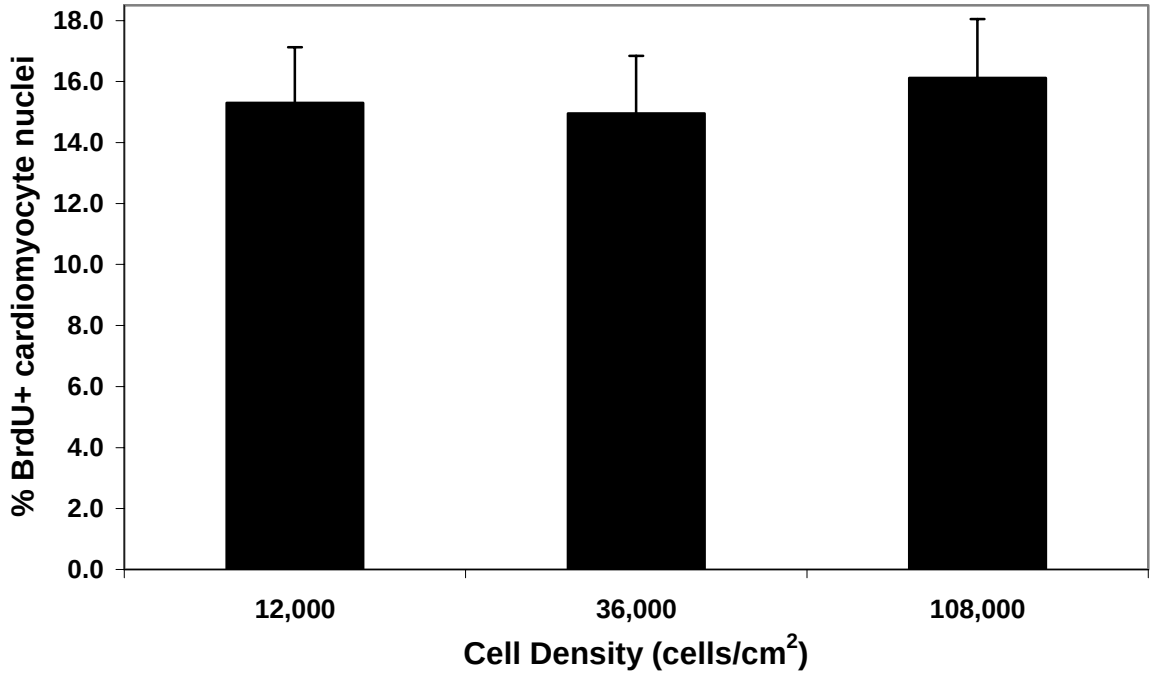
Supplementary Figure 1. *Immunophenotyping of human ES-cell derived cardiomyocytes. Human ES-cell derived cardiomyocytes were derived from embryoid body outgrowth cultures after 18 days of differentiation and enriched by Percoll gradient centrifugation after 18 days, as detailed above. In addition to showing strong immunoreactivity for sarcomeric actin, these myocytes immunostained positively for the expected striated muscle markers and yet were negative for specific markers of skeletal muscle. For example, panel A shows a representative field containing occasional clusters of cells immunoreactive for sarcomeric myosin heavy chain (MF-20 monoclonal antibody detected by red deposit). As expected for human cardiomyocytes, while these cells show little or no immunostaining for α-myosin heavy chain isoform (panel B, anti-αMHC BA-G5 clone), they are strongly positive for the slower β-isoform (panel C, anti-βMHC A4.951 clone). Importantly, after this duration of differentiation, embryoid body outgrowth cultures and their Percoll-enriched derivatives were uniformly negative for skeletal muscle markers, including fast skeletal myosin chain (panel D, anti-skm fMHC MY-32 clone). As a positive control for the latter, the inset in panel D shows strong immunostaining of a skeletal muscle C2C12 myotube. All images are representative of at least three replicates. Scale bar = 20 microns.*


a)
sMHC
b)
αMHC
c)
βMHC
d)
skm fMHC
+Con

Supplementary Figure 2. Matrix attachment factor effect on human ES cell-derived cardiomyocyte proliferation. Differentiating H7 human ES cells were differentiated for 19 days before Percoll separation, and fraction IV cells were cultured for an additional 4 days with serum-free culture media. No significant differences in the percentage of BrdU+ cardiomyocytes were found with different matrix attachment factors, although reproducible trends were observed, with gelatin-coated substrates exhibiting the highest levels of proliferation.



Supplementary Figure 3. Cell density effect on human ES cell-derived cardiomyocyte proliferation. H7 human ES cells were differentiated for 19 days before Percoll separation, and fraction IV cells were cultured for an additional 4 days with serum-free culture media. Cell density did not appear to influence the percentage of BrdU+ cardiomyocytes over a range of cell plating densities, suggesting that paracrine stimulation of growth was not involved.



Supplementary Figure 4. Growth factor receptor inhibition. H7 human ES cells were differentiated for 19 days before Percoll separation, and fraction IV cells were cultured for an additional 4 days in serum-free media with inhibitors to growth factor receptors. The inhibitors were added for the final 48 hours of culture prior to fixation. None of the inhibitor compounds significantly inhibited cardiomyocyte proliferation compared to the controls.