Human Embryonic Stem Cells passaged using enzymatic methods retain a normal karyotype and express CD30

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Running Title: hESCs passaged enzymatically retain a normal karyotype

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Abstract

It has been suggested that human embryonic stem cells (hESCs) are susceptible to chromosomal rearrangements as a consequence of single cell dissociation. We compared 2 methods of dissociation that do not generate single cell suspensions (collagenase and EDTA) with an enzymatic procedure using trypsin plus the calcium-specific chelator EGTA (TEG), that does generate a single cell suspension, over 10 passages. Cells passaged by single cell dissociation using TEG retained a normal karyotype. However, cells passaged in EDTA did acquire an isochromosome p7 in 3 replicates of 1 experiment. In all of TEG, collagenase and EDTA cultures, cells retained consistent telomere length and potentiality. We conclude that single cell dissociation can be used to maintain karyotypically and phenotypically normal hESCs. However, competitive genomic hybridization suggested that subkaryotypic deletions and amplifications could accumulate over time, reinforcing that present culture regimes remain suboptimal. In our cultures the cell surface marker CD30, reportedly expressed on embryonal carcinoma but not karyoptically normal ESCs, was expressed on hESCs of both normal and abnormal karyotype, but was upregulated on the latter.

Introduction

Embryonic stem cells (ESCs) are pluripotential cells with the capacity both to self-renew, and to give rise to all differentiated cell types of the conceptus. The isolation of human embryonic stem cells (hESCs) (1,2,3) has opened up the potential for ESC-based regenerative medicine. The control of murine ESC self-renewal, intrinsically by the Oct4

gene (4-6) and extrinsically by Leukemia inhibitory factor (LIF), has been extensively studied in the mouse and is now well understood (7-9). Although Oct4 is also an intrinsic regulator of human ESC self renewal (Matin et al 2004; Hay et al. 2004) the extrinsic processes that govern self-renewal of hESCs do not require exogenous LIF and are not yet well understood. A practical consequence of this deficiency is that current systems for routine culture of undifferentiated hESCs are sub-optimal and vary both between labs and between cell lines. Most hESC lines are feeder-dependant and require exogenous bFGF. The critical factor or factors provided by feeder cells are secreted, as demonstrated by the ability of conditioned medium to support the undifferentiated growth of hESCs without cell-cell contact (10). However the dependence on undefined and non-human factors for hESC propagation is a barrier to maintaining pluripotential, karyotypically normal cells capable of achieving regulatory acceptance. Noggin combined with high levels of bFGF supports long term undifferentiated proliferation of hESCs in the absence of fibroblast feeders or conditioned medium (Xu et al., 2005). Normal karyotype was retained for up to 32 passages. Encouragingly, totally defined, serum free culture conditions can also support existing hESCs over at least 13 passages while retaining normal karyotype (Ludwig et al., 2006). However, of 2 new hESC lines isolated using this defined culture system, one was XXY and another acquired a trisomy 12 after 7 months of culture, and it is premature to conclude that this system is optimal. An hESC line has recently been derived in xeno-free conditions using human serum and human foreskin fibroblasts (Ellerstrom et al., 2006). This line was shown to be karyotypically stable at least to passage 22. While these advances are encouraging, concerns remain about the possible

long term acquisition of karyotypic or genetic abnormalities under the varying conditions of culture employed across the world.

A critical aspect of practical hESC culture is the method of passage. Most hESC lines require mechanical disaggregation in their early passages, suggesting sensitivity at that stage to enzymatic disruption. In the original report of the isolation of hESCs, collagenase was used to passage the cells, once established, and supported the long-term growth of karyotypically stable, pluripotential cells (Thomson et al 1998). More recently, however, the same group, in collaboration with that of Peter Andrews', reported karyotypic abnormalities in hESCs passaged using collagenase (Draper et al 2004). Collagenase-passaged cells were also reported to contain aneuploid cells by Rosler et al (2004) with 20% of their cultures (H1, H7 and H9) containing some aneuploid cells. Others have reported aneuploidy when using enzymatic or chemical, but not mechanical, methods of passaging (Mitalipova 2005). Trisomy 12 has been reported in early passages of hESCs derived and maintained in a defined culture system (Ludwig et al., 2006). These authors and others (Brimble et al) have suggested that the poor clonal efficiency of hESCs leads to selective pressure for the accumulation of particular chromosomal rearrangements, and that this occurs as a consequence of generating a single cell suspension.

In this paper we compare the karyoptypic stability and potentiality of hESCs passaged with different enzymatic and chemical based methods of disaggregation (collagenase, trypsin and the Ca^{++} chelating agent EGTA, trypsin and the $Ca^{++}Mg^{++}$ chelator EDTA and EDTA alone). Both collagenase- and trypsin-based methods support long term

culture without gross chromosomal changes. These data show that it is possible to routinely passage hESCs using trypsin-based methods that result in a single cell suspension without detecting gross karyotypic instability. We show that single cell suspensions generated with trypsin can be transfected, and clones isolated and expanded that retain a normal karyotype. However, gross karyotypic normality does not detect small deletions or duplications that can be detected over as few as 10 passages by competitive genomic hybridization. In one of the experiments, cells passaged with EDTA did become karyotypically abnormal (duplication of the short arm of chromosome 7).

It has been suggested that CD30 expression is diagnostic for transformed hESCs (Herszfeld, et al., 2006). CD30 is a member of the tumour necrosis factor superfamily that is a surface marker for malignant cells in Hodgkin's disease (Durkop et al., 1992) and is also expressed in embryonal carcinoma (EC) cells that share many of the properties of ESCs but are karyotypically abnormal (Durkop et al., 2000).
We screened both our normal and abnormal lines for CD30 expression and discovered that although CD30 is up-regulated in the karyotypically abnormal line, CD30 expression is also detected in cultures of karyotypically normal hESCs.

Materials and Methods

Cells and Media

H1, H7 and H9 human ES (hESC) cells (p17) were a gift from Geron Corp, USA. hESCs were cultured as described previously by Xu et al. [13] on growth factor reduced Matrigel substrate (Becton Dickinson) in mouse embryonic fibroblast conditioned hESC medium (Knockout DMEM and 20% Knockout Serum Replacement supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 100μM β-mercaptoethanol; Invitrogen) with 4ng/ml recombinant human basic fibroblast growth factor (bFGF) (Sigma).
K562 cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum and 2mM L-Glutamine. The human Embryonal Carcinoma (hEC) cell line N-Tera 2 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum plus 2mM L-Glutamine , 0.1 mM non-essential amino acids and 1mM sodium pyruvate,. Human Trabecular Bone (HTB) cells were isolated as outlined in (Sottile 2002) and grown in the same medium as the N-Tera 2 cells.

Plating eficiency of hES cells passaged under different regimes

Three passage regimes were compared: (1) Collagenase IV (2) Trypsin/EGTA (TEG) – 0.25% trypsin in 92.7mM NaCl, 0.845mM di-sodium hydrogen orthophosphate (Na_2HPO_4), 1.58mM potassium dihydrogen orthophosphate (KH_2PO_4), 4.46mM potassium chloride (KCl), 5mM D-Glucose, 22.28mM Tris-HCl, 0.0009% phenol red, 0.25% trypsin, 1.05mM ethylenebis(oxyethylenenitrilo)tetraacetic acid(EGTA) and

0.000105% polyvinylalcohol). (3) EDTA– 0.2mg/ml ethylenediaminotetra acetate (EDTA) in Ca^{++}- Mg^{++}-free PBS. H9 hESCs were seeded at 1 x10^5 cells/well in 6-well plates at passage 40. These cells had been cultured exclusively by passage with collagenase since their isolation. Each treatment was replicated 3 times and 3 wells were set up for each replicate. Crucially, the initial seeding at passage 1 for all replicates of all 3 treatments was from a single collagenase-treated flask (passage 40). One well per replicate was counted 16-18 hours after plating to allow calculation of the plating efficiency (number of cells plated down/number of cells seeded x100%). Note that, due to cell divisions occurring within that period, this slightly overestimates plating efficiency in each treatment. Once the remaining two wells were confluent one well was treated with TEG to obtain a single cell suspension that could be counted (the collagenase treatment, and to a lesser extent EDTA, resulted in clumps of cells rather than a single cell suspension). On the basis of this cell count the sister well was then passaged. Three wells of a 6-well plate were seeded with approximately 1x10^5 cells. The experimental design is summarised schematically in Figure 1a.

The treatments were as follows; For collagenase , wells were washed with 2ml KO-DMEM, 0.5ml collagenase IV (200 units/ml) was added and plates were incubated at 37ºC for approximately 4 minutes. The collagenase was then removed and the cells were scraped into 1ml CM. Clumps were gently titurated with a 5ml pipette three times. For the TEG treatment, wells were washed with 2ml KO-DMEM, and incubated with TEG at 37 ºC for approx 2 minutes. KO-DMEM was then added and the cells titurated and centrifuged at 500g for 2' before resuspension in CM. For the EDTA treatment, wells were washed with 2ml KO-DMEM , and incubated with 0.5mM EDTA in PBS at 37 ºC

for approximately 3- 5'. The EDTA was then removed and the cells were scraped into 1ml CM. Clumps were gently titurated with a 5ml pipette three times. After 10 passages the cells were expanded to generate stocks for cryopreservation, genomic DNA preparation isolation and karyotype analysis.

For the second experiment, H9 hESCs were seeded at 3 $x10^5$ cells/well in 6-well plates at passage 34. These cells had been cultured exclusively by passage with collagenase since their isolation. Each treatment was replicated 3 times. Again, the initial seeding at passage 1 for all replicates of all 3 treatments was from a single collagenase-treated flask (passage 34). When the wells were confluent the cells were passaged 1:2 to a new well of a 6-well plate.The treatments were as described above and in addition Trypsin EDTA (TED) 0.25% trypsin in 92.7mM NaCl, 0.845mM di-sodium hydrogen orthophosphate (Na_2HPO_4), 1.58mM potassium dihydrogen orthophosphate (KH_2PO_4), 4.46mM potassium chloride (KCl), 5mM D-Glucose, 22.28mM Tris-HCl, 0.0009% phenol red, 0.25% trypsin, 1.05mM ethylenediaminotetra acetic acid(EDTA) and 0.000105% polyvinylalcohol). Wells were washed with 2ml KO-DMEM, cells were incubated with TED at 37 ^oC for approx 2 minutes. KO-DMEM was then added and the cells titurated. The cells were spun down at 500g for 2' and the pellet resuspended in CM.

Calculation of doubling time

H9 cells passaged by TEG disagregation and by collagenase, and HM1 murine ES cells passaged in TEG were seeded at $1x10^5$ cells/well in 2, 6-well plates for each of 2 replicates. At each time point 2 wells were trypsinised in each replicate and the cells were counted using a hemocytometer. Collagenase treated cells do not generate a single cell

suspension and cannot be counted with a hemocytometer. Instead, additional sacrificial wells were seeded for counting following trypsinisation and then discarded. There is therefore additional error in collagenase-treated cultures associated with variation in initial cell number across wells. For each cell type, linear regressions of time were fitted to the logarithm of the cell numbers. Doubling time was estimated as log(2) divided by the slope of the regression. Confidence intervals for doubling times were obtained by replacing the slope by its estimated confidence limits from fit of the regression.

DNA preparation

Genomic DNA was prepared from cell cultures by overnight incubation at 55ºC in lysis buffer (100 mM Tris-Cl, 5 mM EDTA, 200 mM NaCl, 0.2 % SDS, 100 µg/ml proteinase K, pH 8.5), followed by incubation with Rnase A (325 µg /ml) at 37 ºC for 1 hour and further treatment with proteinase K (100 µg /ml) at 55 ºC for 2 hours. DNA was then precipitated by addition of isopropanol, washed with 70 % ethanol and resuspension in TE buffer. Genomic DNA for CGH analysis was further purified by phenol/chloroform extraction prior to precipitation.

Comparative Genomic Hybridisation (CGH) analysis

The CGH procedure was modified from the original methods of Kallioniemi et al., (1992,1994).Target human metaphases were obtained from phytohemagglutinin-stimulated blood lymphocytes cultures (72hs). Chromosome spreads were prepared one day before hybridisation and left overnight at 43 ºC. Before CGH analysis the slides were treated with pepsin (50µg/ml in 0.01 M HCl), denatured with formamide and serially dehydrated in ethanol. hES genomic DNA was labelled by nick translation with

SpectralGreen (Vysis). SpectralRed labelled normal human genomic DNA (Vysis) was used as reference DNA. Labelled fragments were 500-1000 bp. Equal amounts (400ng-1μg) of hES and reference DNA were mixed and precipitated with 20-30 μg of unlabeled Cot-1 DNA (Roche). DNA samples were dissolved in hybridisation solution and denatured at 75 ºC. Slides were hybridised for 72 hours in a humidity chamber at 37 ºC. After hybridisation slides were washed in SSC at high stringency and dehydrated through an ethanol series. Slides were counter-stained with DAPI in Vectashield (Vector Laboratories). The hybridised metaphase cells were examined using a workstation composed of an epifluorescence microscope coupled to a CCD camera (Olympus) and a CytoVision 2.7 CGH software (Applied Imaging). CGH analysis was developed over 10 complete metaphases. For each metaphase three fluorochrome images (DAPI, SpectralGreen and SpectralRed) were acquired and processed using High Resolution CGH software.

Cryopreservation

Cells were resuspended in human ES media and mixed with an equal volume of media supplemented with an additional 10% serum replacement and 10% DMSO. The cells were transferred to cryovials and stored at –80 ºC overnight and transferred to -150 ºC the next day. An initial cooling step at –20 ºC was found to be detrimental rather than beneficial (data not shown).

Transfection

A linearised plasmid containing a neomycin resistance gene flanked by a PGK promoter and poly-adenylation signal was used to test stable transfection efficiencies. Lipofection

was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions. $1x10^6$ subconfluent cells in 3 wells of a six well plate were lipofected with 5µg of linear plasmid per well. Two different electroporation methods were compared, using $1x10^6$ cells in log phase of growth, treated with TEG to give a single cell suspension. Using a Gene Pulser (BioRad), the cell/DNA mix was electroporated in PBS at room temperature in a 0.4 cm electrode cuvette at 940µF, 200V. Using a Multiporator (Eppendorf) the cells were swelled in hypoosmolar buffer (Eppendorf) for 20 minutes at room temperature, mixed with DNA and pulsed in a 0.4 cm electrode cuvette at 300V for 100µS. Following either electroporation method, cuvettes were left at room temperature for 10 minutes prior to plating the cells onto 15 cm matrigelled dishes. Selection in G418 at 150µg/ml was applied 48 hours after transfection. G418 resistant colonies were fixed with methanol and stained with 10% Giemsa.

Karyotype analysis

Exponentially growing cultures were treated with Karyomax colcemid solution (Invitrogen, UK) at 100 ng/ml for 2 hours at 37ºC. Following disaggregation cells were pelleted and resuspended in hypotonic solution at room temperature. After 10 minutes cells were pelleted and resuspended in 3:1 methanol/acetic acid. Cells were then washed twice with 3:1 methanol/acetic acid, dropped onto a slide and air-dried for 3 days. Resulting chromosome spreads were washed in 2xSSC at 60ºC for 2 hours, partially digested with 0.01% Bacto trypsin (Difco Labs), and stained with 5% Gurr's improved Giemsa R66 (BDH, UK) in Gurr's phosphate buffer.

Cell viability assay/ Propidium Iodide (PI) staining

Cells were disaggregated using TEG and a single cell suspension prepared in FACS buffer (PBS, 0.1% BSA, 0.1% Sodium Azide). Cells were aliquoted at 2-5 x 10^5 cells per tube. Cells were washed and resuspended in in 300μl FACS buffer. The flow cytometer was set up for autofluorescence signals in FL2 channel for unstained cells. Cells for PI staining were incubated with 15μl of staining solution (PI at 50μg/ml in PBS) with gentle mixing for 1 min.PI fluorescence ((FL2 channel; 575/42nm) was then acquired for the stained cells. Data for 5-10,000 ungated events were acquired on the FACSAria and analysed using FACSDiva software((Beckton Dickenson Immunocytometry Systems (BD), UK).

Flow cytometry analysis for the Stem Cell Surface Markers

Single cell suspensions of hESC at 1 x 10^6 cells/sample in staining buffer (PBS supplemented with 10% FBS and 2mM EDTA) were incubated with 50μl of 40% heat inactivated rabbit serum for 15 minutes on ice to block non-specific binding, before labelling with primary monoclonal antibody for 30 minutes on ice. Cells were then incubated in the presence of fluorescent conjugated detection antibody for 30 minutes on ice in the dark. After each step unbound antibody was removed by washing twice with staining buffer followed by centrifugation at 260g for 5 minutes. Cells were resuspended in 0.5-1ml PBS for acquisition of data. Primary monoclonal antibodies were SSEA-4 (MC-813-70, IgG3) and SSEA-1 (clone MC-480, IgM) from Developmental Studies Hybridoma Bank, (DSHB, USA), TRA-1-60 (mab 4360, IgM) and TRA-1-81 (mab 4381, IgM) from Chemicon. Isotype controls IgM and IgG3 (Sigma, UK) were included in all

experiments. Concentrations used are listed in parentheses following each antibody. Secondary detection antibodies; FITC-goat anti-mouse IgG3 (0.5 mg/ml) and R-PE goat anti-mouse IgM (0.25 mg/ml) (Southern Biotechnologies) were used at a dilution of 1 in 100. Samples were analysed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems (BD), UK) equipped with a 488nm argon laser and standard filter set. Dead cells were excluded from acquisition based on their forward and side scatter characteristics using an electronic gate. Data for 40, 000 events per sample were acquired and analysed using CellQuestPro software (Becton Dickinson Immunocytometry Systems (BD), UK).

RT-PCR

RNA was isolated using an RNeasy mini kit (Qiagen) following the manufacturer's protocol. One-step reverse transcription–polymerase chain reaction (RT-PCR) was performed using SuperscriptOne-Step RT-PCR with Platinum Taq (Invitrogen) following the manufacturer's recommended cycling conditions. To detect rare transcript expression 45 cycles were used for each primer pair. Primers were designed to be exon spanning to readily distinguish genomic DNA and cDNA products. CD30 mRNA was amplified using the forward primer 5'-AGCTAGAGCTTGTGGATTCCA-3' and the reverse primer 5'-GTCTTCTTTCCCTTCCTCTTCC-3' to give a product of 464 bp. β-actin mRNA was amplified using the forward primer 5'-GCCACGGCTGCTTCCAGC-3' and the reverse primer 5'- CAAGATGAGATTGGCATGGCT -3' to give a product of 528 bp. Products were resolved using 2% agarose gel electrophoresis.

Flow Cytometry analysis for the cell surface CD30(Ber-H2) epitope

Adherent human embryonic stem cells washed with PBS were incubated with TEG to dissociate cells. Recovered cells were pelleted at 200g and resuspended at between $2x10^6$ and $1x10^7$ cells/ml in PBS (Ca^{2+} and Mg^2 free) with 0.2% BSA. 100ul aliquots were stained with either CD30-PE(Santa Cruz; sc19658) or isotype control(mIgG1-PE; BD Pharmingen; 555749). Cells were incubated in the dark at 4ºC for 30 minutes, washed and finally resuspended in PBS. Flow cytometry was performed using a Becton Dickinson FACSAria and Diva analysis software. Live cells were gated using forward- and side-scatter profiles. Data were acquired for 10,000 live events.

Fixation of cells.

Sample fixation was performed either before surface staining for CD30 or immediately following staining. Cells were incubated with 0.1% paraformaldehyde for 15 minutes at room temperature, washed and finally resuspended in 100ul PBS.

Results

Plating efficiency

The response of H9 hES cells to three methods of disaggregation (collagenase, TEG and EDTA) was followed over 10 passages. Plating efficiencies were obtained for each treatment (Figure 1b). All 3 replicates of cells cultured by passage in collagenase failed to survive the seventh passage. This exemplifies the erratic nature of the plating efficiencies generally observed with this method of passage. The TEG passage regime was less erratic than either EDTA or collagenase, yielding average plating efficiency of

32%. All 3 replicates of the EDTA-passaged cells passaged at particularly poor efficiency at passage 8 (Figure 1b)

H9 hESCs passaged with collagenase and mouse ESCs (HM1 cells) passaged with TEG proliferate with doubling times of 17.3 and 13.1 hours respectively, a statistically significant difference ($p<001$) (Figure 4). Human ESCs passaged with TEG proliferate at a similar rate (13.3 hours) to mESCs (13.1 hours) when measured at subconfluent densities. Doubling time increased as TEG-treated cells attained confluence. Collagenase-treated cells did not attain confluence over the course of the experiment. When the growth rate of collagenase-passaged cells and TEG passaged cells were compared at lower initial density (Figure 4b) TEG-passaged cells again reached confluence earlier and appeared to stop growing. As initial plating densities were identical, this confirms a reduced doubling time in TEG-treated cells leading to earlier confluence. Figure 4c shows the morphology of collagenase- and TEG-passaged cells 90 hours post plating. TEG-passaged cells typically formed larger colonies, while collagenase-passaged cultures, in addition to undifferentiated hESCs, contained a second, differentiated cell type of fibroblastic morphology.

Karyotypes

The H9 hESCs that were used to set up the comparison of different disaggregation treatments had been cultured in our lab for 23 passages with collagenase, and had a diploid karyotype (46XX) (figure 2) at passage 40 in this experiment (Figure 1a). Cells disaggregated for 13 passages using TEG retained a normal karyotype. In contrast, cells disaggregated using EDTA had acquired an abnormal karyotype with all three replicates

having a duplication of the short arm of chromosome 7 and deletion of the long arm (46,XX,i(7q)). This result was confirmed by comparative genomic hybridization (CGH) (Figure 3). Although TEG-treatment led to no gross karyotypic abnormalities, CGH suggested the presence of random deletions clustered near the telomeres of many chromosomes. No similar pattern was observed with collagenase-treated cells or with EDTA-treated cells (data not shown). No specific deletions achieved statistical significance, however, the clustered pattern of subtelomeric deletion was consistent across 3 independent relicates and suggests a mixed population containing cells with many different small deletions. A second vial of H9 hESCs was resuscitated and the different passage regimes were repeated, and extended to include TED. Cells passaged with TEG, TED or collagenase were found to be karyotypically normal. In contrast to the first experiment, cells passaged with EDTA were also found to be karyotypically normal (data not shown). We have also switched H1 and H7 cells previously passaged with collagenase over to our TEG-based protocol (data not shown).

We assessed whether the different forms of passage had any effect on telomere maintenance. All 4 treatments showed similar telomere lengths suggesting that the various methods of passage had no differential effect on telomere length and that the telomeres were being maintained (data not shown).

Stem Cell Markers

We examined the expression of surface markers characteristic of undifferentiated (SSEA3, SSEA- 4,TRA-1-60 and TRA-1-81) and differentiated (SSEA-1) hESCs on TEG-passaged H7 (Figure 5) , H1and H9 (data not shown) hESCs in comparison with collagenase passaged cells. These data show a reduction in the marker of differentiation

(SSEA-1) within the cells passaged using TEG and also show that these cells retain a high level of expression of the markers characteristic of undifferentiated hESCs. This is consistent with our observation that TEG-passaged cells are predominantly of undifferentiated phenotype. The TEG-passaged cells were also positive for Oct-4 and alkaline phosphatase (data not shown).

As concerns have been raised about the viability of hESCs once in a single cell suspension we assessed cell viability after passaging with TEG. The average cell viability, as assessed by propidium iodide staining was 93.4 +/-0.26% (n=3).

CD30 expression

CD30 expression has been reported in transformed hESCs, but not in karyotypically normal sister cultures (Herszfeld et al., 2006), suggesting that its' expression may be diagnostic for karyotypic abnormality. We screened our hESC lines for expression of CD30 transcripts by RT-PCR (Figure 6). Since CD30 expression is also characteristic of embryonal carcinoma (EC) cells and of human erythroid progenitors, we used the human EC line N-Tera 2 and the human erythroid progenitor cell line K562 as positive controls and human trabecular bone cells (HTBs) were used, as a negative control. We detected expression in the positive control cell lines N-Tera 2 and K562 but not in HTBs. The abnormal lines H9-EDTA e1repB and C (lanes 9 and 10) also showed expression of CD30 mRNA. Surprisingly, however, other hESC lines that had been shown to have a normal diploid karyotype at a similar passage also expressed the transcript. This included cells passaged with collagenase, EDTA and TEG (lanes 4-8). Next, we screened the cell lines for expression of CD30 protein by flow cytometry using the same monoclonal antibody as used by Herszfeld. As expected both K562 and N-Tera

2 cell lines showed a high proportion of CD30 positive cells (Figure 7) and HTBs lacked the CD30 epitope. Consistent with the RT-PCR data we detected CD30 expression in all the hESC lines tested. The abnormal cell lines H9-EDTA e1repB and C (viii and ix) did express higher levels than the "normal" hESCs (iv-vii). Herszfeld et al (2006) had reported CD30 flow cytometry data using fixed cells. To test whether fixation affected the level of CD30 staining we examined several cell lines using different staining protocols either with or without fixation. Either fixing the cells and then staining, or fixing and then staining reduced the number of cells that stained positive for CD30 expression (data not shown). Monitoring expression of CD30 on fixed cells, however, still showed that cultures of hESCs passaged with collagenase, TEG and EDTA all contained CD30 positive cells. However, though the proportion of expressing cells was similar, the abnormal hESC lines H9-EDTAe1rep B and C displayed significantly higher levels of CD30 expression than the normal hESC lines.

Potentiality in vivo and in vitro

To test the potentiality of hES cells disaggregated by TEG or collagenase, cells were injected into SCID mice. Four animals were injected with each cell type giving rise to 1 tumour with collagenase-treated cells and 4 tumours from TEG-treated cells. Tumours generated from TEG- and collagenase-dissociated cells gave rise in each case to a variety of tissues derived from the 3 germ layers (Figure 8a). There was no apparent accumulation of undifferentiated cells in either treatment. When submitted to in vitro

differentiation protocols, the TEG-treated cells gave rise to cell types arising from all 3 germ layers (Figure8b).

TEG-treated H9 cells were tested for transfection efficiency in both electroporation and lipofection. A set of conditions was defined using a multiporator (Eppendorf), which gave transfection efficiencies some 5-fold higher than were achieved by lipofection ($1/4X10^{-3}$).

The TEG-passaged cells have subsequently been used in our lab in several transfection experiments. One H9 clonal line that has been generated M2 (Hewitt et al., 2006) was shown to retain a normal karyotype at passage 97, with 80 passages carried out in our lab, 23 using collagenase followed by 57 with TEG (data not shown). The TEG, EDTA and collagenase methods of disaggregation were compared for their effects on the proportion of cells frozen, which survive thaw and replating. TEG treatment led to a significant improvement in freeze thaw survival over collagenase (76.2% versus 15.8%).

Discussion

In this study human ESCs passaged by single-cell dissociation using trypsin/EGTA (TEG) were maintained over 10 passages and retained stable morphology, karyotype and growth rate. Single cell dissociation of hESCs did not lead to karyotypic instability. However, subkaryotypic analysis suggests that TEG passage may be associated with small subtelomeric deletions and amplifications.

Plating efficiency and doubling time

We calculated a doubling time of 17.3 hours for collagenase passaged H9 hESCs, compared with 13.3hours for TEG-passaged cells. A previous report calculated the doubling time of H9 hESCs to be 35.3 hours (Amit 2000). This discrepancy may reflect the differences in culture conditions between labs; notably the growth of cells on matrigel versus a fibroblast feeder layer. The shorter doubling time of TEG passaged cells may be a consequence of the reduction in differentiated companion cells when compared with sister cultures passaged with collagenase.

The high plating efficieny and short doubling time of the TEG-treated cells contrasts with reports that hESCs are sensitive to single cell dissociation (eg: Amit et al., 2000; Draper et al., 2004; Hasegawa et al., 2006). However, Hasegawa and colleagues were able to subclone hESCs that they then showed were adapted to single-cell dissociation (Hasegawa et al., 2006). Furthermore, hESCs cultured in the TEG system can be efficiently sorted by flow cytometry (Hewitt et al., 2006). Hence, it appears that hESCs can be adapted in culture to overcome sensitivity to single cell dissociation.

Single-cell dissociation by TEG supports stable karyotype

In the original report of the isolation of hESCs, long term karyotypic stability was demonstrated when collagenase was used for routine passage of established lines (Thomson et al., 1998). Since then, several reports have described the detection of aneuploid hESCs using similar, collagenase-based, passage regimes (Draper, 2004; Mitalipova, Inzunza, Amit). Of particular note, the gain of chromsomes 17q and 12 were shown to occur on several independent occasions(draper, Ludwig et al., 2006). Mitalipova et al (2005) reported abnormal karyotypes after extended passage in culture

using trypsin based methods, but not when physical methods of disaggregation were used. Buzzard et al (2004) hypothesised that the use of mechanical disaggregation may prevent the types of chromosomal abnormalities previously reported.

H9 hESCs can be passaged using 2 enzymatic methods (collagenase and TEG) for similar numbers of passages to the Mitalipova et al report, and maintain a normal gross karyotype with both protocols. Of the 3 methods compared in this study only TEG generates a single cell suspension, and the karyotypic stability of TEG-passaged cells in our hands does not support the idea that single cell cloning gives rise to karyotypic instability. Notwithstanding concerns about sub-telomeric stability we suggest that the use of the TEG regime for rapid cell amplification, electroporation and freezing offers important advantages. Regardless of the method of passage, caution should be shown when cells are cultured at low density as this may lead to the selection of cells harbouring abnormal karyotypes.

Subkaryotypic abnormalities can accumulate in TEG-passaged hESCs

Comparative genomic analysis confirmed the diploid karyotypes of cells passaged with TEG and collagenase. However, in the TEG treated samples the analysis suggested the accumulation of small subtelomeric deletions, though none of these were statistically significant. Pluripotency of TEG-treated cells was not obviously different from that of collagenase-treated cells when assayed both in vivo (tumours in SCID mice) and in vitro (response to embryoid body formation and removal of bFGF and conditioned medium). Our study has not addressed the possible accumulation of point mutations as recently

reported for late passage hESCs (Maitra et al., 2005). We have studied subkaryotypic abnormalities at the level of CGH and detected small deletions and duplications. This technique detects only those aberrations that are shared by a significant proportion of the population. Hence, random changes that are not fixed in the population would not be detected, and the tendency for accumulation of subtelomeric deletions must give rise to concern about using the TEG passage regime for the preparation of cells for therapeutic use.

EDTA-treated cells can acquire abnormal karyotype

In the first experiment (Figure 1a), EDTA treatment was associated with the appearance, in 3 separate replicates, of the same duplication of the short arm of chromosome 7. The complete absence of cells with normal karyotype in all 3 replicates argues powerfully for a strong competitive advantage of this rearrangement when cells are passaged with EDTA. As the same abnormality was detected in all 3 replicates it is likely that the duplication was present in the starting population. It was noted that during the culture of the cells that acquired the karyotypic abnormality there was one exceptionally poor passage affecting all 3 replicates (Figure 1, plating efficiency of 3% as compared with an average of 24%). Those cells harbouring the duplication of the short arm of chromosome 7 may have had a selective advantage at this point. When we repeated the treatment with EDTA using an earlier passage (p34) of collagenase passaged cells,,we did not observe any karyotypic abnormalities. Draper et al (2004) reported that the gain of chromosome 12 and amplification of chromosome 7q in H1 and H14 hESCs was observed after the cells had been put through clonal selection or switched from a

feeder-layer culture system to feeder-free. Both of these treatments could result in low cell densities that may allow a competitive advantage for cells of abnormal karyotypes. Our observation of a particularly poor recovery among the 3 replicates of EDTA-treated cells at p8, followed by fixation of the isoP7 rearrangement, would be consistent with the presence of a minority population of isoP7 cells that preferentially survived under the conditions at that particular passage.

Summary

The TEG protocol offers advantages of convenience and repeatability, leading to significant reduction in the time required to amplify cells for experimental purposes. TEG-treated cells are also adaptable to electroporation leading to transfection efficiencies approximately 1 order of magnitude higher than those reported by Zwaka and Thomson (2003) (*discuss nucleofection efficiency once have the paper!*). Similarly the TEG treatment provides approximately 5-fold freeze-thaw efficiency gains over the collagenase protocol. Cells switched from collagenase- to TEG-passage have now been used for several other projects. A clonal line, M2, derived from H9 hESCs that had been passaged using collagenase for 23 passages and then TEG for 57 passages also retained a diploid karyotype (Hewitt et al., 2006). Furthermore, we have recently used our trypsin based protocol to passage cells for single cell sorting (Hewit et al., 2006). Clones from these experiments have been expanded using TEG-passage and retained a normal karyotype.

Spontaneous chromsomal aberrations (SCA) were previously shown to be reduced by culture of TEG-passaged hESCs in physiological oxygen partial pressure

(Forsyth et al., 2006). SCAs are precursors to karyotype abnormalities. Of concern was the observation that the rate of SCAs was higher in later passage cells. Others have reported an increase in mutation rate with passage of hESCs (Maitra et al., 2005). These data reinforce the need to thoroughly characterise cells prior to therapeutic use, and to provide failsafe protection for cancerous phenotypes by such means as conditional suicide genes (Shuldiner et al., 2003; Hewitt et al., 2006). Together with the present observation that culture regime can lead to detectable levels of gene amplification and deletion, these observations also underline the need to establish a more thorough assay of 'normality' than karyotype alone.

Following reports elsewhere that CD30 is expressed in transformed hESCs but not in non-transformed hESCs of normal karyotype we assessed its' expression on the hESCs used in this study. In our hands, CD30 is expressed on all hESCs examined but its level of expression is greater in the karyotypically abnormal hESCs. Hence the use of CD30 as a diagnostic tool for normality of hESCs may require quantitative analysis and will need to be backed up by examination of additional markers.

All models of the application of human ESCs in regenerative medicine are based upon massive ex vivo expansion of a few or possibly even a single blastomere(s) into very large cell numbers in vitro. To ensure the safety of the resulting graft a great deal remains to be done in optimising culture conditions, in defining and identifying 'normal cells', and in providing failsafe features that protect against cellular overgrowth post engraftment (eg Hewitt et al., 2006b)

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References

Amit., M., Carpenter, MK, Inokuma et al., Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 2000; 227: 271-278.

Brimble, S.N., Zeng, X.M., Weller, D.A. et al., (2004). Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9 2001. Stem Cells and Development 13 (6) 585-597.

Buzzard JJ, Gough NM, Crook JM, et al. Karyotype of human ES cells during extended culture . Nature Biotechnology 22 (4): 381-382 APR 2004

Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, Meisner L, Zwaka TP, Thomson JA, Andrews PW . (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells Nature Biotechnology 22 (1): 53-54

Durkop H, Latza U, Hummel M, Eitelbach F, Seed B, Stein H. Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's disease. Cell 1992 Feb 7;68(3):421-7

Durkop H, Foss HD, Eitelbach F, Anagnostopoulos I, Latza U, Pileri S, Stein H. Expression of the CD30 antigen in non-lymphoid tissues and cells. Journal of Pathology 2000 Apr;190(5):613-8

Ellerstrom, C., Strehl., R., Moya, K., Andersson, K., Bergh, C., Lundin, K., Hyllner, J. & Semb, H. (2006) Stem Cells online publication DOI:10.1634/stemcells.2006-0130.

Enver T, Soneji S, Joshi C, Brown J, Iborra F, Orntoft T, Thykjaer T, Maltby E, Smith K, Dawud RA, Jones M, Matin M, Gokhale P, Draper J & Andrews PW. (2005). Cellular Differentiation hierarchies in normal and culture-adapted human embryonic stem cells. (2005). Human Molecular Genetics 14:3129-3140.

Hasegawa, K., Fujioka, T., Nakamura, Y., Nakatsuji, N. & Suemori, H. (2006) A method for the selection of human embryonic stem cell sub-lines with high replating efficiency

after single cell dissociation. Stem cells express August 24 2006; doi:10.1634/stemcells.2005-0657.

Hay DC, Sutherland L, Clark J, Burdon T.(2004). Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. Stem Cells 22(2):225-235

Herszfeld, D., Wolvvetang, E. Langton-Bunker, E. et al., (2006). CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells. Nature Biotechnology 24 (3) 351-357.

Hewitt, Z. Priddle, H., Thomson, A.J. and McWhir, J.Ablation of undifferentiated human embryonic stem cells by exploiting innate immunity against the α-galactosyltransferase epitope Stem Cells (2006a)

Hewitt, Z., Forsyth, N., Waterfall, M., Wojtacha, D. Thomson, A. & McWhir, J. Fluorescence-activated single cell sorting of human ES cells. Cloning and Stem Cells. 2006b (in press).

Inzunza J, Sahlen S, Holmberg K, Stromberg AM, Teerijoki H, Blennow E, Hovatta O, Malmgren H. Comparative genomic hybridization and karyotyping of human embryonic

stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. Molecular Human Genetics. 2004 Jun;10(6):461-6.

Ludwig, T.E., Levenstein, M.E., Jones, J. M. et al., Derivation of human embryonic stem cells in defined conditions. Nature Biotechnology 24 (3) 185-187.

Maitra, A, Arking D.E., Shivapurkar, N. Ikeda M, Stastny V, Kassauei K, Sui G, Cutler DJ, Liu Y, Brimble SN, Noaksson K, Hyllner J, Schulz TC, Zeng X, Freed WJ, Crook J, Abraham s, Colman A, Sartipy P, Matsui S-I, Carpenter M, Gazdar AF, Rao M and Chakravarti A.(2005). Genomic alterations in cultured human embryonic stem cells. Nature Genetics 37 (10):1099-1103 .

Matin MM, Walsh JR, Gokhale PJ, et al.
Specific knockdown of Oct4 and beta 2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells
STEM CELLS 22 (5): 659-668 2004

Mitalipova M, Calhoun J, Shin SJ, et al. Human embryonic stem cell lines derived from discarded embryos Stem Cells 21 (5): 521-526 2003

Rosler ES, Fisk GJ, Ares X, et al. Long-term culture of human embryonic stem cells in feeder-free conditions. DEVELOPMENTAL DYNAMICS 229 (2): 259-274 FEB 2004

Schuldiner M, Itskovitz-Eldor J & Benvenisty, N. Selective ablation of human embryonic stem cells expressing a “suicide” gene. Stem cells. 21 257-265 (2003).

Sottile V, Halleux C, Bassilana F, Keller H, Seuwen K. (2002). Stem Cell Characteristics of human trabecular bone derived cells. Bone 30(5): 699- 704

Suemori H, Yasuchila K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N. (2006). Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem Biophys Res Commun. 2006 Jul 7;345(3):926-32.

Thomson JA, Itsokitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Science 1998 Nov 6;282(5391):1145-7

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

Xu, R-H., Peck, R.M., Dong, S.L, Feng, X., Ludwig, T & Thonson, J.A. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nature Methods 2 (3) 185-190.

Zwaka, T.P. and J.A. Thomson 2003. Homologous recombination in human embryonic stem cells. Nature Biot

Figure Legends

Figure 1. Plating Efficiency of H9 hESCs passaged using different treatments. A) Schematic representation of experimental design. All treatments originated from a single flask of H9 cells cultured for 40 passages (P40) using collagenase. A sister culture was karyotyped and confirmed 46XX. Passages within the experiment are numbered P+1, P+2 etc. Cells were counted 16- 18 hours after plating (count 1) and again at time of passage (count 2). B) Cells were passaged using either collagenase (green), TEG (blue) or EDTA (pink). The plating efficiency values are the average of the three replicates and the error bars are the standard deviation.

Figure 2. Karyotypes of H9 cells. (a) collagenase treated cells at the start of the experiment (passage 40, 23 passages with collagenase in our lab) (b) passaged 13 times with TEG (c) passaged 13 times with EDTA. Karyotypes in b and c are representative of 10 spreads analysed for each of 3 independent replicates. No abnormalities were apparent in the parental collagenase-treated cells (a) nor in any of 3 replicates of the TEG-treated cells (b). However, all 3 replicates of the EDTA protocol carried the same isochromosome isop7 (panels c and d). Panel d shows chromosome 7 pairs from 3 different EDTA replicates.

Figure 3. Comparative genome analysis (CGH) H9 hESCs. Panel A summarises 2 independent CGH experiments for 1 replicate of TEG-passaged H9 cells showing a

tendency to accumulate small subtelomeric deletions. When CGH is repeated with the same DNA samples (Ai and Aii) similar, but not identical subtelomeric deletions also appear. Circles are areas showing a tendency for small deletions to accumulate in similar subtelomeric regions. Similar, but not identical subtelomeric deletions were also associated with the other 2 TEG replicates but not with EDTA or collagenase (data not shown). Panel B summarises CGH experiments for EDTA-passaged H9 hESCs showing an abnormality on chromosome 7 with duplication of the p arm and loss of the q arm.

Figure 4.Doubling time of hES cells. Panel A. Growth curves calculated for murine HM1 ES cells and human H9 ES cells by seeding 10E4 to 10E5 cells per well of a series of 6 cm dishes. The y axis is a logarithmic transformation of cell number. The black line is H9 cells following 5 passages by collagenase, blue is murine ES cells (HM1) and red is H9 cells following long term passage by TEG. TEG treated cells were greater in number at the start of the experiment and reached confluence faster as indicated by the non-linearity of those data. Collagenase treated cells grew with a constant doubling time of 17.3 hours while murine ES cells had a doubling time of 13.3 hours. When the experiment was repeated for trypsin-treated and collagenase-treated H9 cells at lower seeding density (panel B) TEG treated cells again reached confluence more rapidly. Panel C shows the phenotype of H9 cells 90 hours after plating at 10E4 per 6cm dish. TEG-treated cells (Di) showed less differentiation and formed larger colonies than did collagenase-treated cells (Dii).

Figure 5. Flow cytometric analysis of Stem Cell Markers on H7 hESCs . A) passaged with collagenase or B) TEG.

Figure 6. RT-PCR analysis of expression of CD30 transcripts in hESCs. A) CD30 product B) □-actin product as a control for RNA integrity. Lanes are 1) no RNA control 2) positive control N-Tera 2, 3) positive control , 4)H9 hESCs passaged with collagenase , 5) H1 hESCs passaged with TEG, 6) H9 hESCs passaged with TEG, replicate A from first passaging experiment, 7) H9 hESCs passaged with TEG , replicate C from first passaging experiment, 8) H9 hESCs passaged with EDTA, replicate 2 from second passaging experiment, 9) H9 hESCs passaged with EDTA, replicate B from first passaging experiment (abnormal karyotype) , 10) H9 hESCs passaged with EDTA, replicate c from first passaging experiment (abnormal karyotype) , 11) negative control HTB cells. Position of molecular weight markers are shown on the left.

Figure 7. Flow-cytometric analysis of CD30 expression.A)i) K562, ii) N-Tera 2 iii)HTB, iv) H9 hESCs passaged with collagenase v) H9 hESCs passaged with TEG vi) H1 hESCs passaged with TEG vii) H9 hESCs passaged with EDTA, these cells were grown from replicate 2 from the second passaging experiment viii) H9 hESCs passaged with EDTA, these cells were grown from replicate B from the first passaging experiment (abnormal karyotype) ix) H9 hESCs passaged with EDTA, these cells were grown from replicate C from the first passaging experiment (abnprmal karyotype). B) CD30 staining

is affected by fixation treatment of the cells. The analysis was performed on at least 3 separate occassions, representative plots are shown.

Figure 8. Differentiation of H9 cells in vivo and in vitro. (A) Sections from tumours generated from, Collagenase- (a-f) or TEG-treated (g-n) H9 cells. Cartilage (a, g), mucus-secreting epithelium (b, m), pigmented epithelium (c), neuroglia (d), sebaceous gland (e, n), adipose tissue (h), smooth muscle (i), primitive neural tissue (j), ganglia (k), sweat glands (l). Bar = 50mm. B. Immunostaining of in vitro differentiated cultures from trypsin-treated H9 cells using primary antibodies against a-sarcomeric Actinin (a), Troponin 1 (b), cardiac Troponin T (c), b3-Tubulin (d), Neurofilament 200 (e), a-Fetoprotein (f).

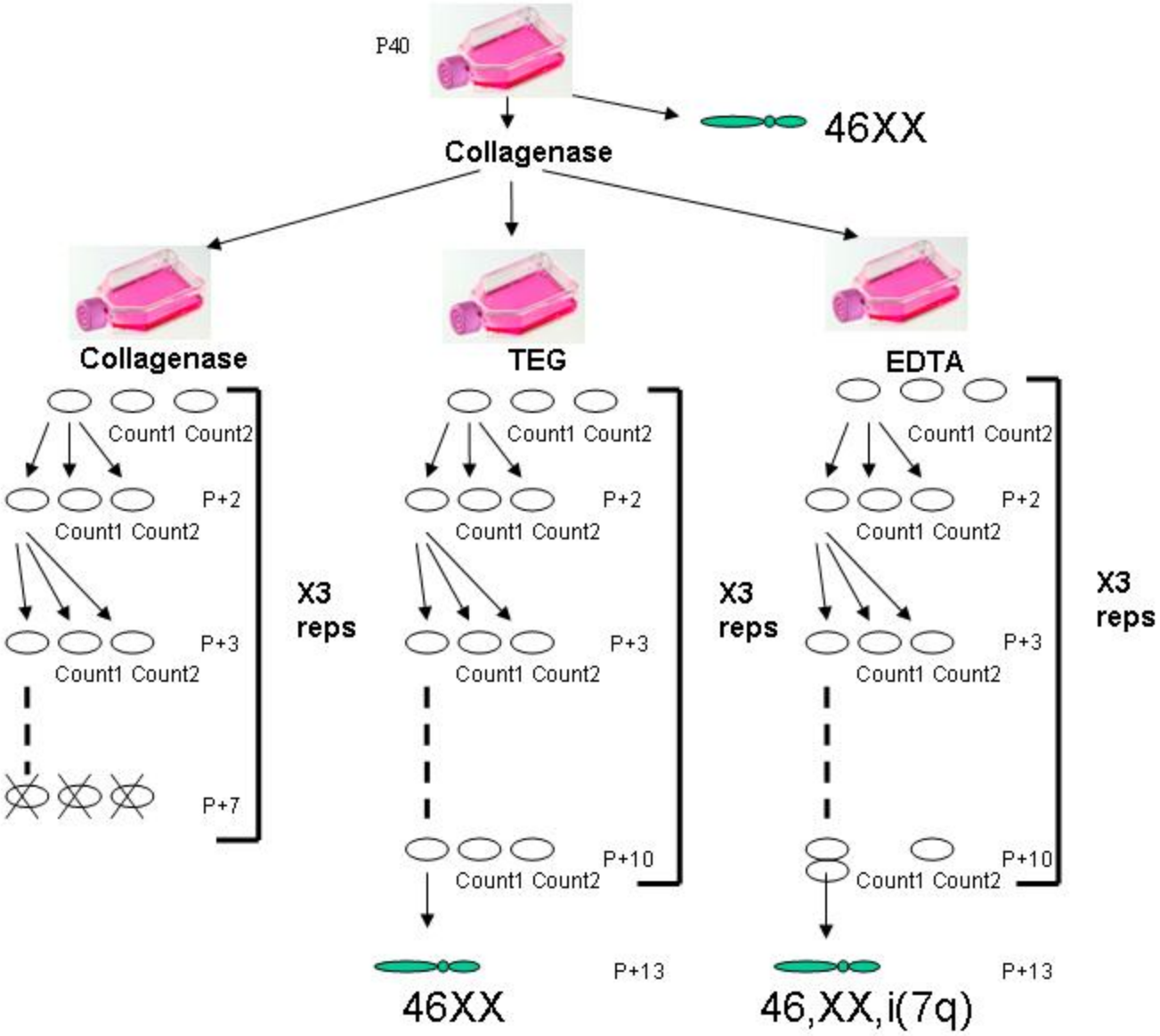

P40
46XX
Collagenase
Collagenase
TEG
EDTA
Count1 Count2
P+2
P+3
P+7
P+10
P+13
X3 reps
46XX
46,XX,i(7q)

Figure 1A

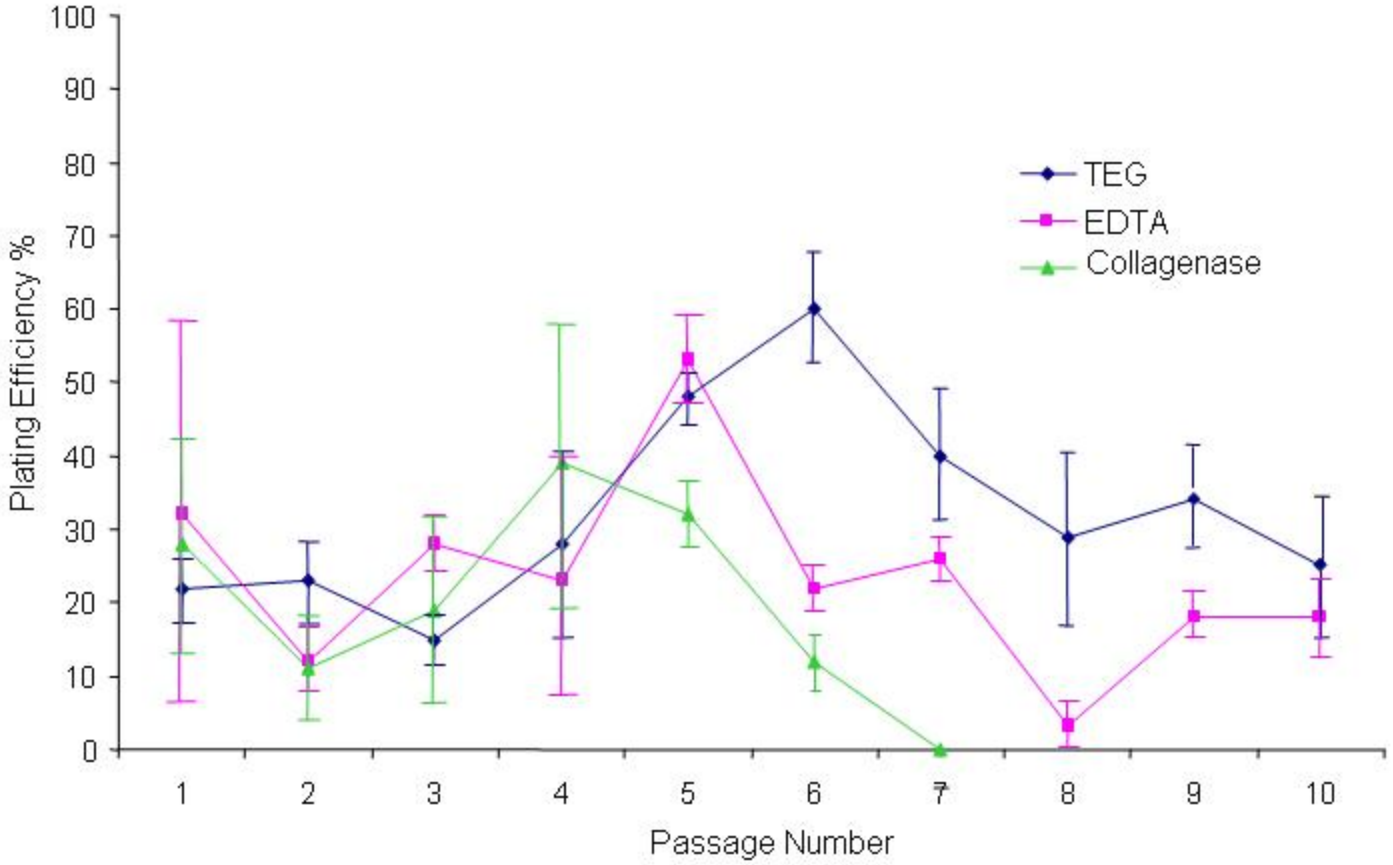
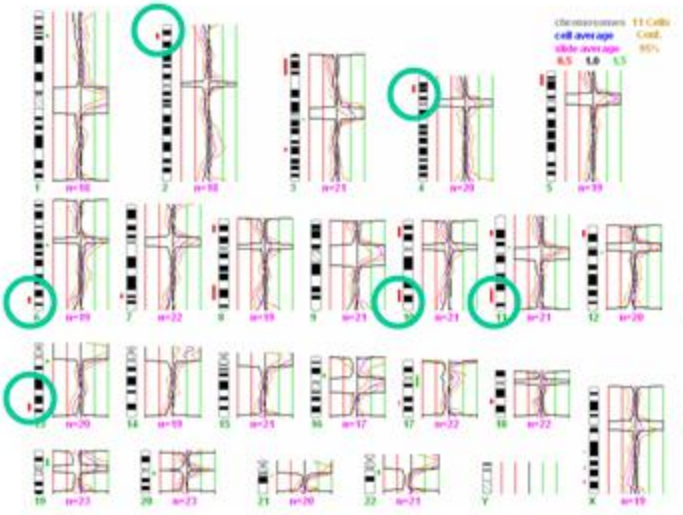

100
90
80
70
60
50
40
30
20
10
0
Plating Efficiency %
TEG
EDTA
Collagenase
1
2
3
4
5
6
7
8
9
10
Passage Number

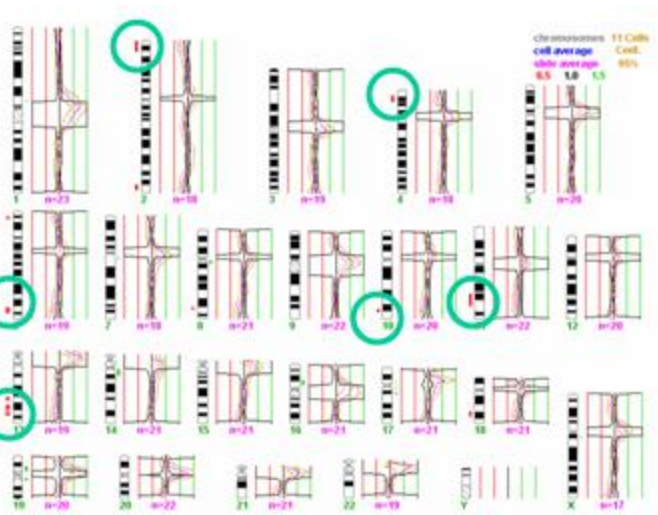
Figure 1B

Figure 2

A. i.



ii.



B.

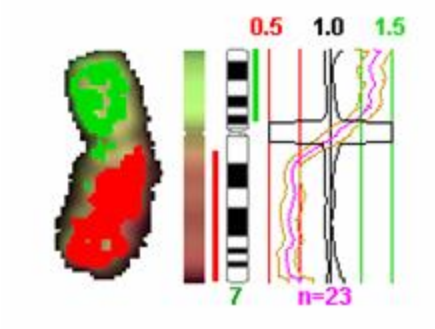


Figure 3

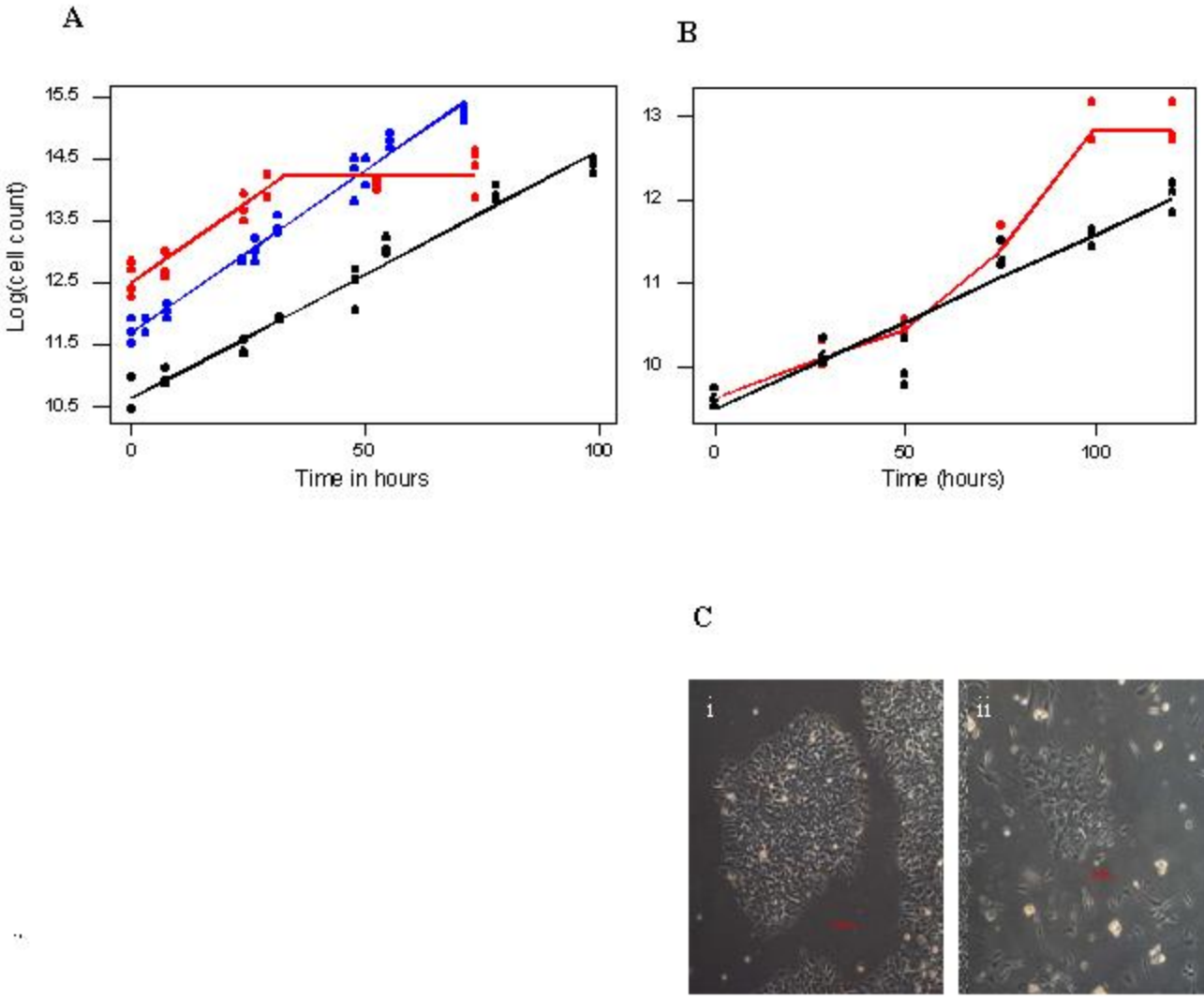
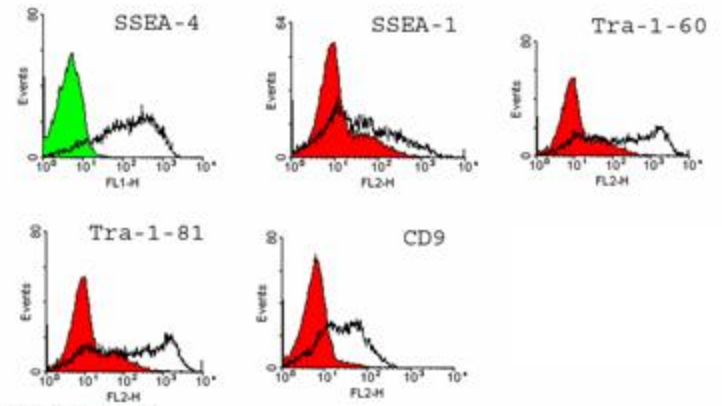

A
B
C
15.5
14.5
13.5
12.5
11.5
10.5
Log(cell count)
0
50
100
Time in hours
13
12
11
10
0
50
100
Time (hours)
i
ii

Figure 4

A) H7 Collagenase



B) H7 TEG

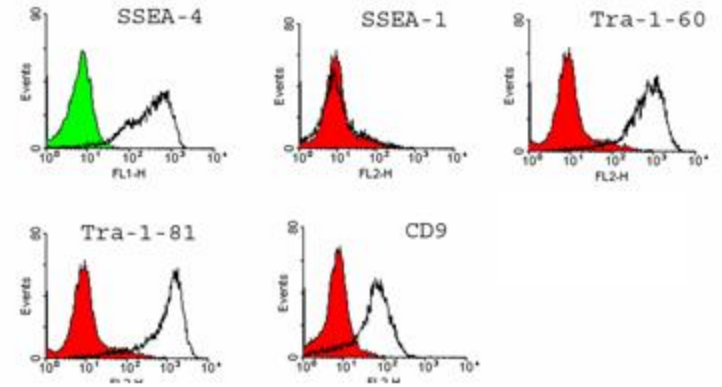


Figure 5

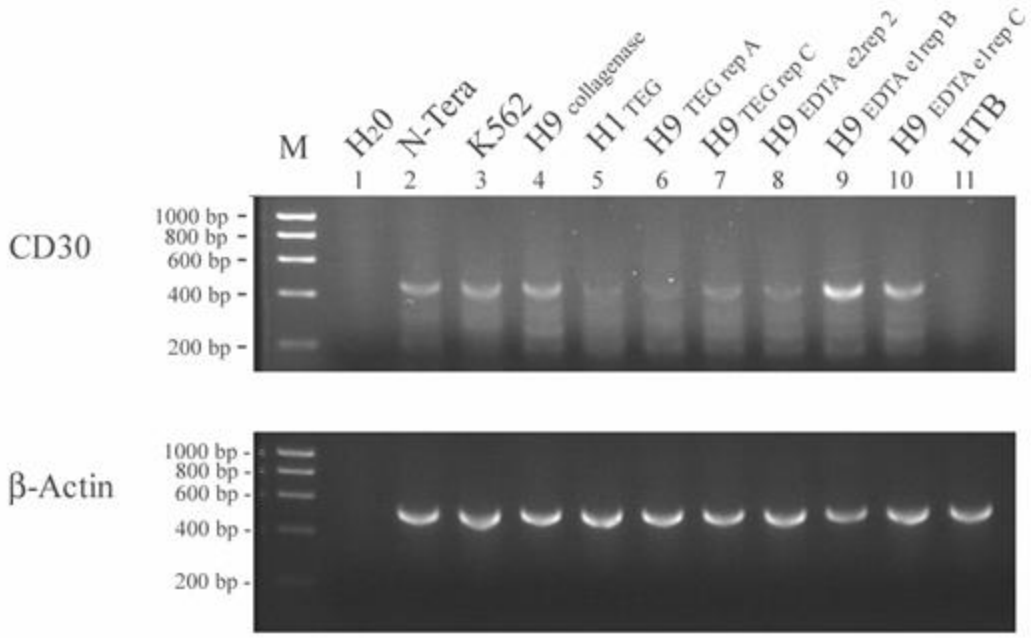

M
H20
N-Tera
K562
H9 collagenase
H1 TEG
H9 TEG rep A
H9 TEG rep C
H9 EDTA e2rep 2
H9 EDTA e1rep B
H9 EDTA e1rep C
HTB
1 2 3 4 5 6 7 8 9 10 11
CD30
1000 bp
800 bp
600 bp
400 bp
200 bp
β-Actin
1000 bp
800 bp
600 bp
400 bp
200 bp

Figure 6

A

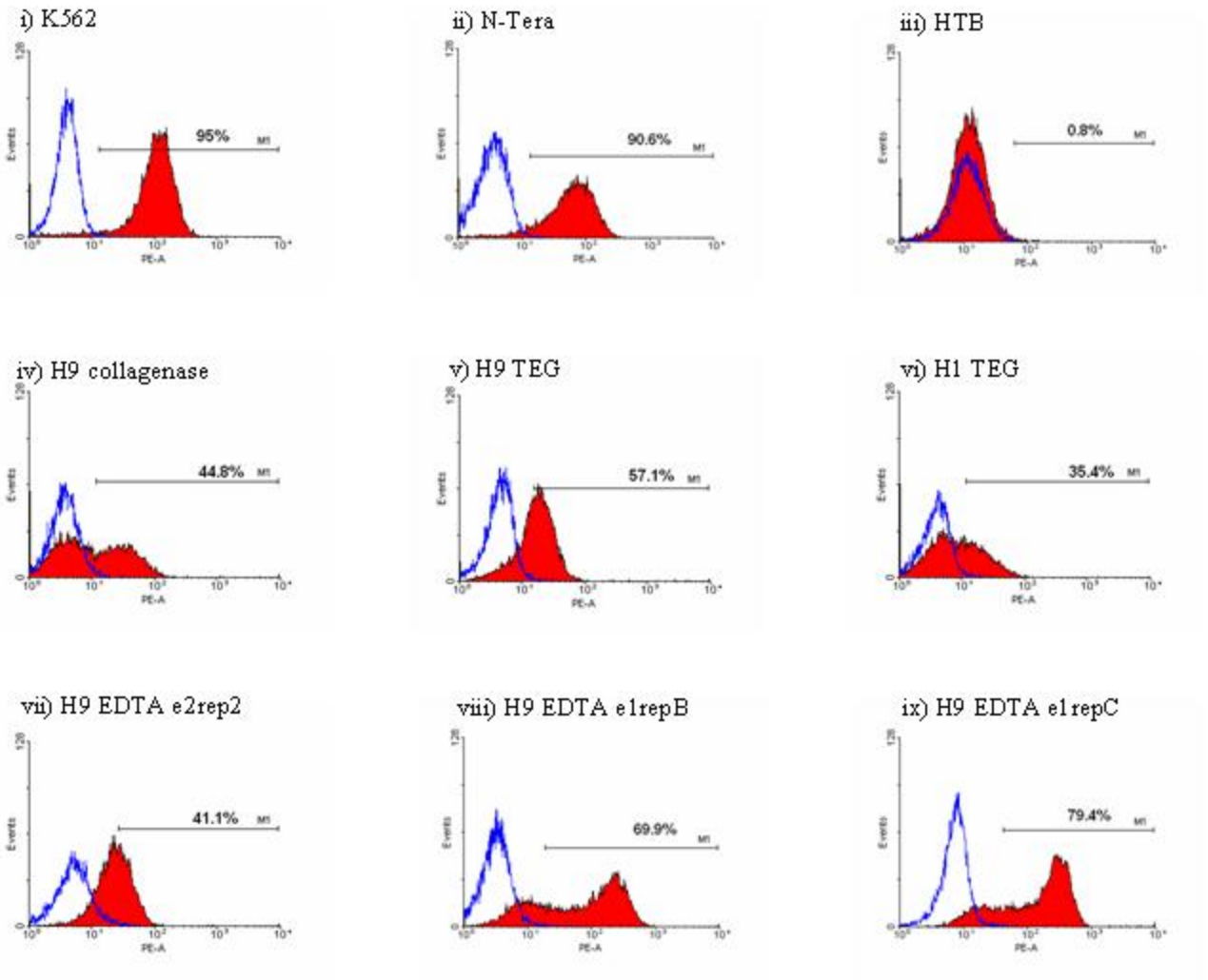

i) K562
95%
M1
ii) N-Tera
90.6%
M1
iii) HTB
0.8%
M1
iv) H9 collagenase
44.8%
M1
v) H9 TEG
57.1%
M1
vi) H1 TEG
35.4%
M1
vii) H9 EDTA e2rep2
41.1%
M1
viii) H9 EDTA e1repB
69.9%
M1
ix) H9 EDTA e1repC
79.4%
M1
Events
PE-A

Figure 7

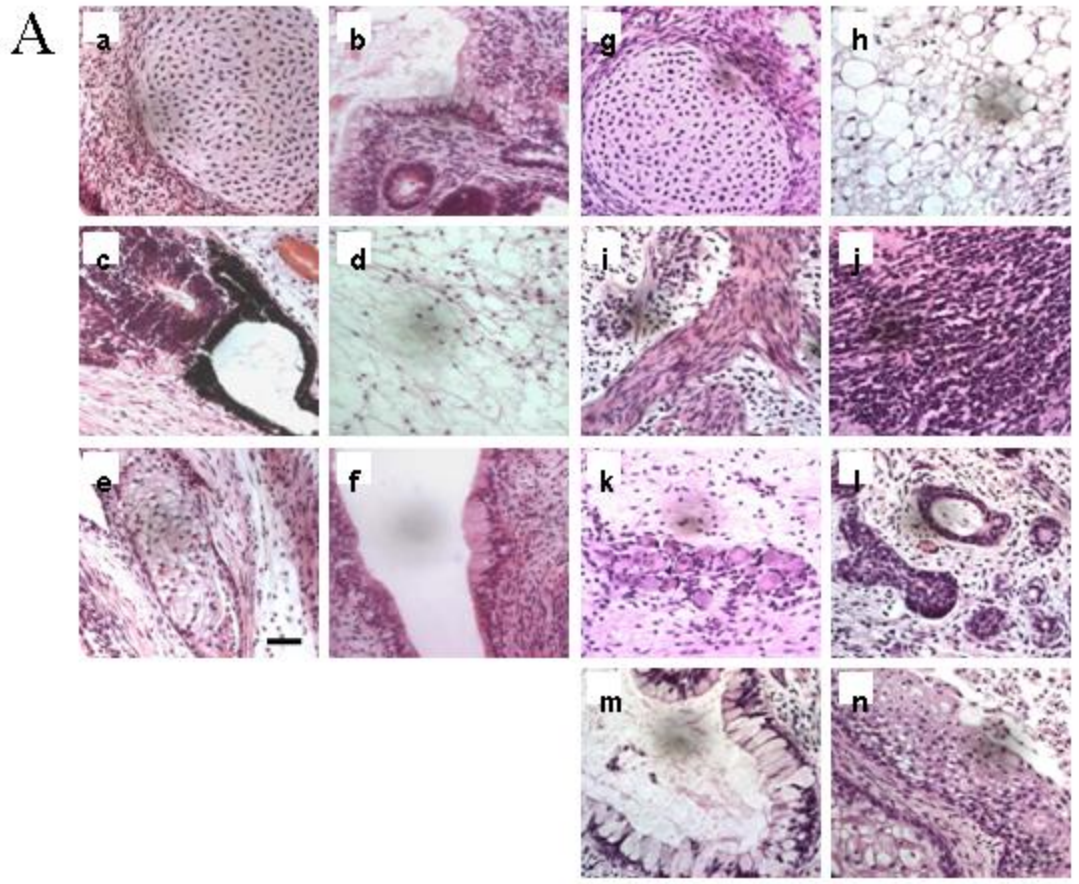


Figure 8