Haematopoietic differentiation of normal and PU.1 mutated murine embryonic stem cells using hydrogel encapsulation in a HARV bioreactor

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Abstract

Leukaemia is a hematologic malignancy consisting in the formation of abnormal hematopoietic cells initiated by a few leukemic stem cells (LSCs) that undergo a poorly regulated and aberrant differentiation process analogous to that of normal hematopoietic stem cells. In recent years, studies of blood neoplasms such as acute myeloid leukaemia (AML) have indicated how disruption of the normal function of transcription factors can affect normal cellular differentiation and lead to cancer. Several studies have shown that abnormalities in the expression of the transcription factor PU.1, along with other cooperative mutational events, can lead to the development of AML, suggesting the involvement of this regulatory network in the pathogenesis and development of the disease. Recent advances in stem cell bioprocess have led to the proposition that, by merging the benefits of animal models and *in vitro* cultures, the use of mouse embryonic stem cells (mESCs) would be a unique tool to overcome the limitations of current approaches to the study of leukaemia and follow a time wise progression of the disease "in a dish". The aim of this study was to provide insight on the behaviour of normal and PU.1 mutated mESCs when cultured *in vitro* using hydrogel encapsulation and a HARV bioreactor, differentiating them towards the hematopoietic lineage. This study provides initial data on the early manifestations of PU.1 related AML and can provide a valuable aid in diagnosis as well as introduce a time lapse of leukaemia development and progression.

Introduction

Haematopoiesis is a highly regulated process that gives rise to all cells of the blood lineage, comprising a delicate balance between self-renewal, proliferation and differentiation. Due to the short life span of mature blood cells, stem cells are required throughout an individual's life to assure the sustainability of the hematopoietic pool. This is a conserved hierarchical process, where hematopoietic stem cells (HSCs), a rare multipotent population residing in the bone marrow, are found at the top of the progenitor hierarchy, giving rise to multipotent and lineage-restricted progenitors that proliferate extensively and differentiate towards mature blood cells (Orkin & Zon, 2008). The hematopoietic, vascular, cardiac, and skeletal muscle lineages develop from subpopulations of mesoderm induced at different time points of embryonic development. All of the steps comprising this elaborate process are tightly controlled by an intricate regulatory network.

In leukaemogenesis, hierarchically, LSCs sit at the top (similarly to HSCs in haematopoiesis), giving rise to more differentiated, heterogenic leukemic blasts. These blasts are known for their high proliferative potential, a blockage in terminal differentiation and defective mechanisms of apoptosis or senescence, leading to their extreme accumulation and to the disease itself. Cancer stem cells share with normal stem cells their capacity for unlimited self-renewal, which led to the proposal that leukaemias may be initiated by transforming events that take place in hematopoietic stem cells (Passegué, Jamieson, Ailles, & Weissman, 2003). On another hand, leukaemias may also arise from the acquisition of stem cell-like characteristics in more committed progenitors (enhanced self-renewal and impaired maturation) due to mutations or selective expression of genes regulating these events. In order to understand the mechanisms and the pathogenesis of this disease it is necessary to identify how these aberrations are acquired, how they affect normal haematopoiesis and how they contribute to the aggressiveness of the disease.

In recent years, studies of blood neoplasms such as acute myeloid leukaemia (AML) have indicated how disruption of the normal function of transcription factors can affect normal cellular differentiation and lead to cancer (Tenen, 2003). Perturbation of the balance of the critical transcriptional factors that regulate haematopoiesis is a defining feature of the majority of leukaemias. It is important to note that leukaemia is not a consequence of nonspecific transcriptional actions, but the end result of a perturbation in their genetic information, usually caused by somatic mutations or chromosomal translocations. Examples of this are the consistent somatic mutations of *Notch* in T cell leukaemias (Weng, Ferrando, Lee, & Morris, 2004), *GATA-1* mutations in megakaryocytic leukaemia (Wechsler et al., 2002) and *PU.1* mutations in myeloid leukaemias (Nicola Bonadies, Pabst, Mueller, & Pru, 2010; Döhner et al., 2003; Mueller et al., 2003; Rosenbauer et al., 2004). Abnormalities in the expression of *PU.1*, a transcription factor of the ETS family known to be indispensable in myeloid differentiation, have been found to lead to the development of AML in mice and its downregulation to be present in human AML patients (Basova et al., 2013; Mueller et al., 2003). This suggests that the regulatory mechanisms involving *PU.1* expression may be involved in the development and pathogenesis of AML.

Studies carried out to determine the leukemic influence of mutations in this transcription factor have been conducted mainly through mouse knockout or knockdown models. These studies suggest that the complete absence of *PU.1* expression is fatal, and its expression below a critical level induces AML (Fisher & Scott, 1998). It has been proposed that the mechanism through which this occurs is related to the failure of *PU.1* mutant cells to express sufficient levels of essential growth factor receptors (Rosenbauer et al., 2004). It was reported that in animals of this model differentiation was blocked at a myeloblast-like stage when in the presence of interleukin-3 (IL-3), interleukin-6 (IL-6) and stem cell factor (SCF). This study also concluded that deletion of *PU.1* or haplo-insufficient expression does not induce leukaemia, but a narrow window of *PU.1* expression seems to be required for AML development.

The *PU.1* DNA-binding domain is known to be involved in protein-protein interactions with other factors, such as *GATA-1*, *c-Jun*, *Runx1* and *C/EBPa*. Particularly, the delicate antagonic expression of *PU.1* and *GATA-1* strongly influences the commitment towards a myeloid or erythroid fate (Rekhtman, Radparvar, Evans, & Skoultchi, 1999). Amongst other regulation mechanisms, *PU.1* comprises a conserved upstream regulatory element (URE) which has been reported to be a myeloid-specific enhancer. Studies have shown that the deletion of the URE reduces *PU.1* expression to 20% in the bone marrow of mutated mice (Rosenbauer et al., 2004) when compared to wild-type (WT) levels. Following studies have described associations between genetic abnormalities of this region and AML biology (N Bonadies et al., 2010; Steidl et al., 2007), stating that as a consequence of this deficient expression, the mutated animals presented cells with altered cytokine response, accumulation of immature myeloid cells and the promotion of recurring chromosomal changes that stimulate the growth of leukemic clones. Furthermore, proving that the reintroduction of *PU.1* was sufficient to restore normal myeloid differentiation of leukemic *PU.1*-knockdown cells, these studies suggest that increasing the expression of lineage determinant transcription factors above a critical level could constitute a therapeutic option to change cancerous cell fate.

When proposing to study the development of leukaemia, in particular AML, it is necessary to take into consideration the fast progression of the disease and its aggressiveness, which implies that it would be very difficult to track the disease in a patient. In this context, animal models of mutated specimens have been used for many years as an attempt to mimic what happens in the human condition. Although these provide a unique insight to the physical manifestations associated with the advances of the disease and allow the study of different stages of its progression, they lack the possibility to obtain a proper time lapse of the illness. Other study approaches rely on the use of human stem cells, a method that raises a lot of ethical questions. So far, studies conducted with primary AML samples have only detected rare cases of direct genetic inactivation of the *PU.1* gene, so to this point how these mutations occur and their influence in the biology of myeloid leukaemias remains unclear.

It is proposed that by merging the benefits of animal models and *in vitro* cultures, the use of mouse embryonic stem cells (mESCs) would be a unique tool to overcome the limitations of current approaches to the study of leukaemia and follow a time wise progression of the disease in the laboratory. Moreover, advances in the bioengineering of stem cells have come to stimulate the process of culture in a three-dimensional (3D) environment, aiming to mimic the natural surroundings of the cells *in vivo*. In fact, studies have shown 3D approaches to be more successful than the standard two-dimensional (2D) cultures in replicating the influence of specific environmental cues in the fate of HSCs (Di Maggio et al., 2011).

In this study, normal and PU.1 mutated mESCs derived from the same murine species were cultured in a system consisting of their encapsulation in calcium alginate beads and culture in a high aspect rotating wall vessel (HARV) bioreactor. The culture process of these cells was coupled with the supply of chemical and biochemical cues to direct their differentiation towards the haematopoietic lineage, including culture in HepG2 conditioned media (HepG2 CM) and the introduction of lineage-specific growth factors into the culture media. The protocol followed had been previously established in the laboratory for the haematopoietic differentiation of only E14Tg2α cells (Fauzi, Panoskaltsis, & Mantalaris, 2012), stressing the importance of also performing a comparison with the results derived from the culture of these cells. Firstly, this study validates the application of the protocol on E14Tg2α cells, studying the influence of the culture with HepG2 CM and also of different feeding schedules on the environment of the cells and on their differentiation. This was a first step required to ensure the correct differentiation of the normal and mutated cells from the mouse model and to guaranty that a significant comparison between cells could be drawn using this approach. The same protocol was then used to differentiate and compare E14Tg2α with wild-type (WT) and URE/URE deletion (URE) mutated ESCs.

This is a novel approach to the study of the development of leukaemia, aiming to follow

haematopoiesis from the embryonic development as a way to search for initial manifestations of the disease. This study provides initial results suggesting differences between the behaviour of normal and mutated cells. The results presented here suggest the interesting use of this platform to further create a time-wise study of the development and progression of the disease and provide a valuable aid in early diagnosis and treatment of haematopoietic malignancies.

Materials and Methods

Cell sources

Embryonic stem cells E14Tg2α (E14 thioguanine resistance; E14Tg2α, ATCC, Middlesex, UK Cells) from passages <20 were used. WT and URE mESCs were derived from the C57BL/6J laboratory mice model. WT refers to the control, as in cells derived from normal non-mutated mice, whereas URE refers to cells derived from mice with a URE/URE deletion, presenting a decrease in PU.1 expression of 80% (Basova et al., 2013)

mESC maintenance media

mESCs were cultured in Dulbecco's Modified Eagles Medium (DMEM) without sodium pyruvate (Gibco) supplemented with 10% fetal bovine serum (ES-FBS, Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin, 2 mM L-glutamine (Gibco), 0.1 mM β-Mercaptoethanol (Sigma-Aldrich) and 1000 U/mL of Leukaemia Inhibitory Factor (LIF) (Chemicon). All components were added to a bottle of DMEM except for LIF and Mercaptoethanol, which were always added freshly to the culture upon medium replacement.

WT and URE maintenance media

WT and URE ESCs were cultured and expanded regularly in Knockout Dulbecco's Modified Eagles Medium (Knockout DMEM) supplemented with 15% fetal bovine serum (ES-FBS, Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin, 2 mM L-glutamine (Gibco), 1% Non-essential amino acids (NEAA), 0.1 mM β-Mercaptoethanol (Sigma-Aldrich) and 1000 U/mL of Leukaemia Inhibitory Factor (LIF) (Chemicon). All components were added to a bottle of Knockout DMEM except for LIF and Mercaptoethanol, which were always added freshly to the culture upon medium replacement.

Hep-G2 Conditioned Media (CM)

Hep-G2 CM consisted of 50% mESC maintenance medium and 50% exhausted medium from Hep-G2 culture. Hep-G2 were cultured in mESC expansion media without LIF or β-Mercaptoethanol. Cells were seeded at a density of 5×10^4 cells/cm^2 in HepG2 expansion media, remained for 4 days without media exchange and then the media was collected. Prior to use, the media was filtered and 0.1 mM β-mercaptoethanol was added (Rathjen, 2004).

Early differentiation media

High glucose Iscove's Modified Dulbecs Medium (IMDM) was supplemented with 15% ES-FBS, 2 mM L-Glutamine, 0.45 mM Monothioglycerol (MTG), 10 mg/mL bovine insulin, 5.5 mg/mL human transferrin, 5 ng/mL sodium selenite (ITS supplement), 50 µg/ml ascorbic acid and 40 ng/mL mouse stem cell factor (mSCF). Only ES-FBS, L-glutamine and Pen/Strep were added to the bottle of media, as the rest of the components were added freshly.

Hematopoietic differentiation media

mESC early differentiation medium was supplemented with 10 ng/mL of mouse IL-3 and 3 U/mL of human erythropoietin (hEPO).

Semi-solid Methylcellulose media

IMDM was supplemented with 1% ES Methylcellulose (Stem Cell Technologies), 15% ES-FBS, 2mM L-glutamine, 150 µM α-Monothioglycerol (MTG), 1% bovine serum albumin, 10 µg/mL Insuline, 200 µg/mL human transferrin (BIT supplement), 150 ng/mL mSCF, 30 ng/mL mIL-3 and 30 ng/mL mouse IL-6.

Culture in 3D

After being expanded in 2D and reaching an adequate number for encapsulation of $1x10^7$ cells, cells were trypsinised from the flasks and ressuspended in 4 mL of a 1.1% alginate 0.1% gelatine solution. The solution is passed through a previously sterilised peristaltic pump system and droped at a low speed onto a 100 mM Calcium Chloride 10 mM HEPES and 0.01% Tween solution, gelling immediately to a bead like shape. The solution with the beads was then left to agitate for 6 minutes, washed twice with PBS and placed in static culture on a flask for one day befoe being transferred to the bioreactor.

The cells were cultured in the bioreactor for 21 days, starting with 3 days in Hep-G2 conditioned media, followed by 8 days in early differentiation media and finally 10 days in hematopoietic differentiation media, after which the cells are decapsulated and plated back on 2D, in semi-solid media methylcellulose media.

In order to release the cells from the beads, they were incubate with a depolymerisation buffer, consisting of 55 mM EDTA and 10 mM HEPES. The cells were incubated at 37ºC with occasional agitation for 10-20 minutes until the bead structure was no longer visible.

MTS proliferation assay

For 2D and 3D proliferation assays it was used the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) following the manufacturer's instructions.

CellTiter Glo proliferation assay

After some experimental evidence, supporting already published evidence (Huang, Chen, & Walker, 2004), that MTS assay may not be the most reliable

and optimized proliferation assay for 3D cultures, the CellTiter-Glo® 3D Cell Viability Assay (Promega) was used following the manufacturer's instructions (Promega Corporation, 2014).

Live/ Dead viability assay

Viability of cells inside the hydrogels was evaluated using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) following manufacturer's instructions. After the incubation period, the beads were washed twice with PBS and their fluorescence was observed through a Leica DM IL inverted phase microscope (Leica, Weitzlar, Germany) and record using analysisD sofware (Olympus, Munich, Germany).

Gene expression studies

Cells were decapsulated from the beads and each sample was prepared with 3-4x10^6 cells. The cells were washed, centrifuged and had the pellets stored at -80ºC until RNA extraction. Samples of RNAs from mESCs were extracted using three kits supplied by Qiagen: QIAshredder kit, RNeasy Mini Kit and RNase-Free DNAse set following manufacturer's instructions. After extraction and RNA quantification, total RNA was reversed transcribed into complementary DNA (cDNA) using the Reverse Transcription System (Promega, Southampton, UK) following the manufacturer's recommendations. The cDNA samples were then used immediately for PCR or frozen at -20ºC.To perform PCR amplification, the Sensifast SYBR HI ROX Kit from Bioline was used, following the manufacturer's instructions. Each sample was added onto a 96 well PCR plate and the strands were amplified for 1 cycle at 95ºC for 2 minutes followed by 40 cycles of 5 seconds at 95ºC, 10 seconds at 60ºC and 15 seconds at 72ºC. RT-PCR results were then analysed using the comparative CT method (Applied Biosystems, 2008; Schmittgen & Livak, 2008). The primers used to assess the expression of genes related to the differentiation process were ordered from Invitrogen based on sequences retrieved from the literature. Their sequence can be found in Table I.

Table I - List of primers used for RT-PCR analysis

Genes	Forward/ Reverse sequence
GAPDH	AGCCACATCGCTCAGACACC
	GTACTCAGCGGCCAGCATCG
PU.1	AGAGCATACCAACGTCCAATGC
	GTGCGGAGAAATCCCAGTAGTG
Gata-2	CCCTAAGCAGCGCAGCAAGAC
	TGACTTCTCCTGCATGCACT
Flk-1	CAACAAAGCGGAGAGGAG
	ATGACGATGGACAAGTACCC

Flow cytometry

Cells were either trypsinised from the flasks or released from the beads as previously explained. All the markers used were supplied by BD Biosystems and the manufacturer's instructions were followed for the staining of the cells. The samples were then read in the BD LSRFortessa analyser, compensation parameters were established using compensation beads from BD Biosystems and recorded results were analysed using the software FlowJo.

Immunocytochemistry

Cells were plated onto gelatinised 4 well chamberslides at a density of 5×10^4 cells/cm^2 in mESC maintenance media or WT and URE maintenance media. After 3 days of culture, the cells were fixed by incubation with 4% PFA for 20 minutes at room temperature and then permeabilised by incubation with 0.2% Triton-X-100 (VWR International Ltd.) for 20 minutes at room temperature. Afterwards, the cells were incubated with 3% blocking goat serum (Santa Cruz Biotechnology, Heidelberg, Germany) diluted in primary diluent (0.05% bovine serum albumin (BSA) (Sigma) and 0.01% sodium azide (NaN_3) (Sigma) in PBS) for 30 minutes at room temperature. Working solutions of the antibodies were prepared in primary or secondary diluent (0.05% BSA in PBS) solutions, immediately before staining. Following the blocking process, the samples were incubated with primary antibody at 4ºC overnight. The following day the cells were washed once and incubated with the secondary antibodies for 1 hour at room temperature in the dark. If double staining was performed, the steps since incubation with the primary antibody were repeated until here. The samples were then washed with PBS and mounted using Vectashield with 1.5 mg/mL 40,6 diamidino-2-phenylindole (DAPI) (Vector Laboratories, Peterborough, UK) and fluorescence was observed using the Olympus BX51 microscope (Olympus UK Ltd) and images were captured using the DP50 camera (Olympus UK Ltd).

Wright-Giemsa staining

Cells were collected from the flask and approximately 2x10^5 cells were loaded into the well of a cytospin cytocentrifuge set and were centrifuged for 3 minutes at 100g. When completely dry, the cells were stained with Wright Giemsa stain (Sigma-Aldrich) through dipping in 50% Wright-Giemsa stain for 30 seconds and washing by dipping in deionised (DI) water for approximately 10 minutes. Cells were then visualised on the Olympus BX51 microscope (Olympus UK Ltd) and images were captured using the DP50 camera (Olympus UK Ltd).

Colony forming assays

A number of 3x10^5 cells/ml cells were seeded into 35 mm culture plate (VWR International) containing semy-solid methylcellulose media, to assess the haematopoietic clonogenic capacity of the cells. The formation of different hematopoietic colonies was identified at 14-17 days of incubation in a fully humidified chamber at 37ºC.

Results

Effect of HepG2 conditioned media in the culture of mESCs encapsulated in hydrogel and cultured in a HARV bioreactor

E14Tg2α cells were cultured for 21 days in calcium alginate beads in a HARV bioreactor, using HepG2 conditioned media and lineage specific growth factors to induce the differentiation of mESCs towards the hematopoietic lineage.

First, the proliferation rates of the cells were measured over 5 days of culture in the bioreactor, comparing mESC maintenance media with HepG2 conditioned media using the MTS proliferation assay.

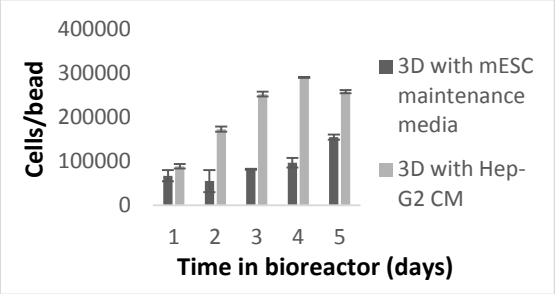

Figure 1 - Proliferation of encapsulated cells cultured in mESC maintenance media and HepG2 CM in the bioreactor

From this first experiment, it was observed that the cells maintain a high viability in culture even after 7 days and that the proliferation rates inside alginate beads and a HARV bioreactor are actually increased in HepG2 CM when compared to regular mESC maintenance media.

Another experiment conducted in this study has compared two different feeding schedules and their influence on the biochemical characteristics of the cells' environment throughout the culture period in the bioreactor. This has led to the conclusion that changing the media every three days did not result in the accumulation of extreme concentrations of toxic metabolites when compared to media changes every two days and did not affect cell proliferation. It was also concluded that the employment of this strategy did not lead to the complete depletion of the main nutrients required for cell maintenance and proliferation, opposite to what was suggested in previous studies. These experiments also resulted in the differentiation of Tg2α cells towards the haematopoietic lineage. To summarize, these results led to the reasoning that the best approach to use for the differentiation of ESCs derived from the normal and PU.1 mutated mouse models should be the use of HepG2 CM, calcium alginate encapsulation and culture in a HARV bioreactor, using the protocol previously established in the laboratory and media changes every 3 days.

Comparative analysis of URE mutant, WT and Tg2α using HepG2 CM, 3 day feeding strategy and alginate encapsulation and HARV bioreactor

When doing the comparative study, the first thing to assess was the initial state of the cells. This was done my measuring their pluripotency through the expression of Oct3/4 and SSEA1 using flow cytometry and immunocytochemistry analysis. The results can be seem in figures 2 to 5.

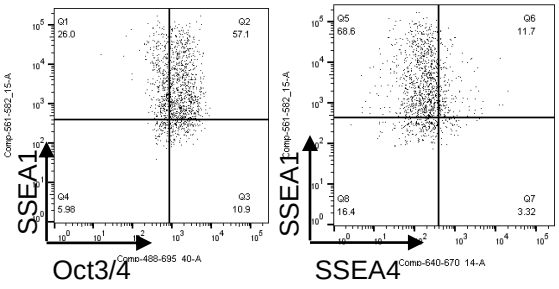

Figure 2 - Day 0 analysis of Tg2α cells

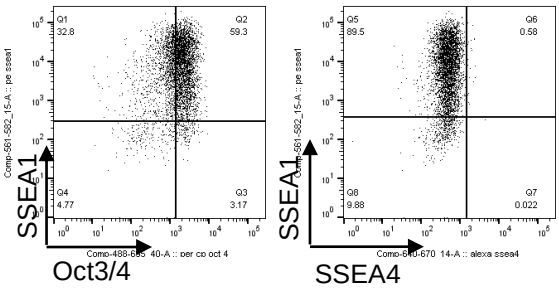

Figure 3 - Encapsulation day analysis of WT cells

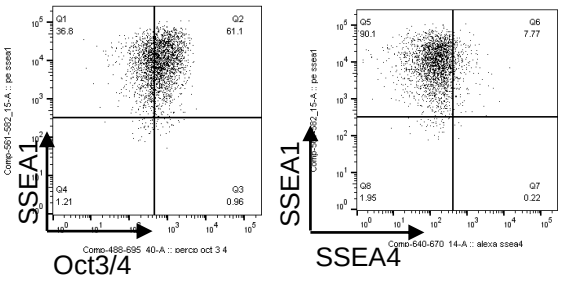

Figure 4 - Encapsulation day analysis of URE cells

mESCs are known to express the nuclear markers Oct3/4 and the surface marker SSEA1 (Cui et al., 2004) in their undifferentiated state, and start to express SSEA4 once they start to differentiate (Abcam, 2015; Gang, Bosnakovski, & Figueiredo, 2007).

In comparison with Tg2α cells, WT and URE cells presented low expression of pluripotency markers at day 0 of culture, so the assay was performed again on the day of encapsulation. From this data it is possible to conclude that by the day of encapsulation all cell types presented over 50% of Oct3/4+SSEA1+ expression, indicative of their pluripotent state.

To further confirm these results, immunostaining assays were also performed on the cells, staining for Oct3/4 and SSEA1 expression.

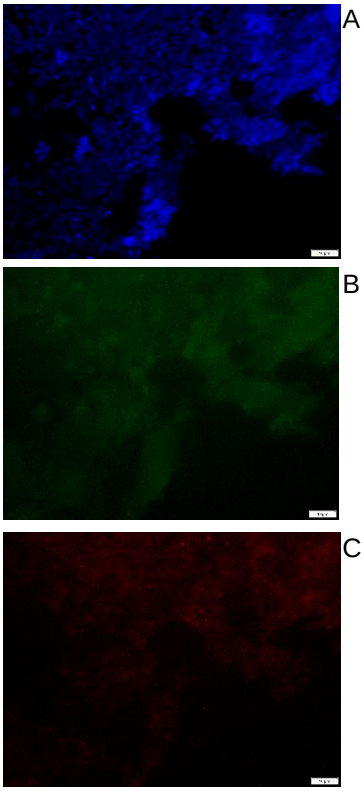

Figure 5 - Immunostaining for DNA with A - DAPI (in blue), B - Oct3/4 expression with FITC (in green) and C - SSEA1 expression with Texas Red (in red).

Oct3/4 is a nuclear marker, so it is possible to see its expression in green in the same areas as DAPI is staining in blue. These results are indicative of the pluripotency of the cells but the pictures taken should be improved. It is suggested that on following assays the pictures be taken in different conditions or the staining process be optimized. The results shown represent URE cells but similar results were observed for the other cell types.

Throughout the experiment, the cells were assessed for their proliferation capacity and their viability in culture, using the CellTiter Glo assay and the Live/Dead assay, respectively. The results can be seen on figures 6 and 7.

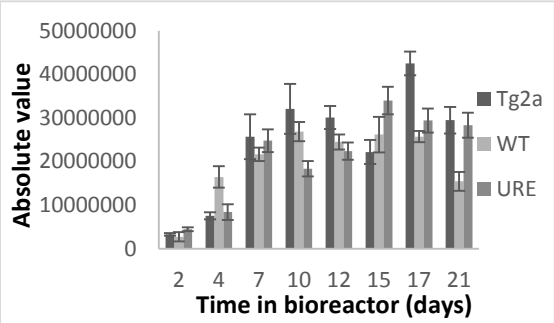

Figure 6 - Proliferation of Tg2α, WT and URE cells

The observation of the absolute values obtained from the proliferation assay and its direct proportionality to the number of cells in culture, suggests from figure 6 that the 3 types of cells exhibit exponential growth on the first 7 days of culture, followed by a stagnation in proliferation. By day 21 a decrease in cell numbers is observable for Tg2α and WT, but not significantly for URE ($p>0.05$).

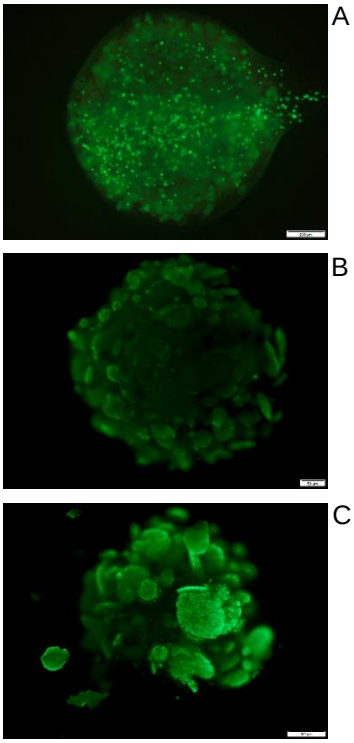

Figure 7 - Live/ Dead staining of cells inside the beads on days A – 4, b – 15 and C – 21 of culture in the bioreactor. Live cells stained with green and dead cells stained with red.

The Live/Dead assay performed on the cells indicates that all cell types were able to not only grow and proliferate in this system but also to form increasingly larger colonies inside the beads. Furthermore, since it is barely possible to see red expression in the pictures, it is evident that cell morbity inside the beads was not significant.

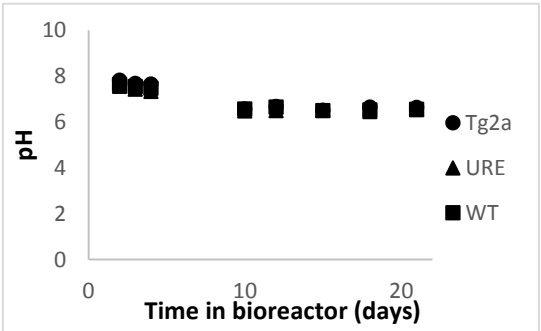

Figure 8 - pH levels in the exhausted media throughout culture period in the bioreactor

Studies performed with mESC in order to evaluate the effects of changes in pH and osmolality in culture during differentiation show that although a reduction of the pH levels to 6.8 does negatively impact the number of embryoid bodies produced at the end of the culture, it also shows the upregulation of pluripotency genes Nanog and Oct-4 when

compared to higher pH values (Chaudhry, Bowen, & Piret, 2009).From the plotting of the pH values in this graph it is noticeable that no significant differences were present in the culture media of the different cell types. The pH values were maintained between 6.5-7.5 and decreased as the change to IMDM media and to more sparse media changes was conducted.

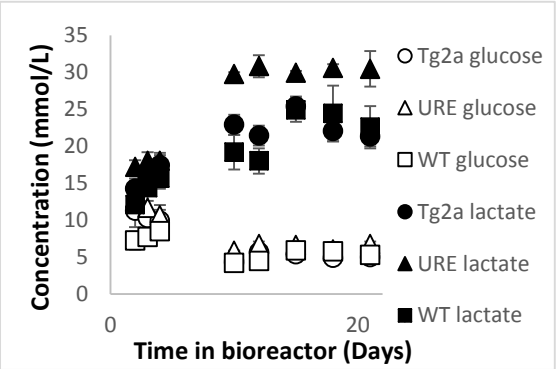

Figure 9 - Glucose and lactate levels present in the exhausted media throughout the culture period for the different cells

From the analysis of Figure 9, it is evident that the 3 cell types show a similar consumption of glucose throughout the culture in the differentiation media, but as Tg2α and WT show similar lactate production, URE presents much higher levels (on the order of 30 mM). Moreover, Tg2α and WT show a similar trend in their lactate production, while URE just exhibits a constant level of production throughout the culture period. It is necessary to compare these results with the glutamine consumption to search for any possible correlations and a further insight to the metabolic pathways of the cells.

The analysis of the graphs in 10 show for Tg2α and WT the same trend in the consumption of glutamine and production of ammonium that is found in the previous figure for the production of lactate, suggesting a probable correlation between the two. The same is observable for URE, as the levels of glutamine and ammonium present in the media remain constant. The difference here is found in glutamate levels, which do not comply with this pattern for URE cells. URE cells appear to produce high levels of glutamate when cultured in HepG2 media. These results seem to suggest that non-mutated cells, Tg2α and WT, may experience a shift in their metabolism during the culture period, as implied by the changes in the levels of metabolites in the media. The differences found when comparing the results of the mutated cells would be interesting to further delve into, as it is known that mutated cells do exhibit different metabolic requirements, in particular cancerous cells.

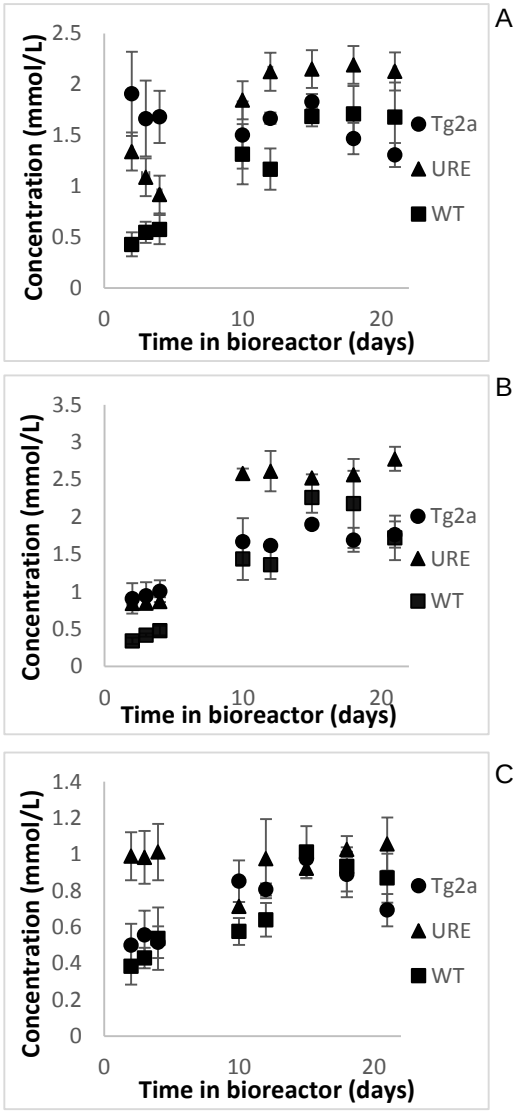

Figure 10 - Levels of A - Glutamine, B - Ammonium and C - Glutamate in the exhausted media throughout the culture period of the different cells

After 21 days of differentiation in the bioreactor, the differentiation efficacy of the protocol was measured through the assessment of the expression of haematopoietic markers in the cells through flowcytometry. The results are shown in figures 11 to 13.

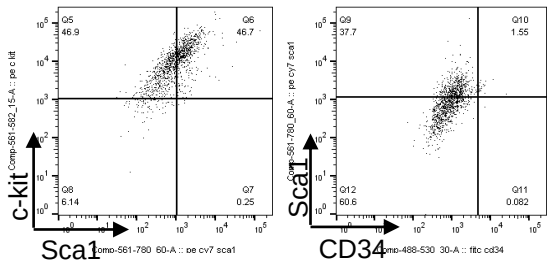

Figure 11 - Flow cytometry analysis of Tg2α cells after 21 days in the bioreactor for the expression of c-kit, Sca1 and CD34 haematopoietic markers

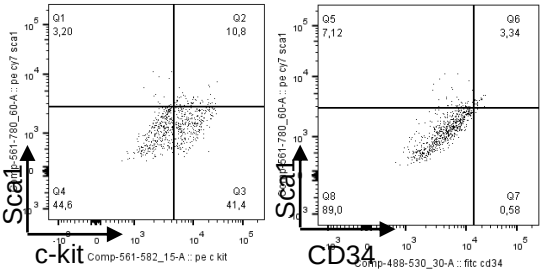

Figure 12 - Flow cytometry analysis of WT cells after 21 days in the bioreactor for the expression of c-kit, Sca1 and CD34 haematopoietic markers

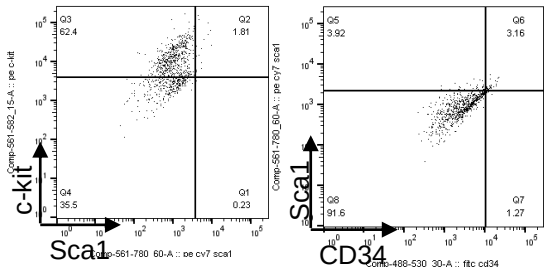

Figure 13 - Flow cytometry analysis of URE cells after 21 days in the bioreactor for the expression of c-kit, Sca1 and CD34 haematopoietic markers

From the analysis of the Tg2α cells, it is evident that most of the cells express c-kit, 45% are c-kit+Sca1+ and not even up to 2% express CD34. These results show that a high number of cells have differentiated towards the haematopoietic lineage. It was expected that the WT cells behaved very similarly to the Tg2α cells, since both cell types are derived from normal non-mutated laboratory mice, but WT cells seem to present a lower degree of haematopoietic differentiation than Tg2α. In these cells, only 10% show c-kit+Sca1+ and the majority of the cells do not express Sca1 at all. It is not possible to say if they have expressed it before and have lost the expression at this point, since no previous analysis was conducted with these cells, which should be performed on a next experiment. Similarly to what Tg2α cells show, only 3% of the cells express CD34, indicating that the cells are showing a lower degree of haematopoietic differentiation than it was before shown to be obtainable with this protocol. Observing the results from the analysis of the URE cells, even lower differentiation results can be seen. Most of the cells do not express Sca1 at all, similarly to WT cells, but in this case not even up to 2% of the cells express c-kit+Sca1+. Once again, the expression of CD34 is very low and close to 3%. These results suggest that the different cells are not exhibiting the same differentiation patterns and do not express haematopoietic markers in the same way after culture in the same conditions. On a first note, the experiments should be repeated and it should be ascertained why are WT cells behaving unequally from Tg2α cells. Moreover, these results suggest that it would be interesting to see the expression of these haematopoietic markers along the culture, for instance at day 12, to verify if they are being expressed before and not anymore at the end of the 21 days. Also, it would be interesting to plate the cells at day 12 and distinguish between adherent and suspension and maybe check their morphology as well.

The cells collected on day 21 were also analysed for the genetic expression of the mesoderm marker Flk-1 and for the expression of PU.1 and GATA2, to verify if their expression was opposed. These was determined by PCR and the results are shown in figure 14.

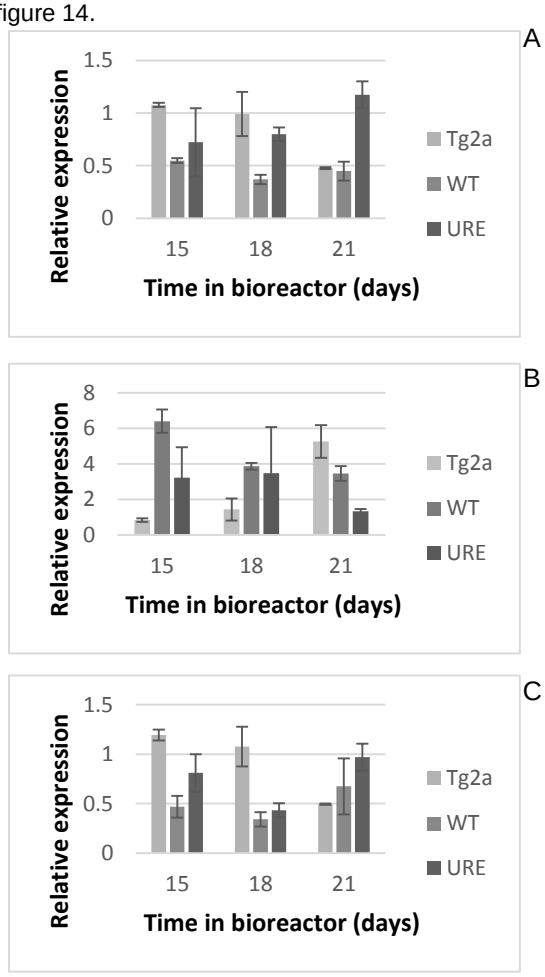

Figure 14 -A - Flk1, B – PU.1 and C – GATA2 expression levels on Tg2α, WT and URE cells on days 15, 18 and 21 of culture in the bioreactor

For Flk1 expression in Tg2α, the expression on day 21 is significantly lower than on the previous days ($p<0.05$) but the expression of the previous days is not significant between each other. For the expression in WT none of the values are significantly different from each other neither for URE. Although the expression levels of Flk1 do not appear to change between days for WT and URE they are significantly different between each other, apart from day 15, suggesting that they express the Flk-1 gene at different levels, and URE expresses it at higher

levels. Flk-1 is a mesoderm marker, so the fact that URE cells express high levels of it are consistent to the previous flowcytometry results, suggesting that these cells have yet to fully differentiate to haematopoietic progenitors and are at a more primitive stage than the other cell types. For PU.1 expression, there is a significant increase in expression in Tg2α on the last day of culture but for the other cell types the changes in expression are not significant. No significant differences in PU.1 expression were found between WT and URE in any of the days, although a 20% reduction in expression was expected in URE. Despite this, the expression values of PU.1 on day 21 are significantly lower in URE than in Tg2α. Regarding GATA2 expression, there is a significant decrease in expression in Tg2α on day 21, confirming that as PU.1 expression increases, GATA2 expression decreases. The difference in the expression values is not significant for WT but there is a significant increase of expression in URE from day 18 to day 21 of culture. Unfortunately, once again it is not possible to see a clear difference between the expression levels of WT and URE cells, although the expression levels on day 21 are significantly different between URE and Tg2α, meaning that the cells from the mutant model are expressing higher levels of GATA2. These results confirm the antagonic expression of PU.1 and GATA2, since the lowered expression of PU.1 in the mutated cells corresponds to an increased expression of GATA2.

To further characterize the cells from the URE/URE mutant, the cells were cultured in 2D and the cells in suspension were collected and stained with Wright-Giemsa. The pictures taken of the stained slides can be seen in figure 15.

When comparing with literature (Mcgarry, Protheroe, & Lee, 2010), it is possible to identify from the image cells with similar morphology to erythroid progenitors, indicating that after 21 days of differentiation the URE cells are exhibiting signs of haematopoietic differentiation and a shift towards the erythroid lineage. This is very interesting, because the results previously published suggested that a following step of culture in methylcellulose was required in order for the cells to fully mature and differentiate and these results come to suggest that it would be interesting to analyse the morphology and the types of cells that are being produced after the bioreactor culture. On further experiments it would be interesting to compare these results with the results from WT and Tg2α after 21 days, to see what are the differences regarding the type of progenitors that are being formed.

To assess the capacity of the cells to form haematopoietic progenitors. The colonies formed were observed throughout several time points and were scored and photographed on day 14. Examples

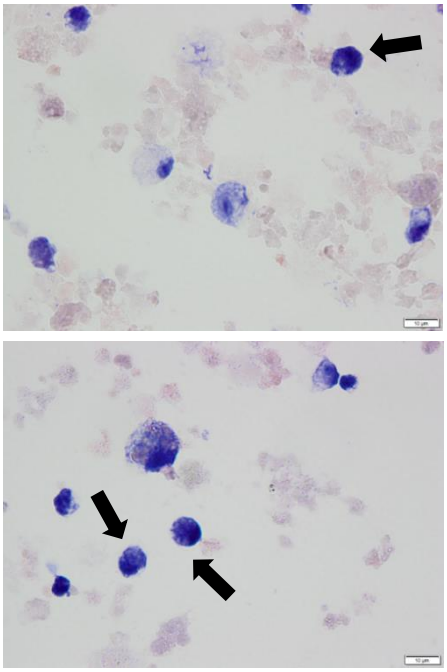

Figure 15 - Pictures of Wright-Giemsa stain on URE cells after 21 days of culture in the bioreactor. Scale: 10 µm

of the colonies formed can be seen on figure 16.

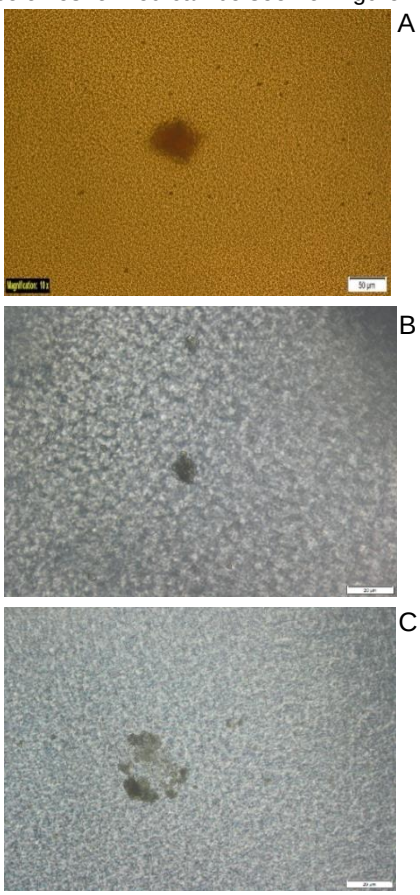

Figure 16 - Examples of colonies formed after 14 days of culture on methylcellulose of A - Tg2α, B - WT and C - URE cells

From the analysis of the methylcellulose plates it was found that for Tg2α, there were some embryoid-body like (EB-like) colonies present but there were also some burst-forming unit-erythroid (BFU-E) colonies identifiable. Moreover, some of the BFU-E colonies showed signs of haemoglobinization (see figure 16-A). When comparing WT results with the ones observed for Tg2α, it is possible to see that these cells have not matured as much, since no pink-redish collonies can be observed. Also, there was a higher number of EB-like colonies found instead of erythroid progenitors. Analysing the results found in the culture of URE in methylcellulose, the morphology of the colonies formed is very interesting and distinct from the ones found in the other two cell types. It following experiments it will be recommended to further analyse and characterise these colonies. From this assay, it is possible to conclude that WT and URE cells show the presence of very immature cells that still result in the formation of EB-like colonies and that URE cells show colony morphology different than the other cell types.

Conclusions

From the first stage of this project, it was possible to conclude and confirm the efficacy of the use of HepG2 CM as an early haematopoietic differentiation enhancer on E14Tg2α cells. The cells exhibited high viability and increased proliferation when cultured in HepG2 CM. It would be interesting to assess the effects of this media in the WT and URE cells studied in this project, in order to determine the influence of this approach on the final differentiation efficacy of the protocol.

From the comparison between the different cell types, it was possible to observe that the differentiation efficacy shown for Tg2α cells was not the same for WT cells, which should have ideally shown similar results. Although this seems to be the case, only one experiment was conducted with each cell type, so it is advised that they be repeated as to confirm these results. One of the interesting results coming from this project was to verify that the reduced expression of PU.1 in the cells of the mutated model express higher levels of GATA2 during the process of haematopoietic differentiation. These results suggest that when conducting following experiments it would be very interesting to follow the expression of PU.1 and GATA genes from the pluripotent stage of the cells onto their differentiated state and not just on the final stage of the bioreactor culture. The results drawn from the flowcytometry analysis suggest that it would also be interesting to follow the changes in the expression of surface markers associated with haematopoietic differentiation throughout the culture period in the bioreactor. Also, one of the most interesting results from these experiments was the fact that it was shown that it is possible to induce the differentiation of the cells into a haematopoietic and erythroid fate in the 21 day culture period by the Wright-Giemsa results. This staining procedure should be repeated for Tg2α and WT cells at this period of the culture. To summarise, this work has provided initial results promising the application of this model to the study of the development of leukaemia, by following the differentiation process of mutated cells and comparing it with non-mutated ones. It is a simple, controlled model that allows for easy and multiple time point analysis, making it useful for the establishment of time wise changes in the characteristics of the cells. Overall, it was possible to show that mESCs comprising a mutation in the URE of the PU.1 gene show differences in their haematopoietic differentiation process and validated the continued study and analysis of this mutation.

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