Understanding the role of miRNA expression in the differentiation of hematopoietic stem cells from pluripotent stem cells

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Abstract

Hematopoietic diseases such as leukaemia can be treated by a haematopoietic stem cell transplant, but suitable donors are in short supply. Hematopoietic cells derived from pluripotent stem cells have been suggested as a possible alternative, however hematopoietic progenitors made this way have so far been unable to either engraft in sufficient numbers or differentiate into all mature blood lineage types *in vivo*.

Recent published evidence suggests this may be due to a failure to down-regulate key miRNAs. Microarray data comparing hematopoietic progenitors derived from bone marrow or from pluripotent stem cells was studied and key miRNAs were selected for validation.

This combined analysis showed that several miRNAs fail to down-regulate in pluripotent cell derived progenitors, unlike the equivalent cells in bone marrow. These miRNAs play a role in suppressing epithelial-mesenchymal transition and their high expression may be a cause of the observed failure to engraft.

Five of these miRNAs were chosen as candidates for inhibition (miR-200b, miR-200c, miR-148a, miR-205, and miR-424). Methods of miRNA inhibition were investigated, and the effects of the inhibition of the chosen miRNAs on hematopoietic development from hESCs and hiPSCs were assessed using gene expression, marker expression and colony assay techniques.

Although miRNAs were successfully inhibited using lipofection both individually and in multiplex these experiments did not significantly improve the efficiency of hematopoietic development, possibly due to high levels of redundancy among tumour suppressors. The limitations of the inhibition technique, i.e. the short timeframe of inhibition and inability to down-regulate more than 5 miRNA at once may be insufficient to overcome this problem.

Differentiation in hypoxic conditions was tried in the hope of making more global changes to miRNA expression, and these experiments yielded an increase in CFU-GEMMs and cells expressing HSC markers, which may be helpful in future attempts at hematopoietic stem cell generation.

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Table of abbreviations	
AGM	Aorta gonad mesonephros
ANCOVA	Analysis of covariance
ANOVA	Analysis of variation
BM	Bone marrow
CAFC	Cobblestone area forming cells
CB	(umbilical) Cord blood
CFU	Colony forming unit
EB	Embryoid body
EHT	Endothelail hematopoietic transition
EMP	Erythro-myeloid progenitors
EMT	Epithelial mesenchymal transition
GVHD	Graft vs. host disease
H9	hESC line
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
HSC-HE	Haemogenic endothelium with HSC potential
HUVEC	Human umbilical vascular endothelial cells
LT-HSC	Long-term repopulating HSC
miRNA	micro-RNA
MOI	Multiplicity of infection
MPP	Multi-potent (hematopoietic) progenitors
NHDF	Normal human dermal fibroblasts
NSG	NOD-SCID-gamma null mice
P-HPC	Pluripotent stem cell derived hematopoietic progenitor cells
PSC	Pluripotent stem cell
qPCR	Real time quantitative polymerase chain reaction
SB-Ad3	hiPSC line
ST-HSC	Short term HSC

1. Introduction

1.1 Brief introduction into hematopoietic stem cell transplantation

Bone marrow transplants are a lifesaving treatment for leukaemias and bone marrow deficiencies [11]. The cells which are transplanted are called hematopoietic stem cells (HSCs), which produce all the blood cell types found in adults including erythrocytes, which carry oxygen around the body, and the white blood cells which make up the immune system. HSC are extracted from donors' bone marrow or from banks of frozen umbilical cord blood and transferred to the patient, where they migrate to the bone marrow and engraft, reconstituting the hematopoietic system with healthy cells. Because the transplant produces immune cells the donor and recipient must be genetically matched for histocompatibility antigens, or the transplanted immune system will recognise the patients' own cells as foreign, and attack them. This phenomenon is known as graft vs. host disease (GVHD) and it can be chronic or deadly. Unfortunately perfect genetic matches are rare and can take months to find. Umbilical cord blood cells are immunologically naive so they have a lower risk of GVHD, but multiple donors are required because a single umbilical cord does not have enough HSCs to treat a patient [12], and it is extremely difficult to expand HSCs in culture. Due to these problems alternative sources of HSC are being sought; one of the most promising of these is to generate them from pluripotent stem cells.

1.2 Pluripotent stem cells

Pluripotency: Pluripotent stem cell (PSC) is the term used to describe both embryonic and induced pluripotent stem cells (ESC and iPSC respectively). These cells resemble those found in the pre-implantation (blastocyst stage) embryo, they are uniquely useful due to their ability to proliferate indefinitely in culture and their capacity to give rise to any adult cell type, a quality known as 'pluripotency'. PSCs have many potential uses in both basic research and in medicine. For example differentiation of PSCs *in vitro* can shed light on early development, studying the behaviour of differentiating stem cells from patients with genetic diseases can illuminate their mechanisms, and drug discovery can be aided by *in vitro* testing on key cell types derived from pluripotent cells. Finally, the use of PSCs opens up the field of regenerative medicine, where replacement tissues, including HSCs, may be grown to replace damaged ones.

Derivation and culture of pluripotent stem cells: Embryonic stem cells are derived from the inner cell mass of blastocysts [13] (see **Figure 1.1**). Cells in this early embryonic stage are unique, as at any later stage the cells will be limited in the number of times that they can divide and committed to a particular lineage. Once differentiated, cells are unable to change to

another cell type. However, in 2007 Shinya Yamanaka [14] and Jamie Thomson [15] showed that human adult cells can be reprogrammed back into the pluripotent state by expressing 4 key pluripotency factors (*OCT4, SOX2 KLF4* and *c-MYC*) introduced into the cell as viral transgenes. These cells are called iPSCs and have similar morphology and gene expression to ESCs as well as indefinite ability for self-renewal. Since 2007 techniques for reprogramming have progressed, using non-viral or non-integrating techniques with the aim of increased efficiency and reduced risk of mutagenesis. It has also been discovered that other factors can be substituted for the original 'Yamanaka factors' and in some cases some factors can be eliminated entirely. These techniques are described in **Table 1.1**.

Figure 1.1

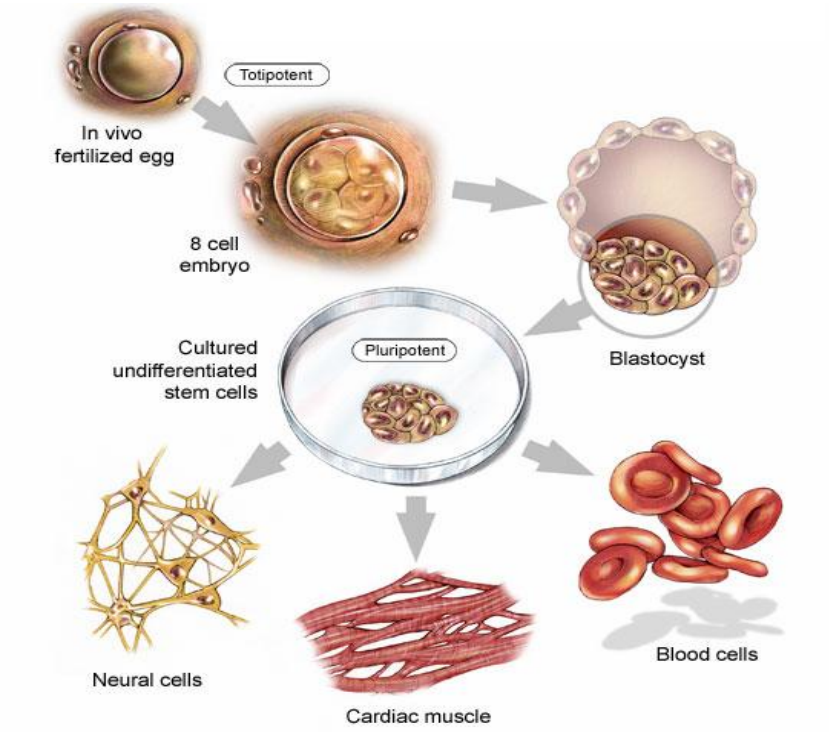


Figure 1.1: Schematic diagram showing derivation and potential of ESCs (adapted from Ref. [4]). A fertilised oocyte is allowed to develop to the blastocyst stage and then the ICM is removed and cultured. These pluripotent cells can be induced to differentiate into many cell types including neurons, cardiac cells and blood cells.

Table 1.1: Techniques for reprogramming			
Type of vector	Factors (O-*OCT4*, K-*KLF4*, S-*SOX2*, N-*NANOG*, L-*LIN28* and M -*c-MYC*		References
Integrating virus			
Retroviral	OKSM	Established technique, good efficiency, random integration of genes, incomplete	[16]

		silencing	
Lentiviral	OSNL	Higher efficiency than retroviral vectors, allows reprogramming of non-dividing cells, random integration of genes, incomplete silencing	[15, 16]
Adenoviral	OKSM	Low likelihood of integration, low efficiency	[17]
Non-integrating virus			
Sendai virus	OKSM	Good efficiency, non-integrating	[18]
Non-viral methods			
Episomal plasmids	OSKMNL	Very low efficiency	[19]
piggyBac transposon	OKSM	Medium efficiency, precise transgene deletion, labour intensive	[20]
miRNA	miR-200c, miR-302 family	Very low efficiency	[21]
Synthetic mRNA	OKSML	Medium efficiency, no antiviral response, time and labour intensive	[22]
Protein	OKSM	No risk of immune response associated with RNA or DNA, low efficiency, expensive	[23, 24]
Small molecules	CHIR, FSK, PD0325901, VPA, Tranylcypromine, TTNP, B616452, DZNep,	Low cost, no immune response, not yet proved in human cells	[25]

Both types of hPSCs will spontaneously differentiate unless carefully cultured. Human PSCs (hPSCs) form round, flat colonies which were originally kept on a feeder layer of mouse embryonic fibroblasts alongside media supplemented with serum and bFGF. More recently feeder cells and serum have been replaced by chemically defined animal free equivalents, which reduces variation and allows the cells to be used clinically.

Once a line of PSCs is established researchers can differentiate the cells into the lineage or cell type that they wish to study. This is done by mimicking the conditions which promote differentiation to a particular tissue in the developing embryo, usually by exposing the cells to various combinations of cytokines.

PSC could be used to make HSC for transplant: If an efficient technique for deriving HSC from PSC was developed the proliferative capacity of PSC would allow unlimited numbers of HSC to be generated, solving the problem of limited HSC numbers. The PSC would still need to be genetically matched to the patient, but any cells produced this way would be completely immunologically naive, reducing the need for a perfect match. It would also be possible to bank large numbers of PSC lines with many combinations of histocompatibility antigens. Finally, as reprogramming techniques improve in safety and efficiency it may eventually be possible to derive iPSC from a patient's own cells, which would completely remove the risk of GVHD.

Challenges must be overcome before PSCs can be used clinically: PSC derived treatments have considerable clinical potential, but there are barriers to their use in regenerative medicine. The most concerning is the possibility of transplanted cells giving rise to tumours. The constant proliferation of PSCs in culture can lead to genetic [26] and epigenetic [27] mutations which are potentially tumorigenic [28]. Undifferentiated PSCs are themselves tumorigenic; their proliferative capacity and undifferentiated state means they form teratomas *in vivo*, so any PSC derived graft with cells which are not fully differentiated is at risk of creating tumours. Another risk is that an imperfect differentiation protocol may produce cells which appear to be the correct cell type but which are aberrant in some way which causes them to behave unpredictably *in vivo*.

The use of hESCs for regenerative medicine is complicated by ethical and political obstacles as they can only be made by destroying a human foetus, and also by the possibility of immune rejection of the transplant, as it is likely to be difficult to find a perfect genetic match for every patient.

hiPSCs have been suggested as a solution to the ethical problem, as they can be made from cells derived from adult volunteer donors, and also to the problem of immune rejection as they could theoretically be derived from the patient's own cells. However reprogramming techniques are imperfect, and the reprogramming techniques with the highest efficiency use integrating viruses (see **Table 1.1**) which carry a risk of insertional mutagenesis. The stress of

reprogramming itself can also introduce mutations [29]. iPSCs also retain epigenetic markers from their tissue of origin after reprogramming, which can have unpredictable effects on gene expression [30, 31]. Any PSC derived cells will have to be carefully screened for mutations and gene expression before they can be used for transplant.

PSC derived treatments have clinical potential: In spite of these problems stem cell research has progressed to the point that it is possible to conduct clinical trials. The first of these trials was performed by Geron Corporation, using hESC derived oligodendrocytes for spinal cord repair. Although the initial results were positive, showing no adverse effects on the patients even after immune suppression was withdrawn, there was no clear improvement in the patients' condition and the trial was ended for financial reasons [32]. The trial has recently been picked up by another company, Asterias, and is ongoing [33].

The most successful trials of PSC treatments so far have used hiPSC derived retinal pigment epithelium to treat macular degeneration. A phase I trial showed excellent results, with no immune rejection and an improvement in vision for the majority of the patients [34]. Further trials, including a phase II trial are ongoing and of the 13 current clinical trials involving PSCs, 9 of them are treating macular degeneration [35].

A report of a case where hESC derived cardiac progenitors were used to treat heart failure showed positive results [36], and other trials, including one using hESC derived insulin-producing β-cells [33] are due to begin soon. For many of these studies it is too early to say if the treatment is effective but the lack of adverse effects and immune rejection suggests that pluripotent stem cells can be used safely, which means that if an effective protocol for differentiating hPSC to HSC was developed it could be translated to the clinic.

1.3 Hematopoietic differentiation from pluripotent cells

1.3.1 HSCs are at the top of the hematopoietic hierarchy and reside in the bone marrow niche

Several types of hematopoietic cells can be differentiated from PSC. HSC are cells at the very top of the hematopoietic hierarchy and they differentiate through various stages before committing to a particular hematopoietic lineage. 'Long-term repopulating' HSCs (LT-HSCs) are defined by their ability to repopulate transplanted bone marrow and reconstitute haematopoiesis indefinitely. These cells reside in the endosteal part of the bone marrow niche and are quiescent throughout the lifetime of the organism. The niche is complex and many factors are required in order to maintain a HSC population. Some of these are illustrated in **Figure 1.2**. LT-HSCs give rise to 'short term repopulating' HSCs (ST-HSCs), which are

proliferative but have more limited self renewal potential, and are capable of reconstituting haematopoiesis in a transplant recipient for 8-12 weeks [7]. The cells migrate, towards the perivascular bone marrow niche and become multi-potent progenitors (MPs/MPPs) which are capable of differentiating into all cell types but have no self-renewal capacity. Collectively, this group of CD34+ cells are termed hematopoietic stem and progenitor cells or HSPCs. They can differentiate towards any of the hematopoietic lineages and eventually give rise to all mature hematopoietic cells. Combinations of cell surface markers have been developed to distinguish hematopoietic precursors at each stage of differentiation, some of which are shown on **Figure 1.3**, and which are described in more detail in **results section 3.2.5**.

Figure 1.2

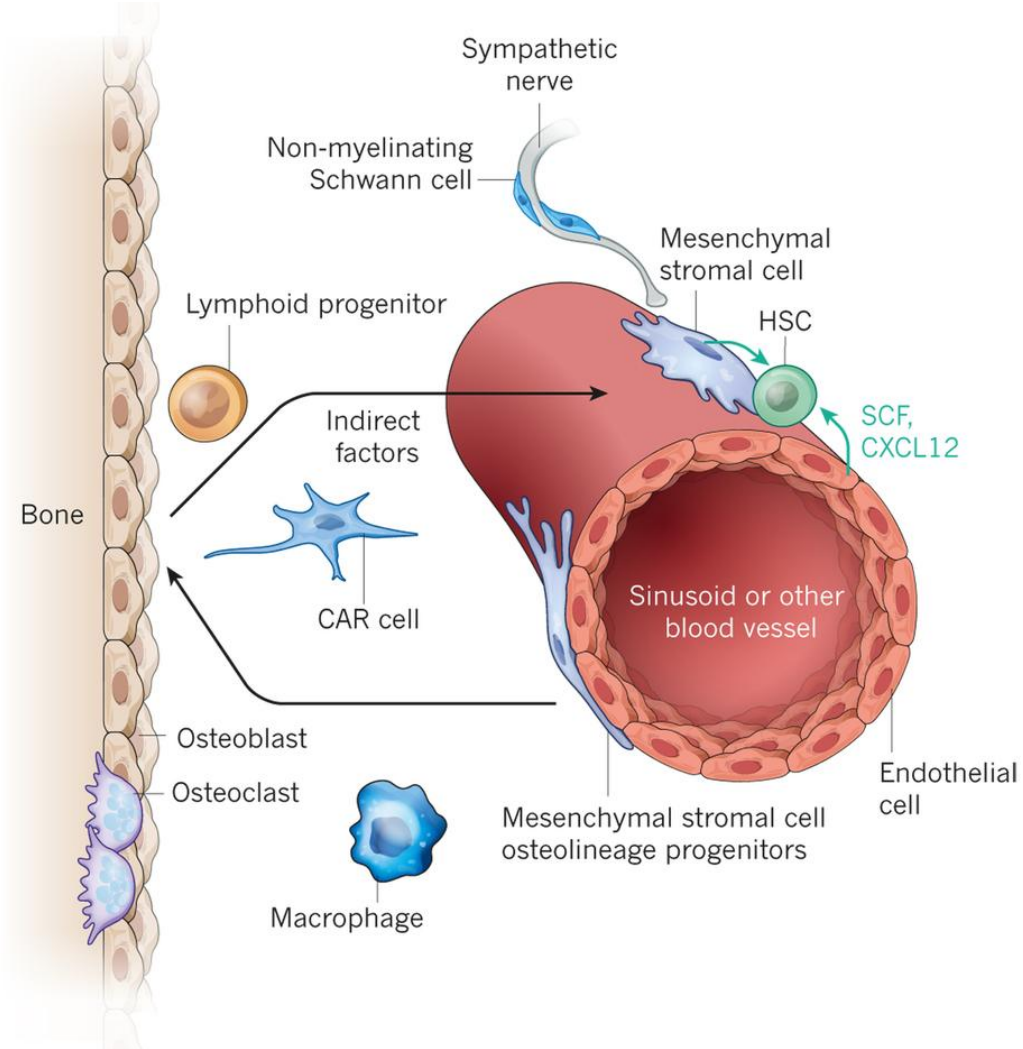


Figure 1.2: Schematic of bone marrow niche derived from Morrison et al review [7]. HSCs are found between the trabecular bone and the blood vessels of the bone marrow and are maintained by signals from osteoclasts, endothelial cells, mesenchymal stromal cells, lymphoid cells, myeloid cells and the sympathetic nervous system. Many factors which control the maintenance and differentiation of HSCs have yet to be elucidated

Figure 1.3

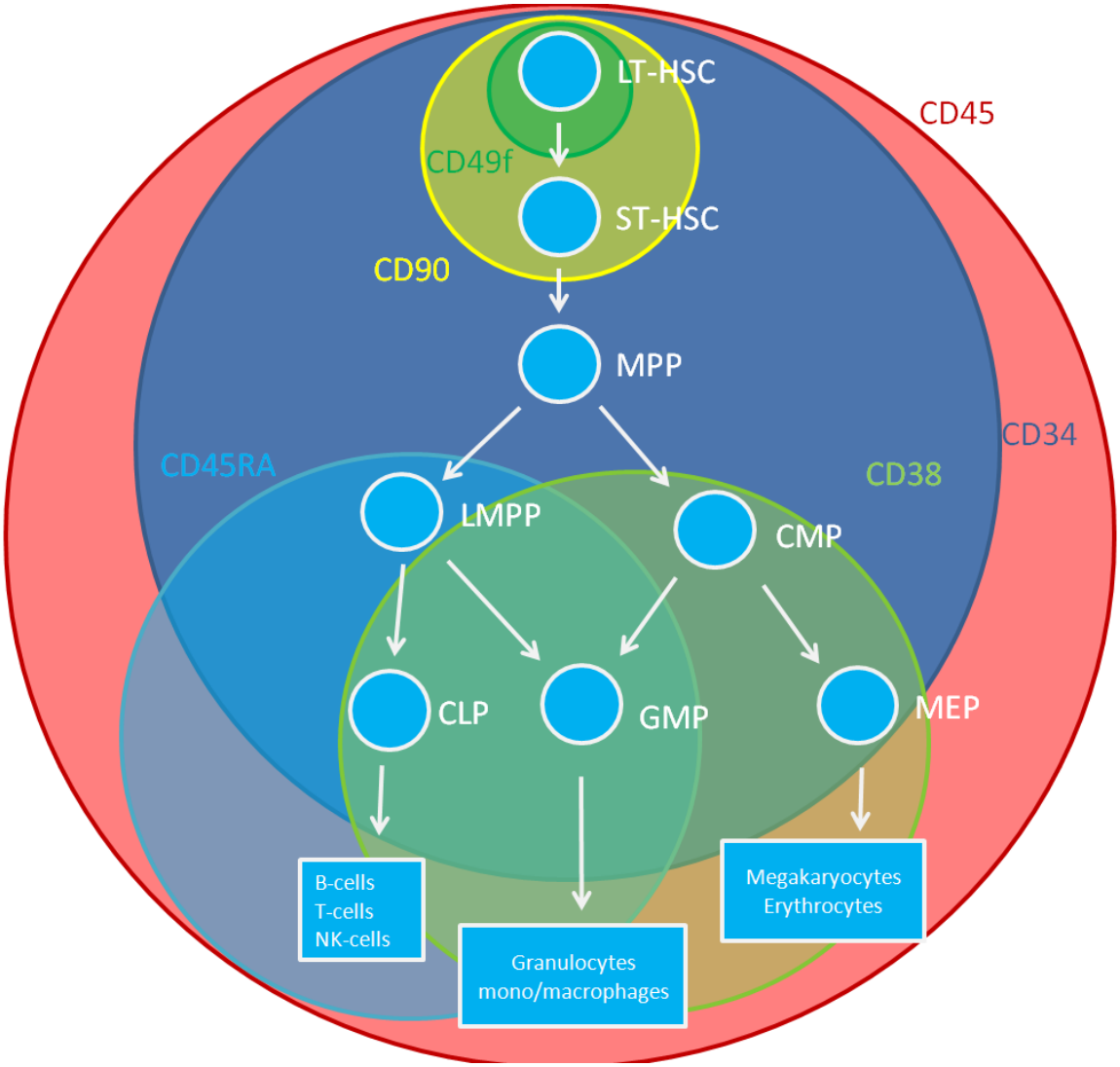


Figure 1.3: Schematic of differentiation from long term hematopoietic stem cells including some of the cell surface markers which can be used to distinguish cell types. LT-HSC= long term repopulating HSC (indefinite self-renewal); ST-HSC= short term repopulating HSC; MPP= multipotent hematopoietic stem cell (non-self-renewing); CLP=common lymphoid progenitor; CMP=common myeloid progenitor; GMP=granulocyte-macrophage progenitor; EMP=erythrocyte macrophage progenitor. LMPP= lympho-myeloid progenitor.

Researchers have attempted to derive HSC from PSC for some time, but without much success. Attempts to make HSC from PSC have followed one of two methods: either the cells are co-cultured with stromal cells which provide an *in vitro* niche and generate signalling molecules which promote hematopoietic differentiation, or cells are allowed to form round aggregates of cells called embryoid bodies (EBs), which differentiate into hematopoietic cells due to exposure to particular combinations of cytokines (methods that have been used to make hematopoietic progenitors are shown in **Table 1.2**). These techniques have been successful in producing hematopoietic progenitors of various kinds, however none have been able to produce HSC, defined as cells able to engraft in a recipient animal and reconstitute its hematopoietic system (see **results section 3.2.1**) without resorting to genetic manipulation [3]. Although it is possible to produce adult hematopoietic cell types *in vitro*, most attempts have produced cells which are foetal in character, and there is typically very low engraftment, which is due to a failure of these cells to migrate to the bone marrow niche. What little engraftment there is appears to be short term and mostly myeloid, rather than multi-lineage production of blood cells [37-42]. For example, hESCs differentiated by co-culture with S17 stromal bone marrow cells engrafted at only 0.16-1.44%, and the cells which did engraft were mostly myeloid [43]. Better engraftment resulted from mimicking the foetal hematopoietic niche by co-culture with stromal cells derived from the aorta-gonad-mesonephros (AGM) [39] which is where HSC arise (see below **section 1.3.2** *Definitive hematopoiesis*), although engraftment was still below the level required for clinical transplantations. Experiments where PSC derived hematopoietic progenitors were transplanted into foetal sheep showed higher engraftment and the capability for secondary engraftment, suggesting that there are factors present in foetal tissues (possibly the AGM and foetal liver in particular [39, 44]) which promote the development of engraftment capacity in hematopoietic progenitor cells derived from pluripotent stem cells (here called P-HPCs).

Table 1.2 : Methods of *in vitro* hematopoietic differentiation

Co-culture	Co-culture with mouse stromal cell lines OP9 or S17	[44-47]
	Co-culture with stromal cells from mouse Aorta-Gonad-Mesonephros (the foetal tissue from which HSCs arise –see **section 1.3.1**) and foetal	[48]

	liver	
	Co-culture with FH-B-hTERT, a human foetal liver-derived cell line, and S17	[48]
Embryoid bodies with a combination of hematopoietic cytokines		[2, 49-57]
Embryoid bodies and mesodermal cytokines, then culture on OP9 stromal cells		[58]
Monolayer	Monolayer culture with hematopoietic cytokines	[61]
Transplant	Transplant of hESCs differentiated on S17 stromal cells into foetal sheep	[45]
	Transplant of hESC derived haemangioblasts into mice	[46]
	Direct transplant of hESCs with hematopoietic cytokines into mice	[47]
	Direct transplant of hESCs with OP9 cells into mice	[48]

Several reasons have been speculated for the difficulty in deriving HSC from PSC: As described above, early attempts to create P-HPCs gave hematopoietic cells with foetal or embryonic characteristics indicating that the cells produced were derived from primitive hematopoietic progenitors rather than HSCs (see below **section 1.3.2** *Primitive Hematopoiesis*), leading some to suggest that the protocols used could only produce primitive cells. Fluorescent marker tracking studies have shown that some of these cells form endothelial cells upon engraftment rather than contributing to blood lineages [41]. However more recent protocols are able to produce definitive hematopoietic cells of the same types found in adults *in vitro* or *in vivo* [8, 49] although they still don't engraft in acceptable numbers. It has also been suggested that these protocols do produce HSCs but that these cells immediately differentiate to non-self-renewing progenitors due to the high concentrations of hematopoietic cytokines and the lack of an appropriate niche in culture. This would explain both the ability of these protocols to produce adult hematopoietic cell types but not long-term

engraftment. A further theory suggests that HSC are produced in these experiments but that they are defective in some way, particularly in their ability to home-to and engraft-in the bone marrow niche. This is supported by the fact that experiments where the cells are injected into the blood stream are much less successful than those where they are injected directly into the bone marrow [37, 46] and in these experiments there is very little colonisation of hematopoietic organs other than the one that the cells are injected into [37].

P-HPCs derived by ectopic gene expression: Hematopoietic development is controlled by transcription factors, and enforcing expression of the correct factors promotes HSC formation. Ectopically expressing *Hoxb4* encouraged mESCs to differentiate into definitive rather than primitive progenitors (see **embryonic haematopoiesis section 1.3.2**) and allowed them to repopulate the hematopoietic system of irradiated mice [50]. Unfortunately *HOXB4* expression did not confer engraftment when it was tried with human cells, although it did increase the yield of hematopoietic progenitors [38]. Other groups have attempted to directly convert other cell types into HSCs using multiple transcription factors; these methods have met with some success (see **discussion section 7.1**) but as they require the introduction of ectopically expressed transcription factors into the genome they are not suitable for clinical use.

To understand the failure of P-HPC to reconstitute the hematopoietic system *in vivo* it is necessary to understand the process of hematopoietic development in the embryo, as differentiation of pluripotent stem cells into hematopoietic lineages closely resembles haematopoietic development *in vivo*.

1.3.2 Embryonic hematopoiesis

Hematopoiesis follows a similar pattern in all vertebrates: Most of what we know about embryonic hematopoiesis comes from studies done in mice or zebrafish, although studies in differentiating hESCs show that a similar pattern is followed in human development. Embryonic hematopoiesis is a complex process, and there are several types of hematopoietic progenitors which arise at different times in different anatomical locations. Briefly, there are three ‘waves’ of hematopoiesis; primitive hematopoietic progenitors are the first to arise, in the yolk sac blood-islands, followed by and erythro-myeloid progenitors and finally definitive hematopoietic progenitors (which includes all HSCs) which arise in the foetal aorta (see **Figure 1.4**).

Hematopoietic ontogeny: All hematopoietic precursors derive from the posterior primitive streak mesoderm in response to Ihh signalling [51, 52]. Some of these cells colonise the yolk

sac and form ‘blood islands’. Early experiments on mouse and chick embryos showed that these clusters are made from cells with both endothelial and hematopoietic potential, which were therefore named ‘haemangioblasts’ [53, 54] which are responsible for the first hematopoietic cells to arise in the embryo. The same cell type can be made using differentiation of PSCs as a model for hematopoiesis [55, 56]. Later in development hematopoietic cells arise from an endothelial tissue called the hemogenic endothelium (HE) [9] via a process called endothelial-hematopoietic transition (EHT) [57, 58]. The cells have endothelial morphology and gene expression and are part of the blood vessel wall, but then begin to express hematopoietic markers before partially detaching and rounding up, forming clusters of cells attached to the blood vessel wall. Eventually they lose contact with the endothelium and join the bloodstream. Wang et al have shown that it is possible to specify HE from hESCs [59]. HE cells with hematopoietic potential are distinct from the rest of the endothelium and are fated to become hematopoietic [60, 61], rather than being bipotential haemangioblasts, so the term hemogenic endothelium has come to replace the term haemangioblast. The hematopoietic ontogeny is shown in **Figure 1.5**.

Figure 1.4

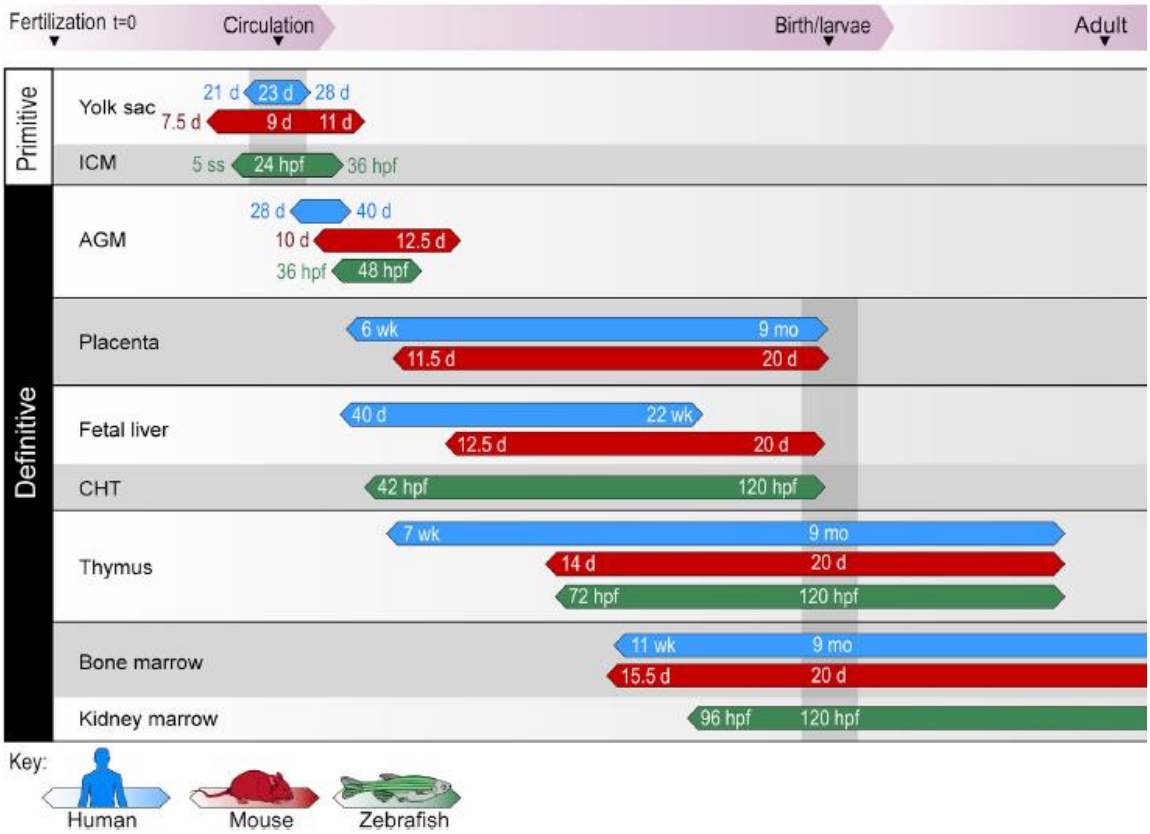


Figure 1.4: Schematic showing the location and timing of hematopoiesis in humans, mice and zebrafish. Figure from Rowe et al review [3]. Human development is shown in blue, mouse in red and zebrafish in green. Each species goes through the same developmental stages of hematopoiesis in the same order: first primitive hematopoiesis, arising in the yolk sac or ICM just prior to the start of circulation, then definitive hematopoiesis, originally in the AGM but later migrating to other hematopoietic organs. The timing of these progressions is different between species and this is shown in the schematic.

Figure 1.5

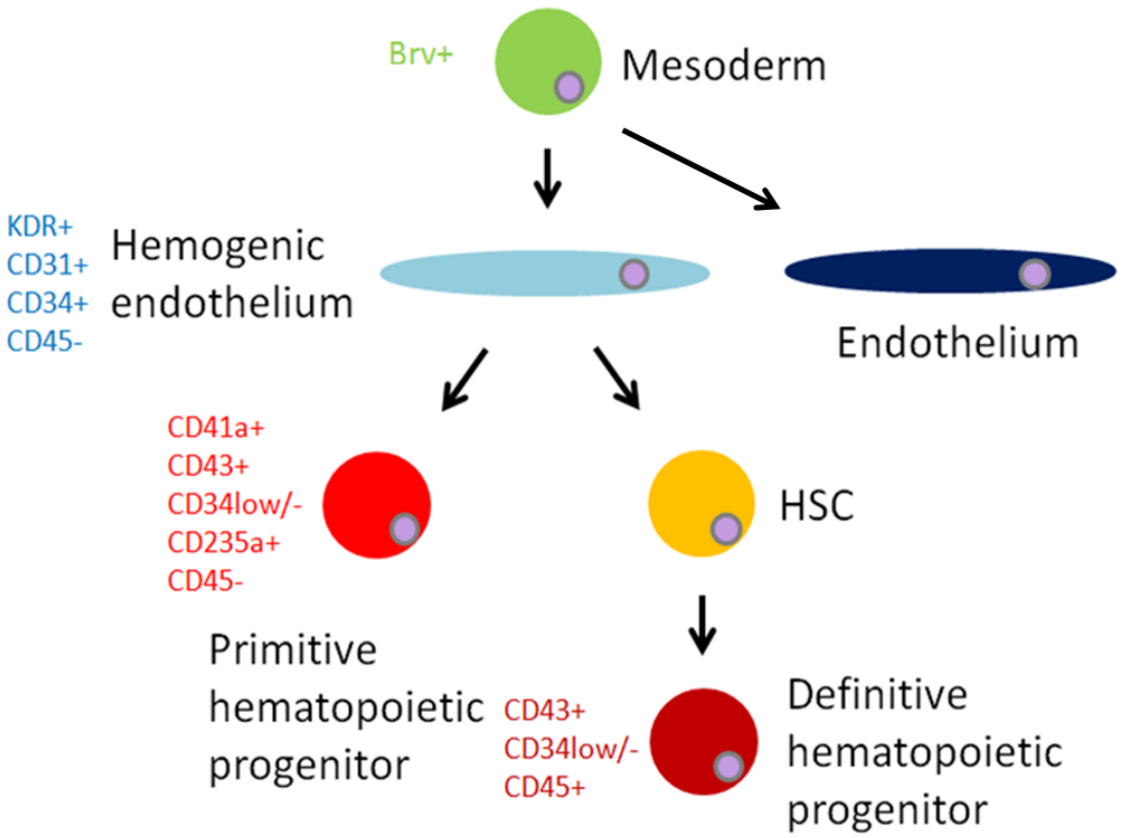


Figure 1.5: Schematic of cell types in embryonic haematopoiesis, showing differentiation from mesoderm to hemogenic endothelium and then definitive and primitive hematopoietic progenitors [9]. Markers for definitive and primitive hematopoietic cells are as shown in Kennedy et al [8]

Primitive Hematopoiesis: The first hematopoietic cells arise from the blood islands in the yolk sac between mouse embryonic day 7.5 (E 7.5) and E 8.5 and are called 'primitive' hematopoietic progenitors [62]. These cells give rise to macrophages and primitive erythrocytes [63], which are larger than erythrocytes found in adults, retain their nucleus, and express embryonic haemoglobins. Primitive hematopoietic cells don't self-renew; they arise transiently to allow oxygenation of rapidly growing foetal tissues.

Primitive hematopoiesis is recapitulated in hESC differentiation models, which show an early wave of primitive erythroid and megakaryocyte and macrophage progenitors [8, 49, 64]. Although primitive and definitive hematopoietic cells are separated both temporally and by anatomical location in the embryo, in PSC models there is considerable overlap so a combination of cell surface markers has been developed to allow them to be distinguished [8, 54, 65] (see **Figure 1.3**).

Erythro-Myeloid Progenitors: The second wave of hematopoiesis also arises in the blood islands, overlapping with primitive hematopoiesis starting at E 8.25 [66]. It is distinguished from primitive haematopoiesis because it produces erythroid and myeloid cells with adult-like morphology [67] which are therefore called erythro-myeloid progenitors (EMPs) [68]. These progenitors are proliferative but they are not capable of self renewal [66]. They continue to arise from vascular tissues throughout the embryo, temporally overlapping with HSC emergence, and like HSCs they migrate to the foetal liver to develop.

EMPs are relatively recently discovered and as yet there are few known markers for distinguishing them from definitive hematopoetic cells. It has been suggested that protocols for generating HSCs are instead generating EMPs, as they produce hematopoietic cells with adult morphology but they produce only a temporary wave of erythroid and myeloid cells when transplanted *in vivo* [39, 40]. They are distinguishable from definitive HSCs because they cannot self renew, and they are unable to produce lymphoid cells.

Definitive hematopoiesis: Definitive hematopoietic progenitors give rise to all blood cells found in the adult, and the definitive wave is the source of all HSCs. HSC development is restricted to a single anatomical location and a narrow time-frame. Experiments where hematopoietic cells from different locations in the foetus showed that cells capable of hematopoietic reconstitution emerge in the aorta-gonad-mesonephros (AGM) between E 10.5 and E 11.5 [69, 70] in mice and at around five weeks in humans [71]. Specifically they arise in the ventral wall of the dorsal aorta [72]. Once they have undergone EHT they form clusters of cells attached to the aortic wall, and then migrate to the foetal liver, where they proliferate and mature. At birth they migrate again to the adult niche in the bone marrow.

Limiting dilution experiments show that there are only around 3 HSCs capable of hematopoietic reconstitution in the embryo at E 11, but by the following day there are more than 60 [73]. This expansion is too rapid to be cell division, which means HSCs are specified at an earlier stage but take time to mature. The immature cells are called ‘pre’-HCSs and are believed to arise between E 8.5 and E 9.5 as shown by Cre-inducible labelling experiments [74, 75]. Taking cells from the AGM and culturing them *in vitro* provides a model system allowing study of the maturation of cells from HE to pre-HSCs to HSCs [76-78].
The narrow time frame and specific location of definitive haematopoiesis, as well as the multi-step process of specification and maturation demonstrate why it is so challenging to derive P-HPCs capable of engraftment and hematopoietic reconstitution. To successfully do so one would have to drive differentiation along the definitive pathway while avoiding

causing primitive and EMP development instead, and allow pre-HSC to mature *in vitro* in spite of the tendency of HSC to spontaneously differentiate outside of their niche.

It is important to be able to recognise HSC *in vitro* and distinguish them from the other cell types. Markers for this purpose are being developed (see results **section 3.2**) but it has been established that only definitive cells are capable of lymphopoiesis [8]. A recent paper suggests that a small number of cells with lymphoid potential arise independently of HSC formation [79], however significant lymphoid potential is still associated with HSCs and still provides a valuable *in vitro* assay for the presence of HSCs

1.3.3 Hematopoiesis from pluripotent cells

A great deal of progress towards the generation of transplantable P-HPCs has been made but the process is still incompletely understood. There are several credible theories for the ineffectiveness of P-HPC at engrafting, but a recent study of miRNA expression suggests that a failure to correctly regulate key miRNAs blocks differentiation and the ability of the cells to home to their niche [37].

In 2012, Risueño et al [37] injected P-HPCs and HSCs derived from volunteer bone marrow into the femurs of immune compromised mice. They found that the HSC would transplant into the femur and engraft, and could later be found in the contra-lateral femur and the spleen, and produced mature hematopoietic cells. In contrast hESC derived cells failed to engraft in significant numbers and the hiPSC derived cells were found only in the femur into which they had originally been injected, indicating a failure to migrate to and colonise other hematopoietic organs, and they failed to differentiate into mature hematopoietic cells. Interestingly they were able to differentiate *in vitro* when removed from the mice, meaning they had hematopoietic potential but that they were blocked from differentiation *in vivo*. The authors examined the expression of a panel of miRNAs, which are key regulators in both pluripotent stem cell biology and hematopoietic differentiation, and found that a number of miRNAs were differentially regulated between bone marrow derived cells and P-HPCs. Therefore it was decided that miRNA expression in hiPSC and hESC derived hematopoietic progenitors should be further investigated in this project.

1.4 miRNAs

Micro-RNAs or miRNAs are a relatively recently discovered group of short non-coding RNA molecules 20-22 nucleotides long, which inhibit gene expression by targeting mRNA

molecules, and an individual miRNA can be used to control whole networks of genes, which makes them particularly important for processes which require a large number of different genes to be controlled at the same time. Multiple studies have shown that miRNAs are expressed in a development- or tissue-specific manner and participate in determination of cell fate, pattern formation during embryonic development, cell cycle control, cell proliferation and apoptosis, regulation of cell signalling and differentiation [1, 80, 81]. This means that manipulating their expression can be useful for controlling cell fate and behaviour.

1.4.1 Biogenesis and mechanism of miRNAs

miRNAs are complimentary to a region of a gene's mRNA transcript, usually the 3'UTR region. They bind to and silence any mRNA to which they are complementary. miRNA biogenesis is shown in **Figure 1.6**. miRNAs are usually transcribed from the genome as part of a longer RNA molecule called a pri-miRNA. They are processed by DROSHA into shorter strands, called pre-miRNA, which are about 70 nucleotides long and form a hairpin loop structure. These are exported into the cytoplasm, where they are further processed by DICER into 20-22 nucleotide double stranded molecules. One of these strands is loaded into the RISC complex, the other is degraded [1]. The RISC-miRNA complex uses several mechanisms to silence target genes (reviewed by Eulialio et al in 2008 [81]); it causes degradation of both mRNA and translated polypeptides and inhibits translation initiation and elongation as well as encouraging ribosome dissociation.

Figure 1.6

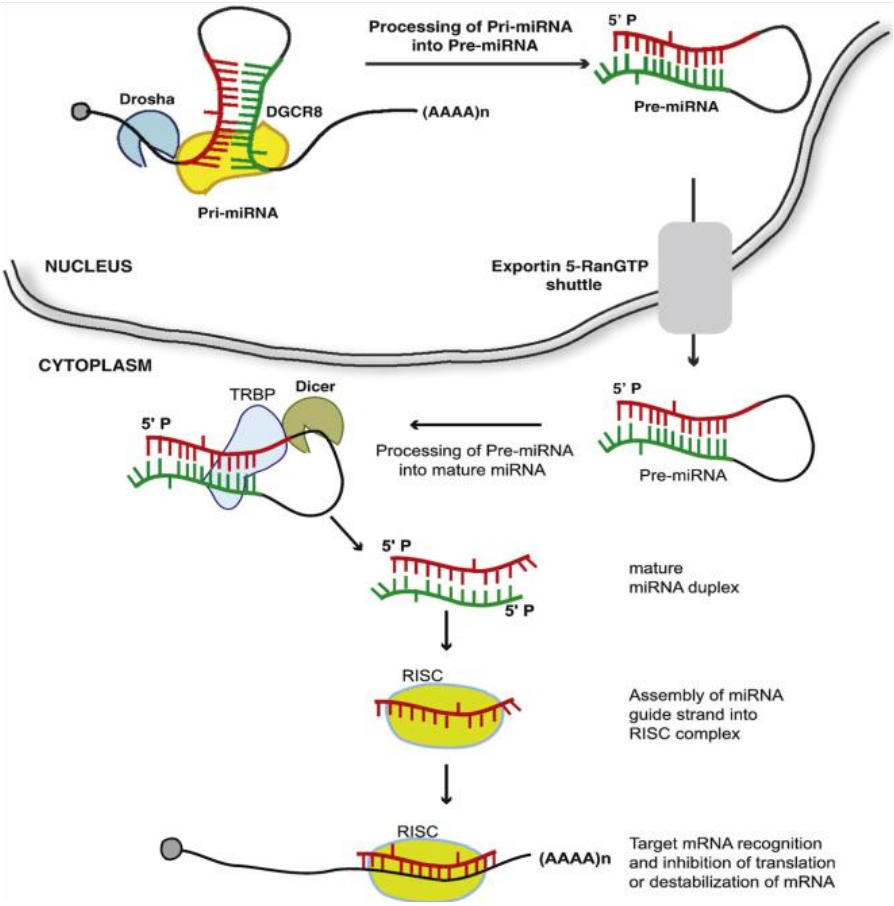


Figure 1.6: Schematic diagram of miRNA biogenesis derived from Mallana et al review [1]. The process of biogenesis is shown from top to bottom: the pri-RNA is transcribed from the genome and forms a stem-loop, and is then processed by DROSHA and DGCR8. It is then transported from the nucleus and further processed by Dicer into a short two stranded RNA molecule which is taken up by RISC and used to guide the degradation of complimentary mRNA.

1.4.2 miRNA terminology and nomenclature.

miRBase, the principal database of known miRNAs lists over 2500 human miRNAs, although many of these have not been validated and are believed not to be real [82]. miRNAs have been named numerically, i.e. miR-1, miR-2 etc, with the exception of some of the very first miRNAs discovered, for example let-7 or lin-4. Duplications of miRNA genes have created miRNA 'families'. miRNAs from the same family with the same seed sequence are distinguished by letters, e.g. miR-200b and miR-200c. miRNAs can be made from both strands of the short stem loop pre-miRNA, but in general only one of them will have a functional role and this one will be the one which is predominant in the cytoplasm, with the other having been immediately degraded. The convention has been to mark the other strand with an asterisk. For example mir-150 is expressed in many hematopoietic cells and the complimentary strand is called mir-150*. In many cases it is not known which of the strands

is functional and in some both appear to have some mRNA inhibitory function so the more precise nomenclature of naming the strands as -3p or -5p has been adopted [83]. For example mir-150 is called mir-150-5p and mir-150* is called 150-3p [84].

1.4.3 miRNAs in reprogramming

miRNAs are a useful target for reprogramming because many genes in a pathway can be controlled by a single miRNA, which can potentially avoid issues of redundancy, and because they can be introduced to cells without genomic integration, avoiding mutagenesis. Ectopic miRNA expression has been explored as a method of reprogramming cells to pluripotency: miR-302 can improve the efficiency of four factor reprogramming [85], and inhibition of miR-145 promotes reprogramming [86]. Fibroblasts can be reprogrammed into iPSCs using miRNAs alone (miR-302, -369 and -200c), although efficiency is low [87]. miRNAs have also been used for direct reprogramming of one mature cell type to another (see **discussion section 7.1**).

1.4.4 miRNAs in hematopoiesis

The process of differentiation from HSCs involves several steps: cells must leave the quiescent self-renewing state of HSCs, then they become very proliferative and undergo a series of progressive specialisations while maintaining their proliferative state. Once their final cell type is determined they undergo maturation and eventually become terminally differentiated and lose their proliferative capacity. All of these steps involve miRNA.

miRNA maintain stemness in HSCs. Knocking out DICER in HSC results in the loss of HSC maintenance as well as loss of erythroid commitment [88]. miRNAs target genes involved in differentiation, maintaining the cells in a less differentiated state [89] and it has been suggested that miRNAs help to balance HSC in a state of multi-lineage potential by preventing the expression of genes which promote a particular lineage. miRNAs also regulate the self-renewal of HSC, for example increasing the expression of miR-125a causes expansion of HSCs [90] and miR-126 maintains HSCs by preventing their entry into the cell cycle [91]. miR-29a promotes self-renewal of HSCs [92]. Increased expression of miR-34a, a miRNA which promotes apoptosis, is associated with myelodysplastic syndrome, a condition in which there is a severe depletion of the HSC population [90, 93-95], as is an increase in miRNAs associated with hematopoietic differentiation such as miR-223 and miR-181 [90, 96].

miRNA are differentially expressed in different hematopoietic lineages and are responsible for the small changes in concentration of cellular factors which control lineage fate decisions

in differentiating hematopoietic cells [97]. miR-181a promotes T-cell development [98]. The miR-23-27 cluster increases myeloid progenitor numbers at the expense of lymphoid progenitors [99], miR-299 promotes granulocyte formation and decreases erythrocyte colonies [100], miR-424 controls the monocyte/macrophage fate decision by targeting NF1A [101] and the megakaryocyte/erythroid fate decision is controlled by miR-150 and miR-223 [102, 103]. These miRNAs ensure that appropriate numbers of each hematopoietic cell type are produced.

Other miRNAs are responsible for regulating the maturation process of hematopoietic cells to the point where they are terminally differentiated and no longer proliferative. The greatest changes in miRNA expression occur when the cells are switching from a proliferative committed progenitor to a terminally differentiated non dividing cell [104]. There is a hierarchy of miRNAs which regulate different stages in this process [105-107]. miRNAs targeting mitophagy must be down-regulated before erythrocytes can undergo terminal differentiation [108] and miR-144 and miR-451, although not required for erythroblast formation are essential for the terminal differentiation of erythrocytes [109]. The final stage of granulocyte maturation requires miR-21 and miR-196b [110], myeloid terminal differentiation requires the down-regulation of miRNAs which promote proliferation [104], and excess miR-34a causes the accumulation of immature B-cells [111].

miRNA are key players at every stage of adult haematopoiesis, but less is known of their role in embryonic haematopoiesis. Experiments in zebrafish have shown that the switch from embryonic to adult erythropoiesis is regulated by miR-144 [112], miR-451 [113] (and miR-223 [114] and miR-126 controls the onset of definitive haematopoiesis via the targeting of *c-MYB* [115]. Few studies of *in vivo* miRNA expression in emerging hematopoietic cells have been done, so little is known about this subject.

miRNAs in hematopoietic differentiation from pluripotent cells: In transplant experiments HSCs can be injected directly into the bone marrow of the femur or introduced instead into the bloodstream, from where they will home to the bone marrow and make their way through the blood vessel wall into the niche. HSC are mobile and will eventually grow to colonise the marrow of bones other than those which they are directly injected into and will produce cells which colonise other hematopoietic organs such as the spleen and thymus. Risueño et al. [37] transplanted hematopoietic progenitors derived from pluripotent cells into mice and found that the hiPSC derived cells could engraft only when directly injected into the femur, and that they would not colonise the contra-lateral femur or the other hematopoietic organs. This is consistent with other published experiments in which P-HPCs have been transplanted into

animals. The same cells showed multipotent hematopoietic potential *in vitro* which suggests that there is a defect in migration or chemotaxis which prevents the cells from reaching their niche. Risueño et al. discovered a possible mechanism for this failure to acquire a motile phenotype when they studied the molecular/genetic profiles of engrafted and *in vitro* P-HPCs compared to HSCs derived from adult bone marrow: they found that there were a number of miRNAs which fail to down-regulate during *in vitro* differentiation, and which are therefore expressed at unusually high levels compared with HSC derived from bone marrow. miRNAs are key regulators of embryonic differentiation and have well documented roles in regulating motility and migration (see **results 4.2.1**). It is therefore reasonable to hypothesise that ectopic expression or inhibition of miRNAs may be helpful in generating functional P-HPCs.

1.5 Aims

The broad aim of this project was to improve the process of generation of human HSCs from PSCs, specifically by altering the levels of key miRNAs.

Hypothesis: the production of functional P-HPCs is impaired due to mis-regulation of miRNAs; genetic manipulation of these key miRNAs could lead to improvements in the yield and functionality of definitive hematopoietic progenitors. The aims of this study were to:

1. Investigate techniques for hematopoietic differentiation, choosing the most effective chemically defined, feeder free method and improve upon differentiation protocols by adjusting differentiation conditions, for example oxygen levels.
2. Identify candidate miRNAs which are differentially regulated in P-HPCs compared to hematopoietic progenitors found in bone marrow and cord blood, by analysis of microarray data and validate these candidates by qPCR.
3. Develop efficient techniques for manipulating miRNA expression in hPSC derived hematopoietic progenitors.
4. Investigate if genetic manipulation of miRNAs improves the efficiency and functionality of hematopoietic differentiation from human pluripotent stem cells as, as measured by colony assays, gene expression and cell surface markers.

2. Materials and methods

2.1 Tissue culture

2.1.1 Culture of human pluripotent stem cells

This project used two cell lines: H9, a hESC line bought form WICell. Inc. and SB-Ad3, a hiPSC line made from adult dermal fibroblasts using Yamanaka factors encoded in a Sendai virus which had been derived by Lako's group. Both of these cell lines have been extensively characterised and validated by Lako's group.

The cells were cultured in STEMPRO® hESC medium (**see Tables 2.1 and 2.2**) on vitronectin coated 6 well plates after being adapted from mTeSR™1 and Matrigel coated plates. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Media was replaced daily. Differentiated cells were removed by scraping the thicker, more opaque, and irregularly shaped differentiated areas of the colonies away using a pipette tip. Typical hESC colonies are shown in **Figure 2.1**. The cells were passaged at a ratio of 1:3 every 4-5 days or when they reached 75% confluence. Prior to passaging each well of a fresh 6 well plate was coated with 1ml of vitronectin solution diluted in PBS to a concentration of 10µg per ml. The plate was then incubated for 1hr at room temperature (20-25^{o}C), and the solution was aspirated immediately before cells were passaged onto the plate. Cells were passaged manually using a StemPro® EZPassage roller to cut the colonies into squares as shown in **Figure 2.2**, which were lifted by flushing and then transferred into the new plates.

Table 2.1: StemPro® hESC media	
DMEM-F12	454ml
StemPro® hESC supplement	10ml
BSA25%	36ml
FGF-β	400µl (final conc. 8 ng/ml)
β-mercaptoethanol (55mM)	909µl

Table 2.2: Products for culture of pluripotent stem cells		
Product name	Product number	Manufacturer
TPP® 6 well plates	92006T	Sigma-Aldrich
StemPro® hESC SF Media	A1000701	Thermo Fisher Scientific
StemPro® EZPassage roller	23181010	Thermo Fisher Scientific
Vitronectin	7190	Stem cell technologies
Stemline® II	S0912	Sigma-Aldrich
Corning® Ultra-low attachment culture dish	CLS3262	Sigma-Aldrich
TrypLE™Express	12604039	Thermo Fisher Scientific
Human FGF-basic	PHG0023	Thermo Fisher Scientific

Figure 2.1

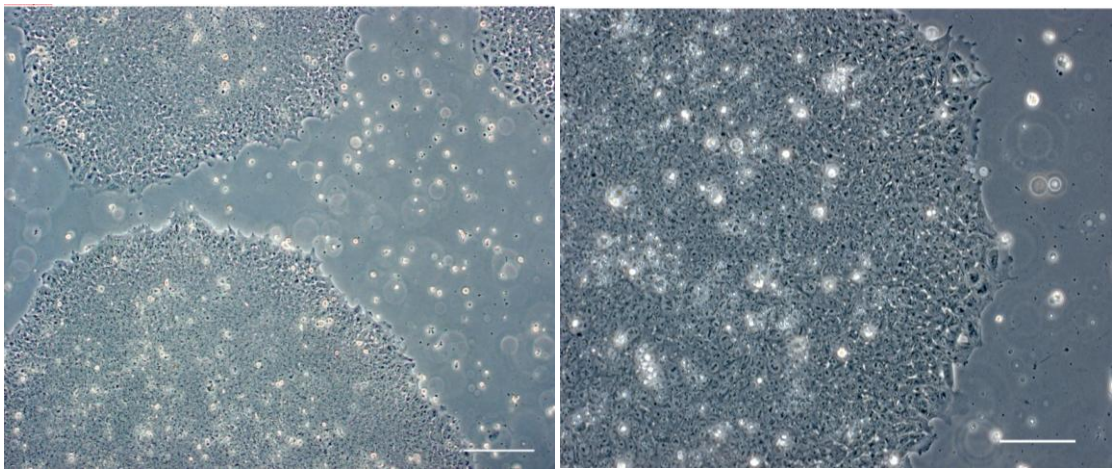


Figure 2.1: Representative micrographs of H9 colonies grown on STEMPRO®. Bar on the left-hand picture represents 200 µm, bar on the right-hand picture represents 100 µm.

Figure 2.2

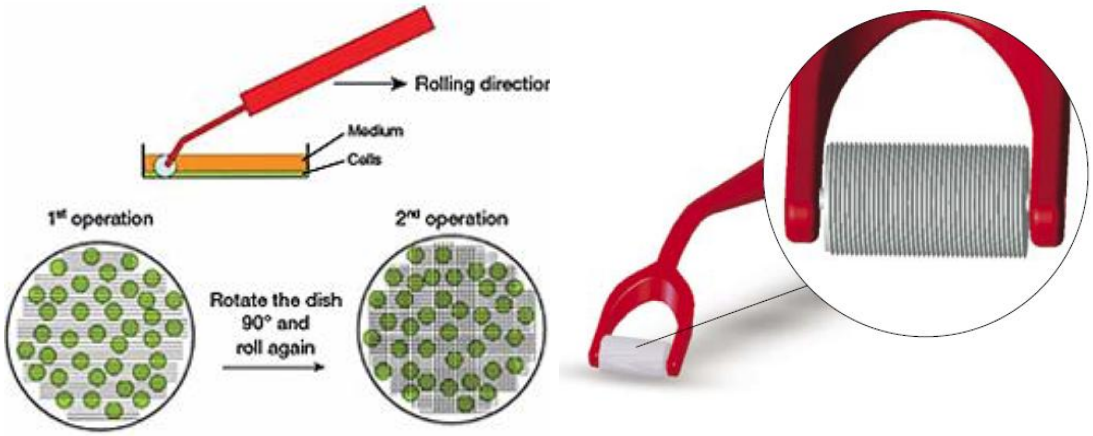


Figure 2.2: Manufacturers schematic of a StemPro® EZPassage roller [5], showing how colonies of pluripotent cells can be divided into evenly sized squares of cells by running the ridged roller over the surface of the plate. These squares of cells can then be lifted by flushing the plate with a pipette and transferred onto a fresh plate.

2.1.2 Hematopoietic differentiation

Several methods of hematopoietic differentiation were used in this project. The microarray data is derived from a population of cells made using the protocol described in Kennedy et al. in 2006 [64], these experiments were done before the start of the project; see **methods section 2.2** for further details.

Figure 2.3

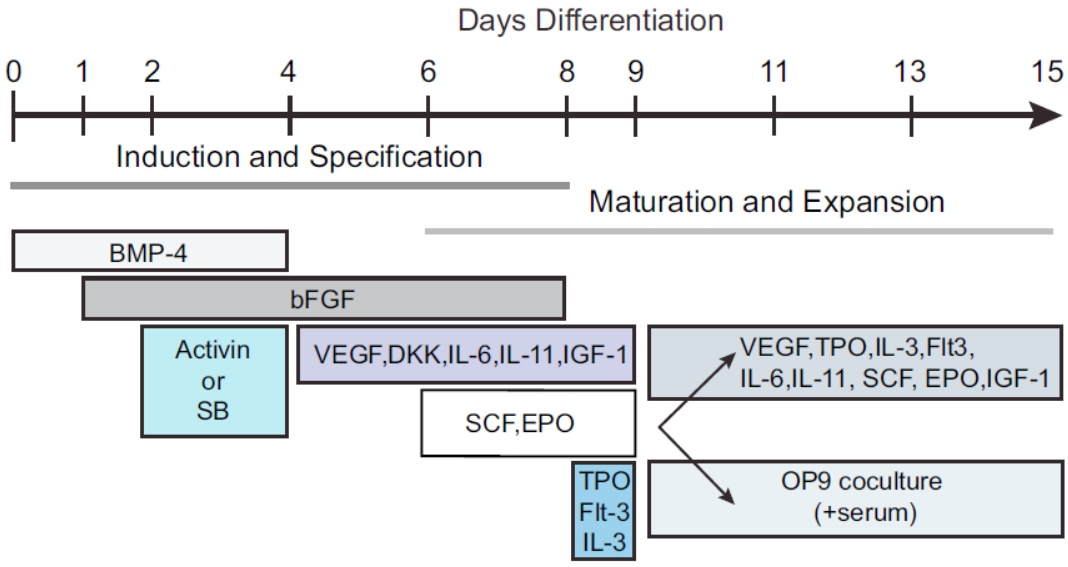


Figure 2.3: Schematic of protocol found in Kennedy et al 2012 [8]. Schematic shows combinations and timing of cytokines used to induce hematopoiesis from human pluripotent stem cell derived EBs. Activin or an Activin inhibitor (SB) can be added between days 2 and 4 to promote primitive or definitive hematopoiesis respectively.

Some of the preliminary work on this project was done using the more recent Kennedy 2012 protocol [8], a method involving embryoid body formation and exposure to hematopoietic cytokines (see **Figure 2.3** for schematic of the process). However the majority of the experiments were done using a protocol developed and provided to us by Dr Jo Mountford and Dr Scott Cowan working at the University of Glasgow [2]. Their protocol was developed to produce large numbers of definitive mature erythroid cells and was modified in our lab to produce hematopoietic progenitors by using only the first 12 days of the protocol, stopping before the cytokines for erythroid specific differentiation are added.

Glasgow protocol:

Day 0, embryoid body (EB) formation: Medium was replaced with StemlineII™ medium with day 0 haematopoietic cytokines (**Table 2.3**). Colonies were cut into squares using the STEMPRO® EZPassage™ Tool, and lifted from the surface of the plate by flushing with a 5ml pipette. They were then transferred to Co-star ultra low attachment 6 well plates at a ratio of 2:1. More cytokine-media mix was added to make the media up to 3ml/well.

Day 2, feeding cells: ½ ml of media was added to each well with cytokines at 6x concentration shown in **Table 2.3**; bringing the total amount of media in each well back to 3ml (this assumes that approx. ½ ml is lost to evaporation).

Day 3, dissociation and plating: Day three EBs were dissociated with TrypLE Express for approximately 15 minutes. TrypLE Express was removed by diluting in a falcon tube with 15ml of PBS, centrifuging and removing the supernatant. A small amount of medium was added to allow the cells to be counted with a standard haemocytometer, they were then plated onto uncoated 6 well plates at a density of 300k cells/well in 3ml/well StemlineII media with cytokines shown in **Table 2.3**.

Day 5, feeding cells: ½ ml of fresh media and cytokines (**Table 2.3**) at 6x concentration were added to each well.

Day 7, feeding cells: Medium was replaced by removing cells and medium from wells, centrifuging and aspirating the supernatant, and then resuspending the cells in fresh media-cytokine mix and returning it to the wells. ½ ml of media cytokine mix (**Table 2.3**) was added to the wells during the centrifugation to prevent the adherent cells from drying out. Media in each well totalled 3ml once suspended cells were replaced.

Day 9, feeding cells: ½ ml of fresh media-cytokine mix (**Table 2.3**) at 6x concentration was added to each well.

Table 2.3: Cytokines used in hematopoietic differentiation

			Final concentration			
Factor	Manufacturer	Product number	Day 0	Day 2	Days 3 and 5	Days 7 and 9
BMP4	R and D Systems	314-BP	10ng/ml	20ng/ml	20ng/ml	20ng/ml
VEGF	Peprotech	100-20	10ng/ml	30ng/ml	30ng/ml	30ng/ml
Wnt3a	R and D Systems	5036-WN-010	10ng/ml	10ng/ml	-	-
Activin A	Peprotech	120-14E	5ng/ml	5ng/ml	-	-
GSK-3b Inhibitor VIII	Calbiochem	361549	2mM	2mM	-	-
FGFα	Peprotech	100-17A	-	10ng/ml	10ng/ml	10ng/ml
SCF	Invitrogen	PHC2113	-	20ng/ml	30ng/ml	30ng/ml
β-estradiol	Sigma-Aldrich	E2758	-	0.4ng/ml	0.4ng/ml	0.4ng/ml
IGF2	Peprotech	100-12	-	-	10ng/ml	10ng/ml
TPO	Peprotech	300-18	-	-	10ng/ml	10ng/ml
Heparin	Sigma-Aldrich	H0878	-	-	5ng/ml	2.5ng/ml
IBMX	Sigma-Aldrich	15879	-	-	50uM	50uM

2.2 Microarray

Microarray analysis was done prior to the start of the project, by Lako's group, in collaboration with Dr David Blesa. Two replicates of undifferentiated H9 hESC line and CD31+CD34+KDR+CD45- subpopulation from day 4 of differentiation were subjected to RNA extraction and hybridisation to an Agilent *G4470C-021827* array containing 866 human and 89 human viral miRNAs using the Agilent protocol 'miRNA Microarray System with miRNA Complete Labelling and Hyb Kit', Version 2.1. Human placenta cells were used as an internal control. This data was compared to the expression profile of human CD34+ bone marrow cells which were hybridised to the same array. The data from this array was obtained from Gene Expression Omnibus (GEO), accession number *GSM595699.*

Bioinformatic analysis of this data was done by David Montaner at Centro de Investigacion Principe Felipe in Valencia (Spain). In brief, the expression data was normalised using quantile normalisation [116] and differential miRNA expression was estimated using Limma package [117] from Bioconductor. Statistical significance (adjusted p values) was used to select the miRNAs that were differentially expressed during human pluripotent stem cell differentiation and adult bone marrow hematopoietic progenitors. The significantly changed miRNAs were organized into patterns, for example "flat-down" which means that miRNA is expressed at the same level from human pluripotent stem cells to hematopoietic progenitors arising at day 4 of differentiation, but is under-expressed in adult bone marrow hematopoietic progenitor cells (see **results section 4.2** for further explanation).

A number of miRNAs were chosen for validation by qRT-PCR on the basis that their target genes might have a function in hematopoiesis. This was determined by analysis of all the mis-regulated miRNAs with information from databases such as Targetscan [118] and miRbase [82] and a literature search on the NCBI database.

2.3 Quantitative RT- PCR (qPCR)

2.3.1 miRNA expression analysis.

The Taqman system of using primers specific to the miRNA of interest was chosen for reverse transcription. See **results section 4.1** for an in-depth explanation of this system and why it is necessary to use it. The primer sequences used in these experiments are proprietary and have not been released in spite of several requests, but the sequences of the miRNAs tested are shown in **Table 2.4**. Small nuclear RNAs RNU44 and RNU48 were chosen as internal controls as they are known to be stably and abundantly expressed across different cell types [119-121]

CD34+ human adult bone marrow and CD34+ cord blood cells purchased from AllCells were used as positive controls for qPCR analysis.

Table 2.4: Sequences of miRNAs studied by qPCR	
	Mature miRNA sequence
miR-9	UCUUUGGUUAUCUAGCUGUAUGA
miR-18b	UAAGGUGCAUCUAGUGCAGUUAG
miR-134	UGUGACUGGUUGACCAGAGGGG
miR-150	UCUCCCAACCCUUGUACCAGUG
miR-150*	CUGGUACAGGCCUGGGGGACAG
miR-148a	UCAGUGCACUACAGAACUUUGU
miR-200b	UAAUACUGCCUGGUAAUGAUGA
miR-200c	UAAUACUGCCGGGUAAUGAUGGA
miR-205	UCCUUCAUUCCACCGGAGUCUG
miR-302a	UAAGUGCUUCCAUGUUUUGGUGA
miR-302a*	ACUUAAACGUGGAUGUACUUGCU
miR-367	AAUUGCACUUUAGCAAUGGUGA
miR-424	CAGCAGCAAUUCAUGUUUUGAA
miR-142	CAUAAAGUAGAAAGCACUACU
miR-146	UGAGAACUGAAUUCCAUGGGUU
miR-223	UGUCAGUUUGUCAAAUACCCCA
miR-181b	AACAUUCAUUGCUGUCGGUGGGU
RNU44	CCTGGATGATGATAGCAAATGCTGACTGA ACATGAAGGTCTTAATTAGCTCTAACTGACT
RNU48	GATGACCCCAGGTAACTCTGAGTGTGTCG CTGATGCCATCACCGCAGCGCTCTGACC

RNA was extracted using the Reliaprep^{TM} RNA cell miniprep system. miRNA was amplified using the TaqMan Micro-RNA Reverse Transcription (RT) Kit with a specific TaqMan Micro-RNA primer. Components of the RT reaction are shown in **Table 2.5** and the PCR program is shown in **Table 2.6**.

Table 2.5: Summary of components for reverse transcription		
Components	Master mix volume (per 15-µl reaction)	Final concentration
100 mM dNTPs (with dTTP)	0.15 µl	10^5 mM
MultiScribe™ Reverse Transcriptase, 50 units/µl	1.00 µl	3.33 units
10X Reverse Transcription Buffer	1.50 µl	
RNase Inhibitor, 20 units/µl	0.19 µl	0.25units/µL
Nuclease-free water	4.16 µl	
Cell lysate	5.00 µl	0.333
RT Primer	3.00 µl	0.2

Table 2.6: PCR Programme for reverse transcription		
Step	Time	Temperature
Hold	30 minutes	16 °C
Hold	30 minutes	42 °C
Hold	5 minutes	85 °C
Hold	∞	4 °C

TaqMan Universal PCR Mastermix II and TaqMan MicroRNA Assays were used for the qPCR, components of the reaction shown in **Table 2.7**. 10µl qRT-PCR reactions were done in triplicate on a 386 well plate in a TaqMan 7900 or a QuantStudio™ 7 machine, using the programme shown in **Table 2.8.**

Table 2.7: Summary of components for qPCR	
Components	Volume per 30-µl reaction
	Triplicates
TaqMan® Small RNA Assay (20 X)	1.8 µl
Product of RT reaction	2.4 µl
TaqMan® Universal PCR Master Mix II (2X), no UNG	18.0 µl
Nuclease-free water	13.8 µl
Total volume	36.0 µl

Table 2.8: programme for qPCR			
	Enzyme Activation	PCR Amplification	
		CYCLE (40 cycles)	
Steps	HOLD	Denature	Anneal/extend
Temperature	95°C	95°C	60°C
Time	10 minutes	15 seconds	60 seconds

Table 2.9: qPCR reagents		
	Product number	Manufacturer
TaqMan® MicroRNA Reverse Transcription Kit	4366597	Thermo Fisher Scientific
ReliaPrep™	Z6012	Promega
TaqMan® MicroRNA Reverse Transcription Kit	4366597	Thermo Fisher Scientific
RNU44 TaqMan® miR assay	4427975/001094	Thermo Fisher Scientific
RNU48 TaqMan® miR assay	4427975/001006	Thermo Fisher Scientific
miR-200c TaqMan® miR assay	4427975/002300	Thermo Fisher Scientific
miR-200b TaqMan® miR assay	4427975/002251	Thermo Fisher Scientific
miR-424 TaqMan® miR assay	4427975/000604	Thermo Fisher Scientific
miR-205 TaqMan® miR assay	4427975/000509	Thermo Fisher Scientific
miR-148a TaqMan® miR assay	4427975/000470	Thermo Fisher Scientific
miR-9 TaqMan® miR assay	4427975/000853	Thermo Fisher Scientific
miR-18b TaqMan® miR assay	4427975/002217	Thermo Fisher Scientific
miR-134 TaqMan® miR assay	4427975/000459	Thermo Fisher Scientific
miR-150 TaqMan® miR assay	4427975/000473	Thermo Fisher Scientific
miR-150* TaqMan® miR assay	4427975/002637	Thermo Fisher Scientific
miR-302a TaqMan® miR assay	4427975/000529	Thermo Fisher Scientific
miR-302a* TaqMan® miR assay	4427975/002381	Thermo Fisher Scientific
miR-367 TaqMan® miR assay	4427975/000555	Thermo Fisher Scientific
miR-142 TaqMan® miR assay	4427975/000464	Thermo Fisher Scientific
miR-146 TaqMan® miR assay	4427975/000468	Thermo Fisher Scientific
miR-223 TaqMan® miR assay	4427975/000526	Thermo Fisher Scientific
miR-181b TaqMan® miR assay	4427975/001098	Thermo Fisher Scientific
TaqMan® Universal mastermix II	4440044	Thermo Fisher Scientific
MicroAmp® Optical 384-Well Reaction Plate	4309849	Thermo Fisher Scientific
GoScript™ reverse transcription kit	A5000	Promega
SYBR™ Green qPCR kit	11762500	Life technologies

2.3.2 Gene expression analysis

For the analysis of gene expression by qPCR RNA was extracted in the same way and 1µg of RNA was used in a 20µl GoScript™ reverse transcription reaction, according to the manufacturer's instructions. The RNA was mixed with oligo(dT) and random primers as

shown in **Table 2.10(i),** then heated at 70^{o}C for 5 minutes, cooled on ice for 5 minutes, then mixed with the reagents for reverse transcription shown in **Table 2.10(ii)**. The program for reverse transcription is shown in **Table 2.11**. Information on the reagents used for this and the qPCR reactions are shown in **Table 2.9**.

Table 2.10(i): components for reverse transcription	
Component	Volume
Experimental RNA	Xµl(1µg/reaction)
Primer Oligo(dT) /random primer	1µl(0.5µg/reaction)
Nuclease-Free Water	Xµl
Final volume	5µl

Table 2.10(ii)	
Component	Volume
GoScript™ 5X Reaction Buffer	4.0µl
$MgCl_2$	1 1.2–6.4µl (final concentration 1.5–5.0mM)
PCR Nucleotide Mix	2 1.0µl (final concentration 0.5mM each dNTP)
GoScript™ Reverse Transcriptase	1.0µl
Nuclease-Free Water	Xµl (to a final volume of 15µl)
Final volume	15µl

Table 2.11: Programme for reverse transcription		
Step	Time	Temperature
Anneal	5 minutes	25 °C
Extend	60 minutes	42 °C
Inactivate RT	15 minutes	70 °C
	∞	4 °C

qPCR was then performed on the cDNA using components shown in **Table 2.12** and primers shown in **Table 2.13.** Controls without cDNA were used to exclude results from wells with contamination. 10µl qRT-PCR reactions were done in triplicate on a 386 well plate in a TaqMan 7900 or a QuantStudio™ 7 machine, using the programme shown in **Table 2.8.**

Primers were designed using the NCBI Primer-BLAST tool [122]. Parameters for qPCR primers are that the primers were 18-22 nucleotides long, specific to the gene of interest, PCR product size was 70-200 base pairs and melting temperature was between 58-62^{o}C. Where possible, primers were designed so that the PCR product spans an intron to reduce the likelihood of genomic DNA contamination. The primers were analysed for primer dimers and secondary structure formation using Thermo fisher's multiple primer analyser software [123]. The efficiency of the primers was determined by melt curve analysis.

Table 2.12: SYBR Green components	
Component	Volume
2x master mix buffer	5µl
Template cDNA	0.4-0.8µl
Primers (F/R)	0.4~0.6µl
Nuclease free water	Xµl
CXR	0.1µl
Final volume	20µl

Table 2.13: Primers for qPCR		
Gene	Forward primer	Reverse primer
GATA1	CAGGCCAGGGAACTCCA	ATCACACTGAGCTTGCCACA
SCL	AGCCGGATGCCTTCCCTAT	CCGCACAACTTTGGTGTGG
HOXB4	GTCGTCTACCCCTGGATGC	CGTGTCAGGTAGCGGTTGTA
GATA2	GGGGACCCTGTCTGCAACGC	GGCAGCTGCACTGAAGGGGG
cMYB	GACAGCAGGTGCTACCAACA	GCTGCATGTGTGGTTCTGTG
HOXA9	CCGAGAGGCAGGTCAAGATC	AAATAAGCCCAAATGGCATCA
RORA	GCACCGCGGCTTAAATGATGT	CTTCTCCTGAAAAAGCCCTTGC
SOX4	ACCACCACTCGCTGTACAAG	AGGTAGACGCGCTTCACCTT
ERG	TCTGTCTTAGCCAGGTGTGG	CGCATTATGGCCAGCACTAT
SOX17	TCTGCCTCCTCCACGAAG	CAGAATCCAGACCTGCACAA
RUNX1	CAATGGATCCCAGGTATTGG	CACTGCCTTTAACCCTCAGC
ZEB1	GCGGAAGACAGAAAATGGAA	GATTCCACACTCATGAGGTC
ZEB2	AATGAAGCAGCCGATCATGG	CAGAACCTGTGTCCACTAC
PU.1	CACAGCGAGTTCGAGAGCTT	GATGGGTACTGGAGGCACAT

All qPCR Results were analysed with SDS 4.2 and DataAssist Software v3.01. Significance was determined by one way ANOVA with three replicates. Information about the reagents used is shown in **Table 2.9**.

2.4 Flow cytometry and cell sorting

Cells were prepared for flow cytometry by dissociating them with TypLE^{TM} Express at 37^{o}C for 15 minutes. The TypLE^{TM} Express was then diluted with PBS and the cells were pelleted by centrifugation, and the supernatant was aspirated. The cells were then re-suspended in 2% BSA-PBS, filtered through a 40µm filter and stained with fluorescent-conjugated antibodies in darkness for 1 hour to avoid photo-bleaching. The cells were then washed and stained with DAPI to eliminate dead cells before being analysed. Unstained populations of cells were used

to control for auto-fluorescence. Consumables used for preparation of flow cytometry experiments are shown in **Table 2.14**, and all antibodies used are in **Table 2.15**.

In the experiments for **results section 3.3** cells were sorted on the BD FACS Aria machine at days 4, 7 and 12 of differentiation, using the combinations of markers shown in **Table 2.16** (fluorescent conjugated antibodies used are shown in **Table 2.15**). FACS was repeated at least three times for each time-point. Multi-colour flow cytometric analysis set-up, colour compensation and cell sorting were done by Ian Dimmik.
Cells were also analysed after miRNA inhibition on the LSRII flow cytometry machine using the combinations of markers shown in **Table 2.16**. Results were analysed using BD FACS DIVA software.

Table 2.14: Products for preparation of Flow cytometry samples

Consumables	Product number	Manufacturer
40µm nylon cell strainers	32540	Corning
DAPI	50-5005	Sysmex
5µl round bottom tubes	352054	Corning
Comp Beads	51-90-9001229/9001291	BD Biosciences

Table 2.15 Antibodies used in flow cytometry

Marker	Flourophore	Product number	Manufacturer
'Kennedy' Antibodies			
CD34	APC	555824	BD Biosciences
CD43	FITC	MHCD4301	Life Technologies
CD41a	APC-H7	561422	BD Biosciences
CD235a	BV421	562938	BD Biosciences
CD45	PercP-Cy5.5	564105	BD Biosciences
'Notta' Antibodies			
CD34	Percp-cy5.5	343522	Cambridge biosciences
CD45RA	APC (660)	550855	BD biosciences
CD38	BV 421	562444	BD biosciences
CD90	PE-Cy7	561558	BD biosciences
CD49f	PE	555736	BD biosciences
'Vodyanik' antibodies			
CD90	PE-Cy7	561558	BD biosciences
CD73	BV 421	562431	BD biosciences
CD34	APC	555824	BD Biosciences
CD43	FITC	MHCD4301	Life Technologies
CD45	PercP-Cy5.5	564105	BD Biosciences

Table 2.16: Markers used in cell sorting				
Time points		Day 4	Day 7	Day12
Markers used		CD31 CD34 KDR CD45	CD34 CD41a CD43 CD235a CD45	
Populations sorted	Negative	CD31- CD34- KDR- CD45-	CD34- CD41a- CD43- CD235a- CD45-	
	Haemangioblast	CD31+ CD34+ KDR+ CD45-	-	-
	Primitive	-	CD43+ CD34+/- CD41a+ CD235a+ CD45-	
	Definitive	-	-	CD43+ CD34+/- CD41a- CD235a- CD45+

2.5 Colony forming unit (CFU) assays

Hematopoietic progenitors were dissociated with TrypLE™ Express and counted. 60000 cells were then filtered through a 40µm cell strainer and transferred to a 3ml aliquot of MethoCult™ media. A syringe with a blunt ended needle was used to mix the cells into the media and transfer it into two 10mm cell culture dishes scored with 2mm grids. See **Table 2.17** for information about the products used in this experiment. These dishes were placed into a larger cell culture dish along with a small tissue culture dish filled with sterilised water as shown in **Figure 2.4.** This was then incubated at 37°C and 5% CO_2. After 14 days colonies in each dish were counted under a light microscope. Numbers were averaged between the two dishes. Representative images of CFU-M, CFU-G, CFU-E, and BFU-E are shown in **Figure 2.5**.

Table 2.17: Components for colony forming unit assay		
Product	Product number	Manufacturer
MethoCult™	4034	Stem cell technology
40µm nylon cell strainers	32540	Corning
Blunt-end Needles	28110/28120	Stem cell technology
3 cc Syringe	28230/28240	Stem cell technology
60 mm Gridded Scoring Dishes	27500	Stem cell technology

Figure 2.4

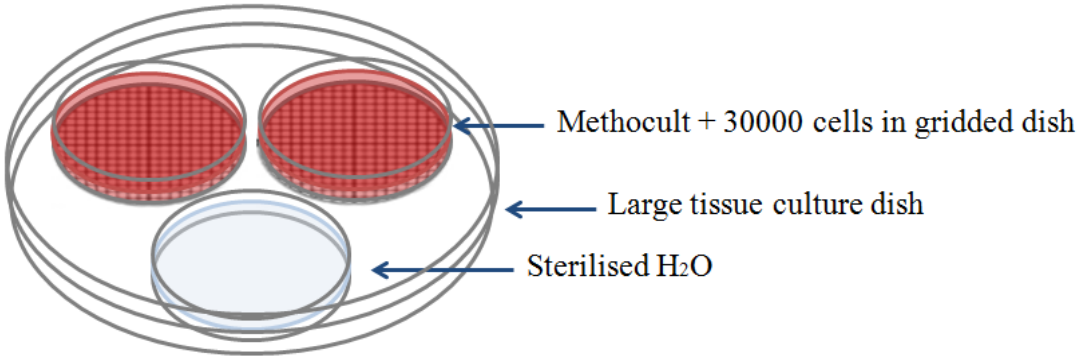


Figure 2.4: Schematic of CFU setup

Figure 2.5

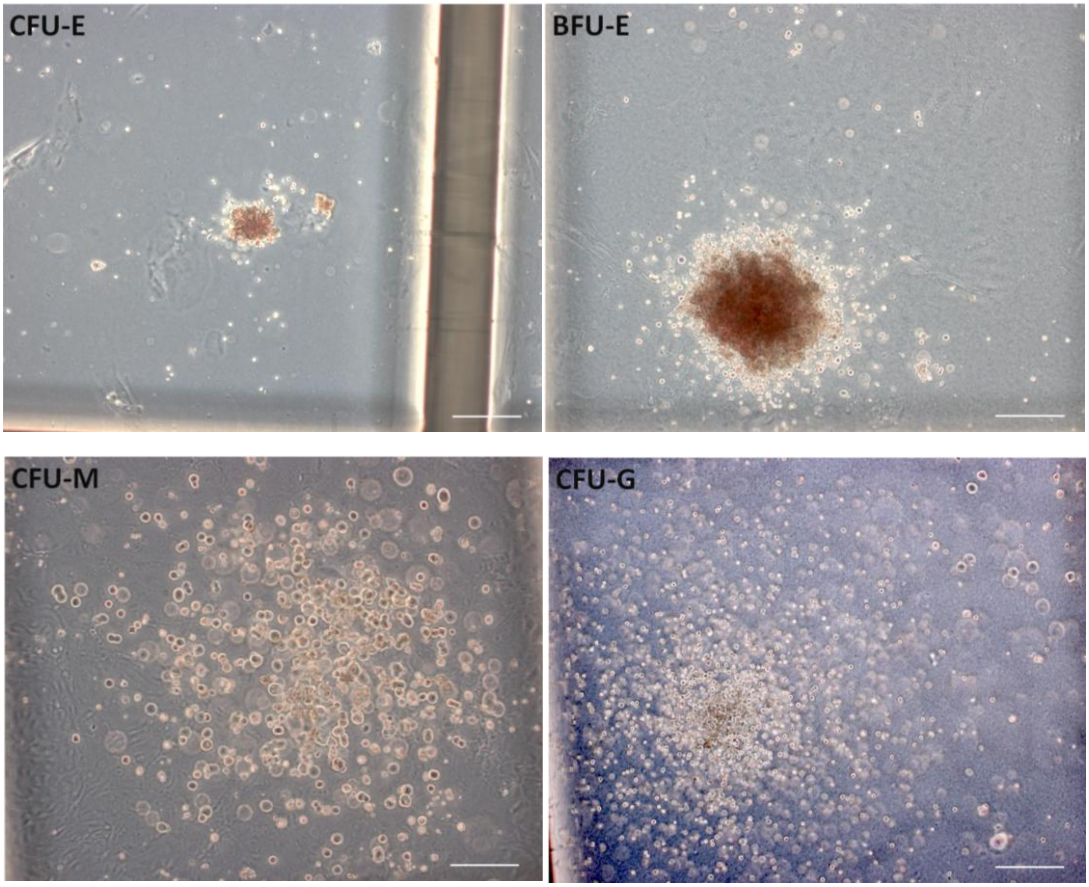


Figure 2.5: Representative micrographs showing typical morphology of the different types of colony forming unit. Top left: CFU-E (hemoglobinised colonies of 8-200 cells), top right BFU-E (hemoglobinised colonies of >200 cells), bottom left: CFU-M (diffuse colonies with large round cells), bottom right CFU-G (colonies made of small cells, sometimes with a dark 'core'). The bars on the images represent 200um.

2.6 CAFC assays

OP9-DL4 cells were cultured at 37°C and 5% CO_2 in T75 flasks in OP9 medium (see **Table 2.18**) which was replaced every three days. The OP9-DL4cells were passaged every 5-7 days to prevent them from becoming confluent, which causes them to become adipogenic. Cells were passaged into 96 well plates approximately 3 days before co-culture, at a density allowing them to achieve 80-90% confluence at the start of the experiment. At day 12 of differentiation hematopoietic progenitors were dissociated using TrypLE™ Express and plated onto the 96 well plate of OP9-DL4 cells in 3x dilutions starting at 5 x 10^4 cells per ml in OP9 medium supplemented with 5ng/ml Flt-3L and 1ng/ml IL-7. They were incubated for 7 days, with ½ of the supplemented medium replaced after 3 days, and then analysed under phase contrast microscopy for cobblestone colonies.

Table 2.18: OP9 medium and cytokines for CAFC		
Recombinant human Flt-3 ligand	308-FK	R&D Systems
Recombinant IL-7	217-17	Peprotech
OP9 medium		
α-MEM	500ml	
FBS	126ml	
Penicillin-streptomycin	5ml	

2.7 Lentiviral transfection

NHDF fibroblasts or differentiating H9 cells were transfected either using Genecopoiea™'s lentiviral particles containing a plasmid with a mir-200c inhibitor, mCherry reporter and hygromycin resistance gene, or with MISSION® Lenti microRNA Inhibitors (Sigma-Aldrich **HLTUD0001)** containing a plasmid with a miR-200c inhibitor and a puromycin resistance gene.

NHDF or H9 cells were passaged into 12 well plates 24hrs before transduction. Cells must be in the exponential growth phase so they were plated at a density allowing them to be no more than 80% confluent. The media was aspirated and replaced with fresh media with 8μg/ml of polybrene. Lentiviral particles were thawed on ice and added to the cells at a range of MOIs (see **results section 5.1**). Wells were transfected in triplicate for each MOI and for the negative scramble-control. Media was changed after 24hrs to remove the polybrene. Cells were analysed after 48 hrs.

For experiments with Genecopoiea™'s lentiviral particles, the percentage of cells expressing mCherry was calculated either by flow cytometry or by counting the percentage of fluorescent

cells photographed using a Zeiss fluorescent microscope. For experiments with Sigma lentiviral particles the cells were treated with 0.5µg/ml puromycin 24hr after transfection.

Multiplicity of infection (MOI) was calculated using the formula MOI = number of viral particles/number of cells. The number of viral particles used in a particular experiment was calculated based on information about the number of viral particles per micro-litre provided on the manufacturer's datasheet.

2.8 Lipofection

Lipofectamine™-RNAi complexes were prepared by diluting 20pmol of mirVana™ miRNA inhibitor or control (mirVana™ miRNA inhibitor negative control or Santa Cruz biotechnology Flourescein conjugate control, see **Table 2.19**) in 50ul Opti-MEM® I Medium and 6µl of Lipofectamine RNAiMax™ in 50ul Opti-MEM® I Medium. Both were incubated at room temperature for 5 minutes, then mixed and incubated at room temperature for a further 20 minutes. For Multiplex reactions 20pmol of each inhibitor was used, while increasing the quantity of lipofectamine to maintain the miRNA-inhibitor to lipofectmine ratio.

The complexes were added to one well of a 12 well plate with 1ml of differentiation medium at day 10 of differentiation, the plate was spun at approx 300g for 10 minutes and then returned to the incubator. Cells were analysed after 48 hours.

Table 2.19: Components for lipofection

	Manufacturer	Product number
Opti-MEM® Reduced Serum Medium	Life technologies	31985062
Control siRNA (FITC Conjugate)-A	Santa Cruz	sc-36869
lipofectamine® RNAiMAX	Life technologies	13778075
miR-424 mirVana® miRNA inhibitor	Life technologies	4464084/MH10306
miR-205 mirVana® miRNA inhibitor	Life technologies	4464084/MH11015
miR-148a mirVana® miRNA inhibitor	Life technologies	4464084/MH10263
miR-200c mirVana® miRNA inhibitor	Life technologies	4464084/MH11714
miR-200b mirVana® miRNA inhibitor	Life technologies	4464084/MH10492
mirVana™ miRNA Inhibitor, Negative Control #1	Life technologies	4464076

2.9 CytoSNP

CytoSNP analysis was performed by Dr Simon Zwolinski (Northern Genetics Service) and Dr David Lee. 1µg of sample DNA was processed on the CytoChip oligo SNP microarray and analysed using BlueFuse Multi software.

2.10 Statistical analysis

The original experimental design in this project intended to use ANOVA to determine the significance of the difference between the control and treated cells. However it is necessary to take into account the effect of passage number on hematopoietic differentiation (see **results section 3.4**). Since this did not become clear until the project was almost completed it was not possible to do all the experiments at the same passage. ANCOVA (analysis of covariance) was used to determine significance instead.

ANCOVA is a generalised linear model which combines ANOVA and linear regression. The control is compared to the treatment while statistically adjusting for the variation caused by the *covariate*, which in this case is the effect of passage number.

This technique for statistical analysis allows the data to be presented in the form of 'estimated marginal means' (EMMs), the means of each factor adjusted for the covariate. This effectively shows an estimate of what the results would look like if all the experiments were done at the same passage.

The figures and the majority of statistics were calculated in GraphPad Prism®, however this software cannot do ANCOVA so the ANCOVA and EMM were done in SPSS statistics. Inhibitions were compared to control data using univariate one-way ANCOVA, p values are calculated as Sidak corrected bootstrapped significance.

3. Results: hematopoietic differentiation

3.1 Differentiation of pluripotent cells into hematopoietic progenitors

This project requires an efficient method for differentiating pluripotent cells into hematopoietic progenitors. Several methods have been used to differentiate pluripotent cells into hematopoietic cells; the main types being co-culture and EB formation with various combinations of cytokines (see **introduction section 1.3**). In order to compare the effectiveness of these methods we need to cover the techniques which can be used to assess the quantities and types of hematopoietic cells which they produce.

3.2 Methods for assessing the effectiveness of differentiation protocols

3.2.1 Transplantation assay

The gold-standard for testing for the presence of HSCs is functional transplantation: that is, hematopoietic cells injected into an animal which home to the bone marrow niche, engraft there and produce all blood lineages, and continue to do so on a permanent basis or over multiple transplantations. Performing this test on human cells presents a challenge, as the animal's immune system will react to the presence of foreign cells. To overcome this researchers have used mice with various types of immune deficiencies. It is essential to use the most immune-deficient mouse possible as any immune response can cause the transplant to be rejected and interfere with the results of the experiment.

'SCID' (severe combined immune deficiency) mice were discovered in 1983 by Bosma et al. [124] They have a mutation in *Prkdc*, the gene responsible for repairing DNA after V(D) J recombination, which means very few T-cells can survive to maturity [125]. Human bone marrow has been transplanted into SCID mice since 1992 [126].

SCID mutations have been made in mice with a NOD (non-obese diabetic) genetic background, which means they are almost without B-cells, T-cells and NK-cells and have impaired innate immunity due to abnormal myelopoiesis and deficiencies in the haemolytic complement system [127-129]. NOD-SCID mice have up to 10-fold more engraftment of human cells compared to mice with the SCID mutation alone [127], but they can still sometimes achieve an immune reaction. This is due to a phenomenon called 'leakiness'; a fraction of lymphocytes spontaneously repair their DNA after V(D) J recombination without *Prkdc*, and these viable NK cells accumulate in the mouse with age, allowing it to reject foreign cells [130].

Ito et al. induced a null mutation in the gene encoding IL2Rγ, which completely blocks the formation of NK-cells, further improving engraftment [131]. These NSG (NOD-SCID-

Gamma chain null) mice are the most immune-deficient mice that have been developed [132] and are now commonly used to assess human HSC potential.

Although transplantation into immune compromised mice remains the gold standard for testing for the presence of HSCs, the ethical issues around animal use and the cost, technical challenges, and time consuming nature of the transplantation assay have meant that *in vitro* assays for hematopoietic cells' potential have been developed. The most informative of these are colony assays, cell surface marker expression and gene expression.

3.2.2 Cobblestone Area Forming Cells (CAFC)

CAFC assays can inform us about the presence of cells with lymphocyte potential, which is used as a stand-in for HSC potential. The bone marrow niche is mimicked using defined mouse stromal cell lines. The stromal cells form a monolayer, which the hematopoietic progenitors migrate underneath, forming 'cobblestone-like' areas of cells which can be distinguished from mature hematopoietic cells because they are non-refractile, appearing dark under phase contrast microscopy [133]. The co-cultures are assayed at different time points after seeding with hematopoietic progenitors, as the cells will differentiate according to their stage of differentiation/position on the hematopoietic hierarchy. At 7-10 days cobblestone areas indicate committed lymphoid progenitors, and cobblestone areas which persist at 5-8 weeks are less mature leukocytes. The presence of these colonies correlates with long term repopulating cells [134], although there is insufficient evidence to say that they actually represent LT-HSC.

The most commonly used stromal cell line is OP9 cells, which are a from a mouse line deficient for M-CSF, making them unable to support macrophage development which allows other hematopoietic cell types to develop. OP9s can be used to support both B and T lymphocytes [135]. OP9s have also been used to support hematopoiesis from pluripotent cells (see **introduction section 1.3**). Notch signalling is critical for T cell development [136] so OP9 cells modified to express delta ligands DL1 or DL4 have been used to support the differentiation of T cells [137]. In co-culture with human cells they are also supplemented with IL7 and FLt3l. This assay is particularly important for this project as T cells are considered to only arise from definitive HSCs and have been used as evidence for HSC presence by Kennedy et al [8], although a recent paper has shown that T-cells may be able to arise independently [79], and long term T-lymphocyte regenerating cells have to been demonstrated to exist in P-HPC experiments. T cells can be tested for with a set of markers including CD4 and CD8.

This technique can be quantitative with the use of limiting dilutions, however the use of animal cell lines which can behave very differently in at different times and in different hands severely limits the reliability of this assay.

3.2.3 Colony forming unit (CFU) assays

CFU assays are the most commonly used in vitro assay for determining the numbers of hematopoietic progenitors in a sample. They are used both in basic research and in diagnosis of haematological malignancies and diseases of the bone marrow [138]. The assay is done by plating hematopoietic progenitor cells in methylcellulose supplemented with hematopoietic cytokines. The methylcellulose is viscous enough to prevent colonies from dispersing, allowing them to be easily counted. Cytokines, including EPO, SCF, GM-CSF and IL3 are used to encourage the cells to differentiate. The test can distinguish several types of hematopoietic colony on the basis of morphology; The most common are lineage restricted colonies such as colony forming unit granulocytes (CFU-G), colony forming unit monocytes/macrophages (CFU-M), colony forming unit erythrocytes (CFU-E) and the larger, less mature burst forming unit erythrocytes (BFU-E), but there are also multi-lineage colony forming units, colony forming unit granulocyte-monocyte (CFU-GM) and colony forming unit granulocyte erythroid monocyte (CFU-GEMM) derived from more primitive multi-lineage precursors [133]. CFU-GEMM colonies are the rarest but the most important for this project as they have multipotent potential and are therefore the ones most similar to HSCs. However CFU assays cannot measure HSC potential directly, as a CFU-GEMM could also be produced by multipotent progenitors or common myeloid progenitors. Although there is some subjectivity in the way the colonies can be scored they are morphologically distinct enough that this is considered a quantitative method.

In the absence of supporting stromal cells the hematopoietic progenitors can't differentiate into lymphocytes, so this assay can only test for erythroid, myeloid and megakaryocyte lineages. Although this assay is limited in that it does not measure lymphoid potential or long term potential it is useful in that it is relatively quick and simple to run, requires far fewer man hours and less technical expertise than CAFCs or transplant assays, and the lack of animal cell lines make it relatively reproducible.

3.2.4 Gene expression

Hematopoietic cells of different types express specific genes. The expression of a particular gene in a sample can be measured by qPCR, allowing a determination to be made about

whether or not it is possible that they are HSCs. In murine embryos gene expression of emerging HSCs has been studied at the single cell level [139]. However, demonstrating the expression of any one gene is not enough to prove that HSCs are present because there are no known genes which are specific to *only* HSCs; many of the hematopoietic genes are expressed at even higher level in more differentiated hematopoietic cells, and other genes reduce in expression as HSC differentiate, so low expression may mean that the cells are terminally differentiated or that they are not hematopoietic at all. It is possible to make comparisons of the level of gene expression for these key hematopoietic genes between P-HPCs and hematopoietic progenitors which are derived from bone marrow or cord blood.

There are several key transcription factors which a HSC must express. HSC are specified in a stepwise manner in the embryo, and this is achieved by the carefully timed switching on of particular genes: *Brachyury* is used to specify mesoderm [140], which is further specialised into hemogenic mesoderm by *KDR* [52, 65], and later into hemogenic endothelium by the expression of *SCL* [9, 141-144] and *TGFβ* [145]. *SCL* and *GATA2* are responsible for promoting the expression of *RUNX1*, which allows EHT to occur and thereby promotes definitive HSC formation in the AGM [71, 146-148].

HOX genes are also essential for specifying hematopoietic cells, there are several involved in hematopoiesis [50, 149, 150], and in general they are highly expressed in HSC but expression decreases as the cells differentiate. *HOXB4*'s role in hematopoiesis has been extensively studied as ectopic expression has been shown to enhance engraftment [151] and can even reprogram mESC derived primitive hematopoietic progenitors into LT-HSCs [50]. *HOXA9* is also a key hematopoietic gene and has been shown to be one of the most differentially regulated genes in HSC compared to P-HPC [150].

There are also several master transcription factors which control hematopoietic cell fate and lineage specification. For example GATA1 promotes erythroid cell fate [109, 152], CEBPA is required for granulocyte/macrophage development [153] and PU.1 is necessary for macrophages and B-cells [99, 101, 154].

3.2.5 Cell surface markers and flow cytometry

The methods described so far have been retrospective, by the time the test produces meaningful results the cell types being tested for are gone, but cells of different types can be distinguished from one another by the proteins or 'markers' which are expressed on their surfaces. Fluorescent conjugated antibodies of different colours bind to the markers on the cells and the combination of the antibodies is used to determine cell type. This can be done in

fixed tissues using immunostaining or in live or fixed single cells using flow cytometry. Blood cells are usually found in suspension and it is often clinically useful to be able to analyse and sort live blood cells so blood is usually analysed using flow cytometry. Fortunately the study of normal and pathogenic blood cell types by flow cytometry is well established, markers for all the blood cell types found in adults have been extensively studies and validated and antibodies for many different blood markers with different fluorescence spectra are available commercially, allowing us considerable flexibility in these experiments.

Markers for LT-HSC: CD34 has long been used to isolate hematopoietic progenitors for transplant but this population, although it contains LT-HSC, is mostly made of more differentiated progenitors. CD45RA [155, 156] and CD38 [157] were found to exclude the CD34+ population with hematopoietic reconstitution potential and the CD90+ population was found to contain all LT-HSCs. This combination of markers alone still cannot isolate LT-HSCs at the single cell level [158], this was only achieved by Notta et al [159] when they discovered that CD49f is exclusively expressed on LT-HSCs within the CD34+CD38-CD45RA-CD90+ population. This combination of markers can isolate single cells capable of reconstituting long term multi-lineage hematopoiesis from bone marrow, although it has not been demonstrated that pluripotent derived cells bearing these markers are capable of the same.

Markers for embryonic hematopoiesis: Markers for human embryonic hematopoiesis are less well studied than those for adult hematopoiesis. In mice the SLAM markers CD150+CD48- represent HSCs [160]. However, although there is some similarity and overlap between the markers on mouse and human hematopoietic cells there are enough known differences as to make it impossible for us to conclude that a combination of markers which are found on one cell type in mice are the same as in human cells [161].

Studies in human foetal tissue are rare and few of them have been done at the time point which is most useful for us, i.e. approx. 6 weeks, when definitive cells start to appear in the AGM. Almost all of our knowledge of the markers for embryonic development hematopoietic cells comes from *in vitro* studies of differentiating PSCs. This presents a problem in that although cells made *in vitro* to those found *in vivo* are comparable in terms of morphology and gene expression they don't necessarily form in exactly the same way. It is also impossible to study the very first markers of HSCs this way, even though it is in these markers we are most interested, since it has not yet been demonstrated that HSCs can be made *in vitro* at all.

Historically, the two most important markers for studying hematopoietic stem cells were CD34 and CD45. CD45 is a 'pan-hematopoietic' marker which is expressed on all hematopoietic cells except mature erythrocytes and can be used to distinguish blood cells from any other type of cell [162, 163]. CD34 is a marker for hematopoietic progenitors, and is expressed on LT-HSCs as well as ST-HSCs, MPPs and some more differentiated hematopoietic progenitors [54]. However when analysing cells differentiated from PSCs it is necessary to use other markers as CD34 is also expressed on endothelial cells and haemogenic endothelium [54] and CD45 does not appear on the very earliest hematopoietic population to arise [65].

The primitive streak mesoderm from which hematopoietic cells eventually arise expresses Brachyury, the subset of cells which will eventually give rise to hematopoietic cells also express endothelial marker KDR [164]. Other endothelial markers such as CD34 and CD31a are expressed on both endothelial cells and the haemogenic endothelium [54, 165]; the earliest markers which can distinguish hematopoietic potential are CD43 [65] and CD41a [148, 166], cells begin to express CD45 as they undergo EHT [167, 168], and lose expression of endothelial markers such as CD31a and VE-Cadherin at the same time. Hematopoietic cells express both CD34 and CD45 for a time and lose CD34 expression as they develop. All CFU-GEMMs develop from the CD34+ population [65].

Experiments by Vodyanik et al [65] where differentiating PSCs were sorted for these cell surface markers and then analysed for their colony forming potential showed that the CD34+CD43+CD235a+CD41a+CD45– cells represented megakaryocyte-erythroid primitive progenitors. The same paper shows that CD34+CD43+CD41a–CD235a–CD45– cells were multipotent myeloid progenitors and Kennedy et al [8] confirmed these results and demonstrated that CD34+/-CD43+CD235a-CD41a-CD45+ cells are definitive progenitors capable of lymphopoiesis.

3.3 Choosing a protocol for hematopoietic differentiation

Protocols for hematopoietic differentiation from pluripotent stem cells have been developed for some time (see **introduction section 1.3**), with progressive improvement in yield and the ability to make more of the different definitive and primitive cell types. Although co-culture methods have proven successful in many ways it is more useful to use an EB protocol with defined factors as this is more reliable than using animal cells and it has more translational potential. Initially the protocol described by Kennedy et al in 2012 [8] (see **methods section 2.1.2**) was chosen, as it a) makes use of defined factors and medium, b) is similar to the one used to generate the microarray data, since it was developed by the same group and c) because

it was shown to produce T-lymphocytes, considered a stand-in for HSC potential, as well as all other hematopoietic lineages.

Unfortunately the protocol proved challenging in our hands, requiring very high numbers of pluripotent stem cells to produce very small numbers of EBs and frequently failing to produce any EBs entirely. This was a serious limiting factor in the early part of the project, making it difficult to produce enough hematopoietic progenitors to experimentally analyse (see **Figure 3.1**).

Fortunately our collaborators Dr Joanne Mountford and Dr Scott Cowan at the University of Glasgow had developed a protocol for the differentiation of erythroid cells [2]. Our lab modified this protocol by differentiating only up to the progenitor stage (a schematic for this protocol is shown in **Figure 3.2**, a more detailed description of the protocol is in **methods section 2.1.2**). This protocol was far more efficient and reliable than the previous one. It can reliably produce hundreds of EBs from a single well of pluripotent stem cells which, once formed can produce hematopoietic cells of all the same types as the Kennedy protocol with at least equivalent efficiency (see **Figure 3.1**). The Kennedy protocol was abandoned early in the project in favour of the one from the Glasgow group, and all the experiments in this project were done using cells made with the Glasgow protocol unless otherwise stated.

Figure 3.1a

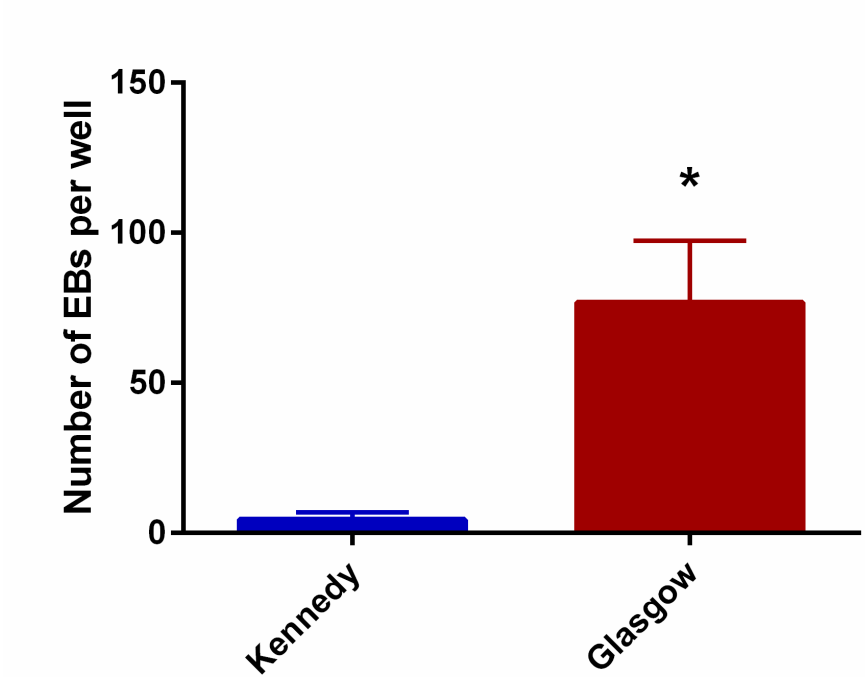


Figure 3.1b

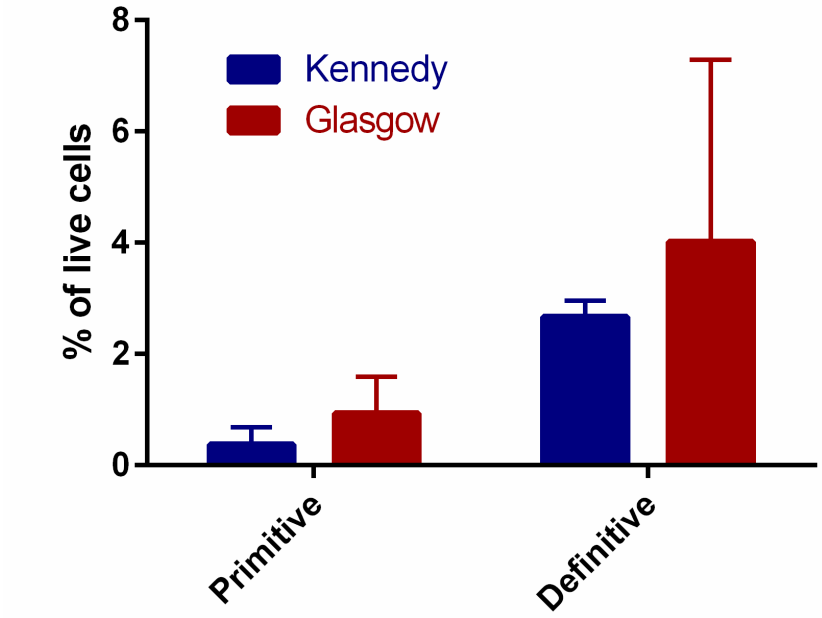


Figure 3.1a: Graph showing mean+SD for the number of embryoid bodies per well of H9 cells at day 3 for each protocol. n = 3

Figure 3.1b; Graph showing comparison between the number of primitive or definitive hematopoietic progenitors produced by each protocol at day 12. Mean+SD n=3. Primitive=CD34low/-CD43+ CD235a+CD41a+, Definitive=CD34low/-CD43+CD45+.

Figure 3.2

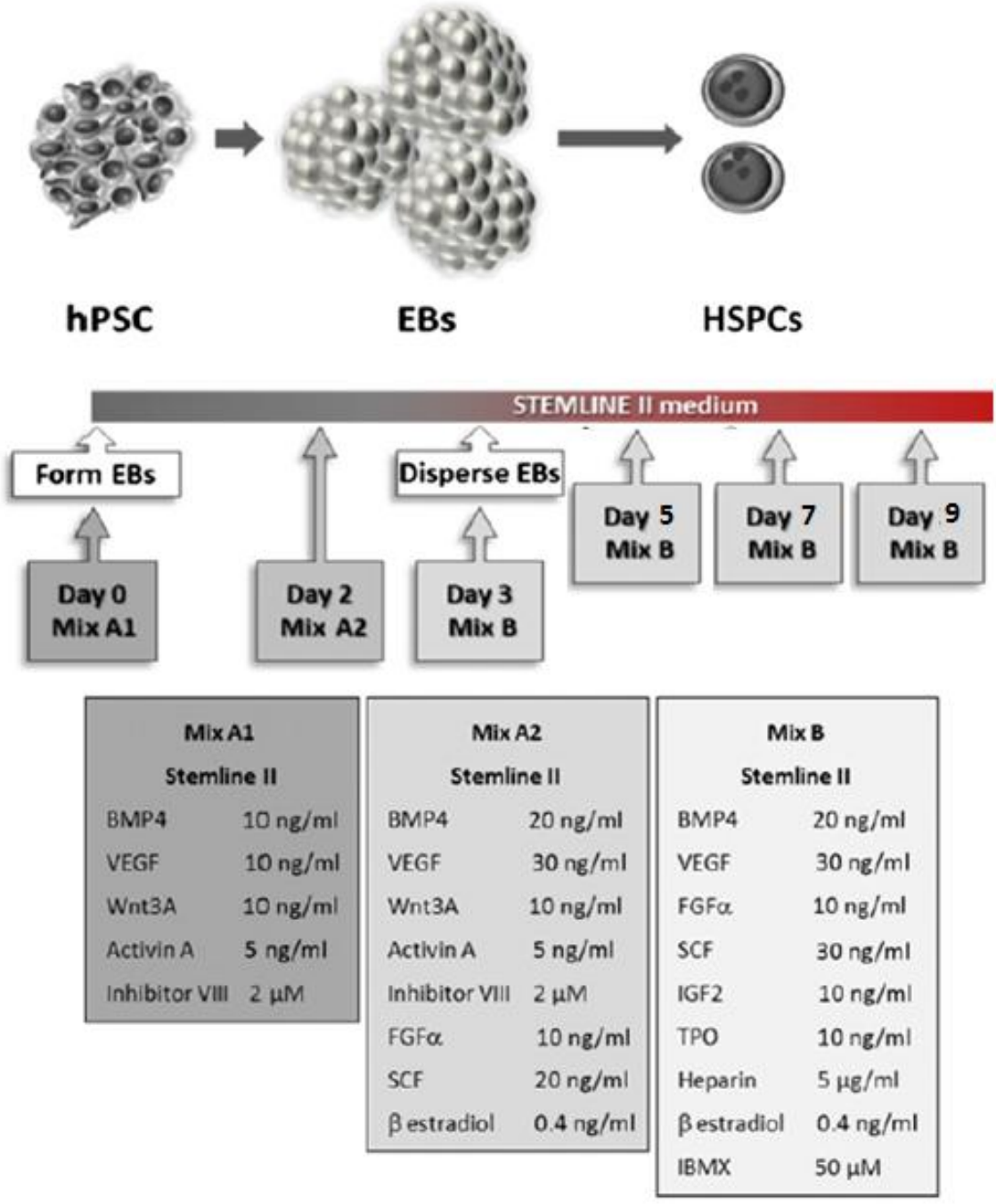


Figure 3.2: Schematic of Glasgow protocol as used in this project, adapted from figure in Olivier et al [2]

The major difference between the protocols is in the way EBs are formed, with the Kennedy protocol dissociating the pluripotent cells into single cells and then re-aggregating them. The advantage of this method is that it allows precise control over the size of the embryoid body using Aggrewell^{TM} plates, which is important because 500-1000 cell EBs are optimal for hematopoietic development [169]. However human stem cells are not able to survive as single cells, and rapidly undergo anoikis when dissociated. This problem can be overcome using

ROCK inhibitor [170], but inhibiting the ROCK pathway inhibits hematopoiesis [171]. Without ROCK inhibitor the cells failed to form reasonable numbers of EBs. In contrast, the Glasgow protocol uses the EZ-roller to cut out uniformly sized squares of cells in the correct size for EBs (see **methods section 2.1.2**) , and as the cells are not split into individual cells it avoids the problem of anoikis.

The Glasgow protocol also used a slightly different combination of cytokines so there was a question of whether the Glasgow or Kennedy cytokines should be used after using the Glasgow method for EB formation. Work by my colleague Dr Jarmila Spegarova showed that the Glasgow cytokines produced a slightly higher yield of progenitors (data not shown).

The Glasgow protocol is able to produce all the major blood lineages: lymphocytes, as shown by our CAFC results (see **Figure 3.3**), and granulocytes, monocytes and erythroid cells, as shown by our CFU data (see **Figure 3.5**). Use of the cell surface markers described by Kennedy et al (see **results section 3.2.5**) shows that their protocol can produce both primitive and definitive hematopoietic progenitors. There is also a small population of cells which express CD34+CD38-CD45RA-CD49f+CD90+ (see **results section 3.2.5**) at day 12 (see **Figure 5.7**). Although it is assumed that the hematopoietic progenitors produced by these cells are foetal in character and it is not known what combination of markers the earliest HSCs that arise in the human embryo express, and this combination of markers cannot unambiguously be said to represent HSCs in this context, it is encouraging that this population exists in our cultures. The efficiency, defined nature, and success at producing diverse hematopoietic progenitors makes this protocol ideal for assessing the effect of miRNA expression on HSC potential.

Figure 3.3

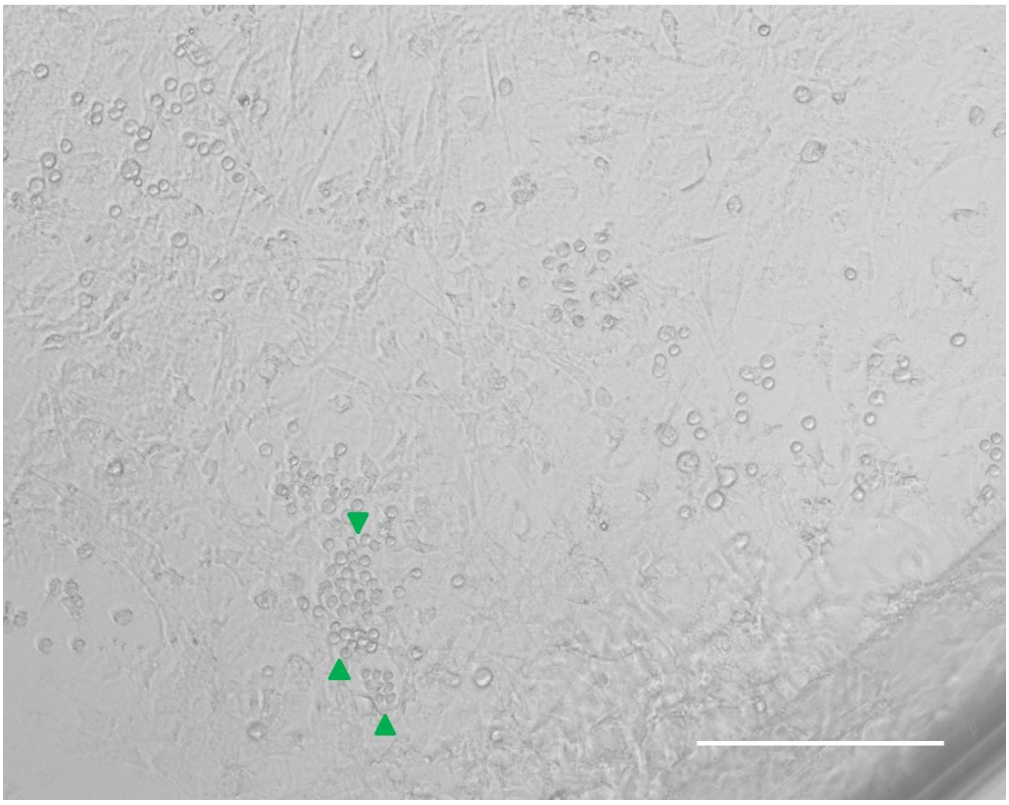


Figure 3.3: Representative micrograph of CAFC assay. Colonies with cobblestone morphology can be seen (green arrows). Bar represents 200 μm.

The Glasgow protocol recapitulates embryonic hematopoietic development: First, mesoderm is formed, then endothelial cells (KDR+CD31a+CD34+CD45-), followed by primitive hematopoietic cells (CD43+CD34+/-CD41a+CD235a+CD45-), and finally definitive cells (CD43+CD34+/-CD41a-CD235a-CD45+) (see results **section 4.2**). The dissociation and plating of the progenitors at day three allows colonies of endothelial-hematopoietic progenitors to grow, and clusters of rounded hematopoietic cells can be seen arising from the adherent endothelial colonies –the same morphological progression which has been observed in developing hematopoietic cells in the foetal aorta [58] (see **introduction section 1.3**), which provides an immediate visual confirmation of hematopoietic development (see **Figure 3.4**).

In this project both a hESC line and a hiPSC line have been used (H9 and SB-Ad3 respectively). This is important because several papers have shown differences in the effectiveness of hematopoietic differentiation between ESCs and iPSCs [37, 38]. The H9 and SB-Ad3 lines in particular were chosen because they are established, well-characterised lines

which are known to be easy to culture and can be reliably differentiated into many cell types (see **methods section 2.1.1**).

Figure 3.4a

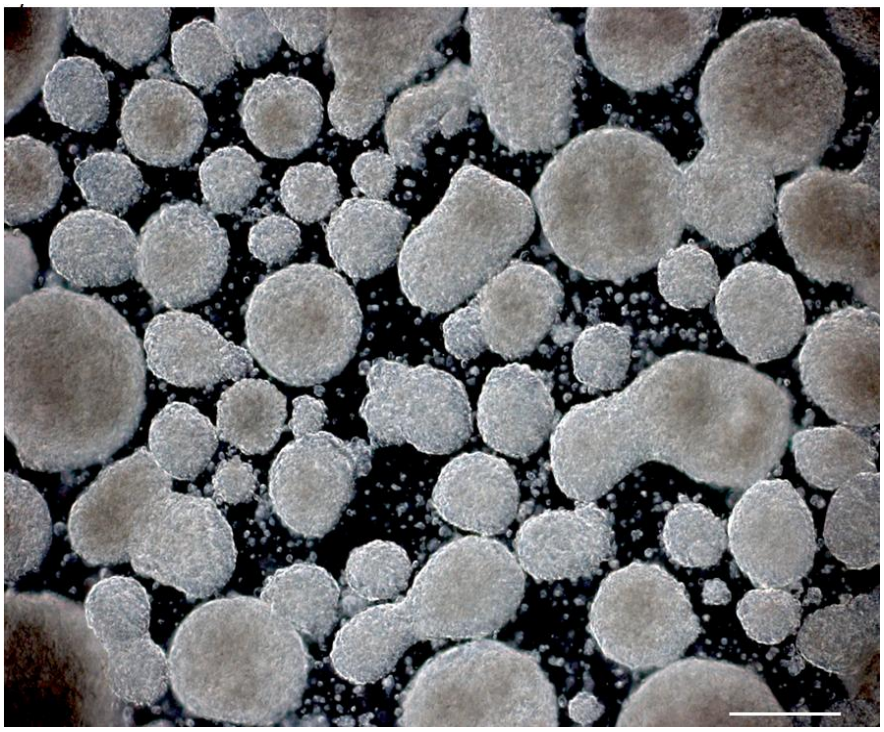


Figure 3.4b

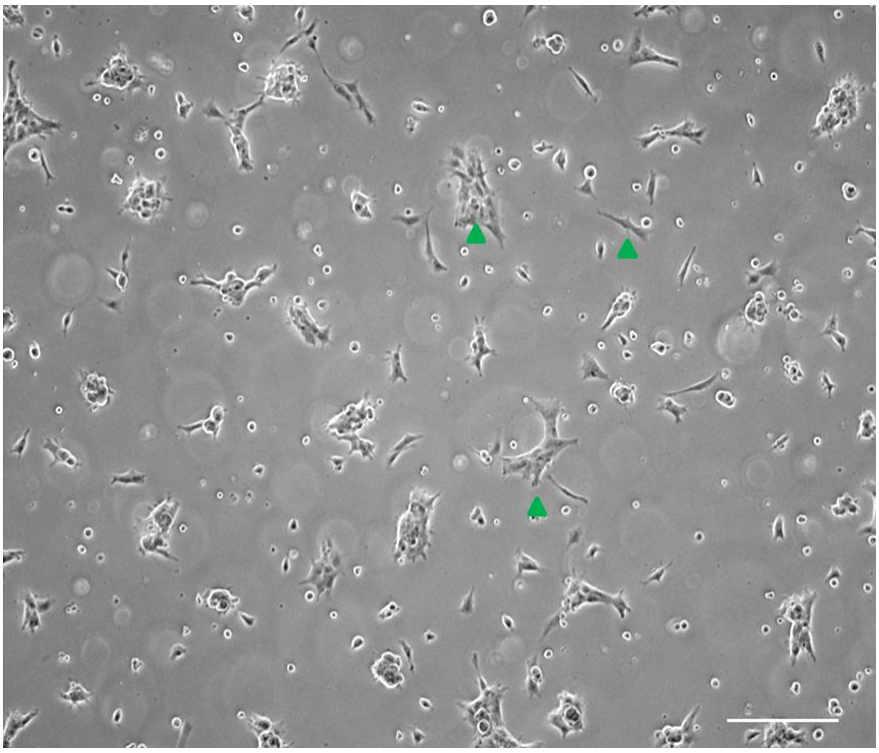


Figure 3.4a: Representative micrograph of day 3 H9 EBs. Bar represents 500 µm **3.4b:** Representative micrograph of day 4 colonies. This image is of SB-Ad3 derived cells but both H9 and SB-Ad3 show the same morphology. Small colonies of endothelial-like cells can be seen (green arrows). Bar represents 100 µm

Figure 3.4c

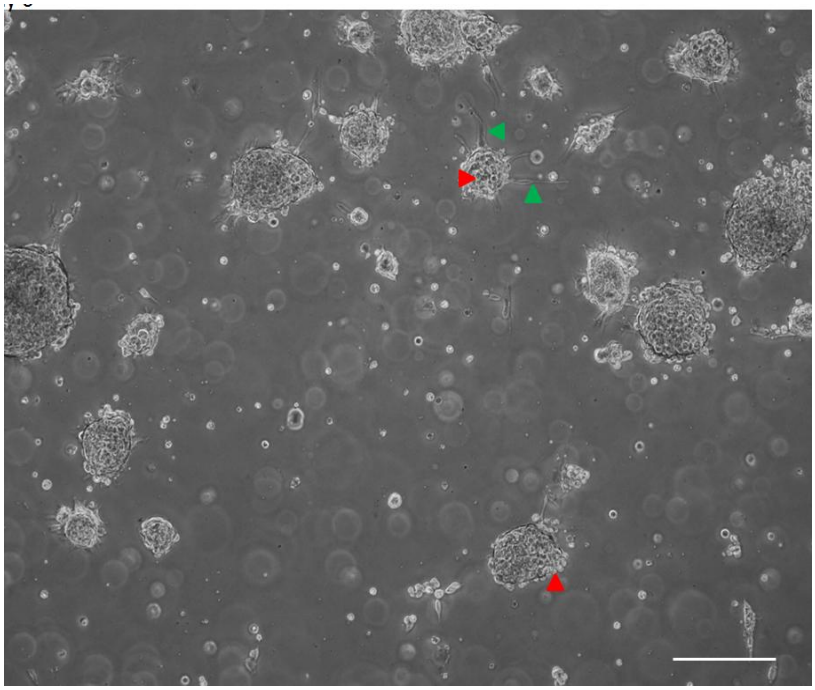


Figure 3.4d

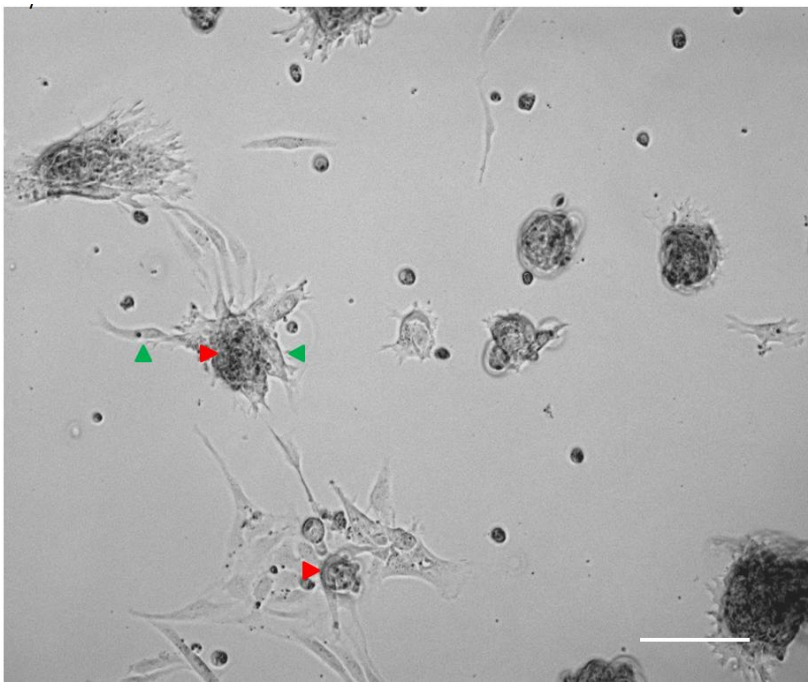


Figure 3.4c: Representative micrographs of day 6 H9 derived hematopoietic colonies. SB-Ad3 colonies have the same morphology. The bar on **3.4c** represents 200 µm, **3.4d** represents 100 µm. Colonies at day 6 are larger; cells with endothelial morphology can be seen around the edges of the colonies (green arrows) and some cells are beginning to round up and 'bud off' the colonies (red arrows), similar to *in vivo* hematopoietic cluster formation.

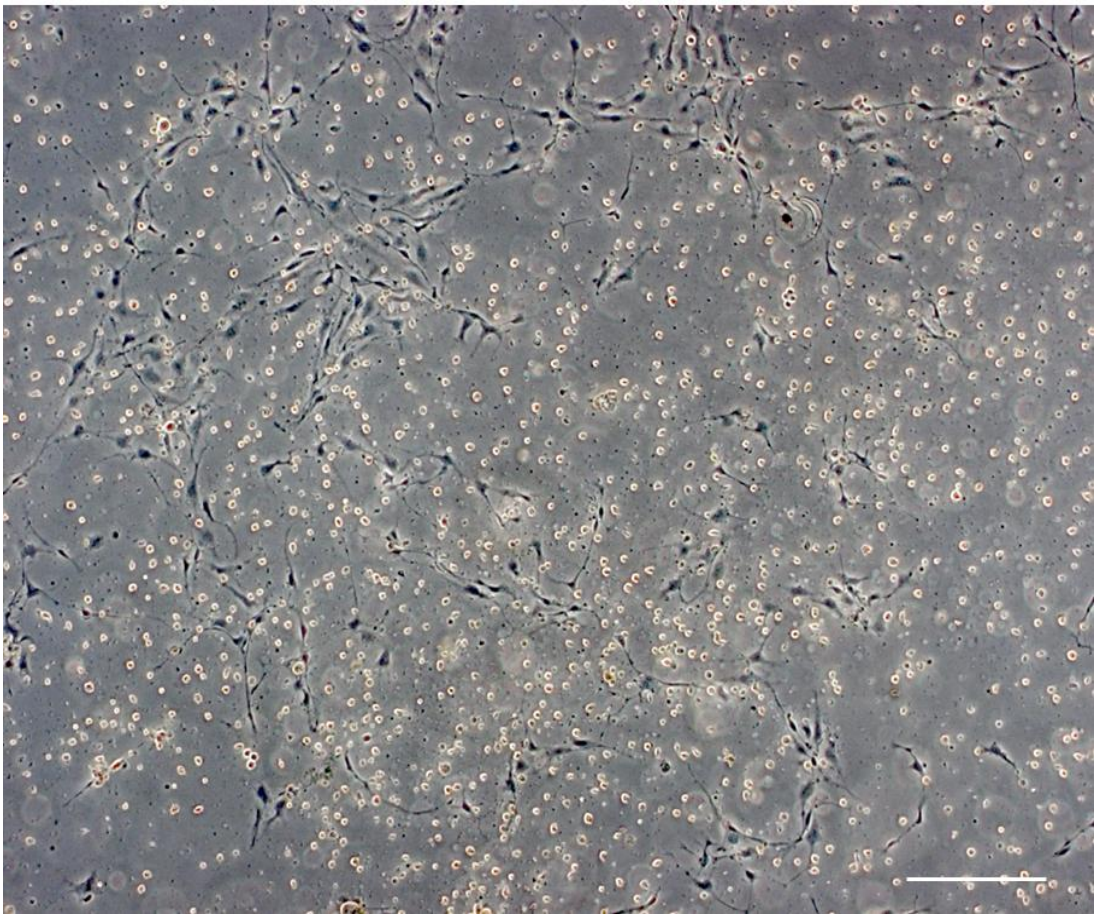


Figure 3.4e: Representative micrograph of day 12 hESC derived haematopoietic cells. The majority of cells are in suspension at this point, in both the H9 and SB-Ad3 experiments. Bar represents 200 μm.

3.4 The effect of passage number on hematopoietic differentiation

CFU assays: During the course of this project, in spite of using an improved protocol it became clear that the results of many of our experiments were inconsistent, giving variable results from one repeat to another. While searching for an explanation for this problem a pattern in the colony forming assay results became clear; fewer hematopoietic colonies are formed from pluripotent stem cells at later passages (see **Figure 3.5**). The trend is clear in both the H9 and SB-Ad3 cell lines, numbers of all colony types are reduced at later passage, although not all of them have statistical significance. The trend is particularly obvious in the erythroid colony potential as day 12 hematopoietic progenitors can produce up to several hundred per plate at passage <40 but consistently none or almost none at passage >60. Although there are is not enough data to show significance for all of the different colony types there is a clear downward trend in the number of cells with any type of hematopoietic potential.

Figure 3.5a

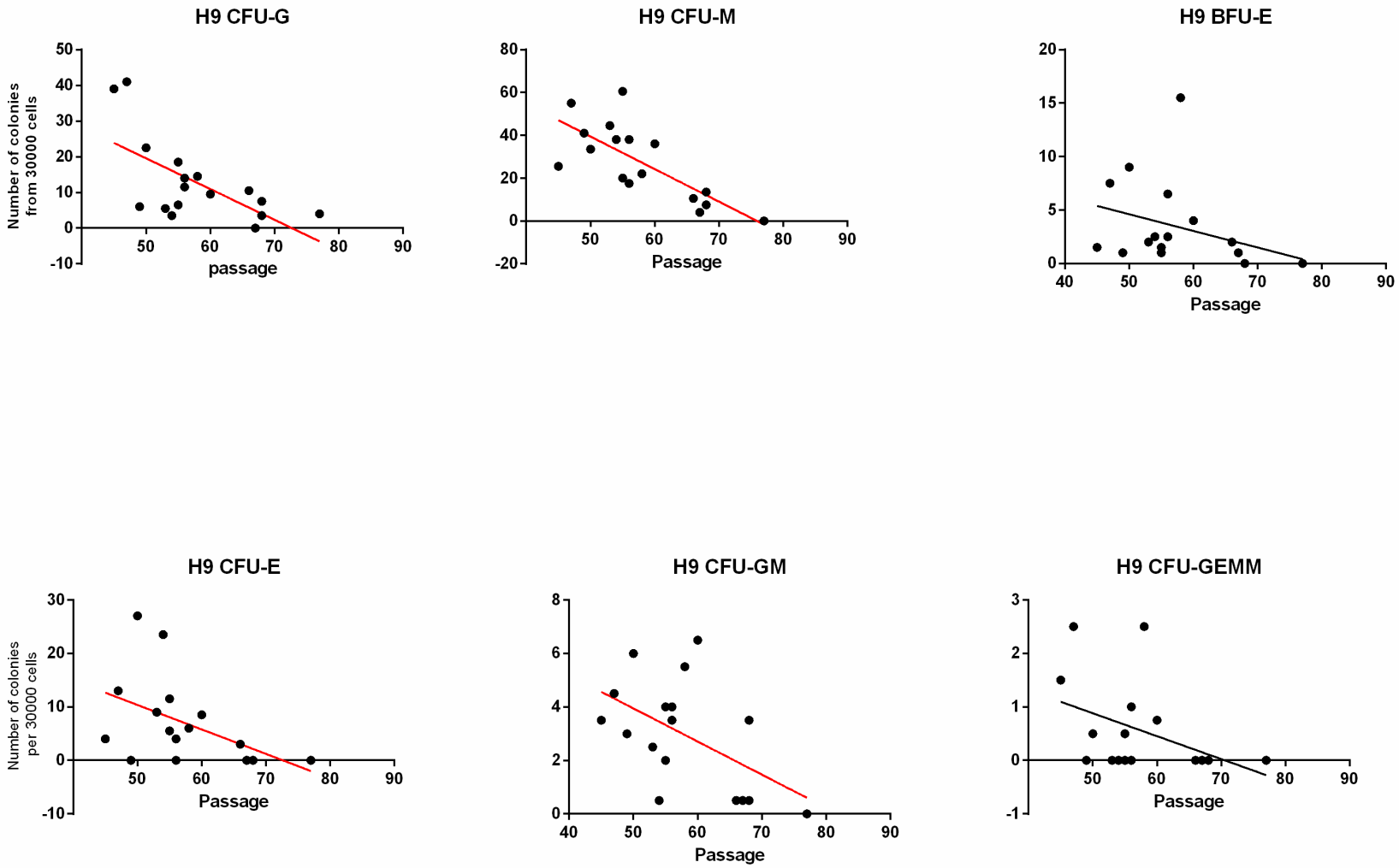

H9 CFU-G
Number of colonies from 30000 cells
passage
H9 CFU-M
Passage
H9 BFU-E
Passage
H9 CFU-E
Number of colonies per 30000 cells
Passage
H9 CFU-GM
Passage
H9 CFU-GEMM
Passage

Figure 3.5b

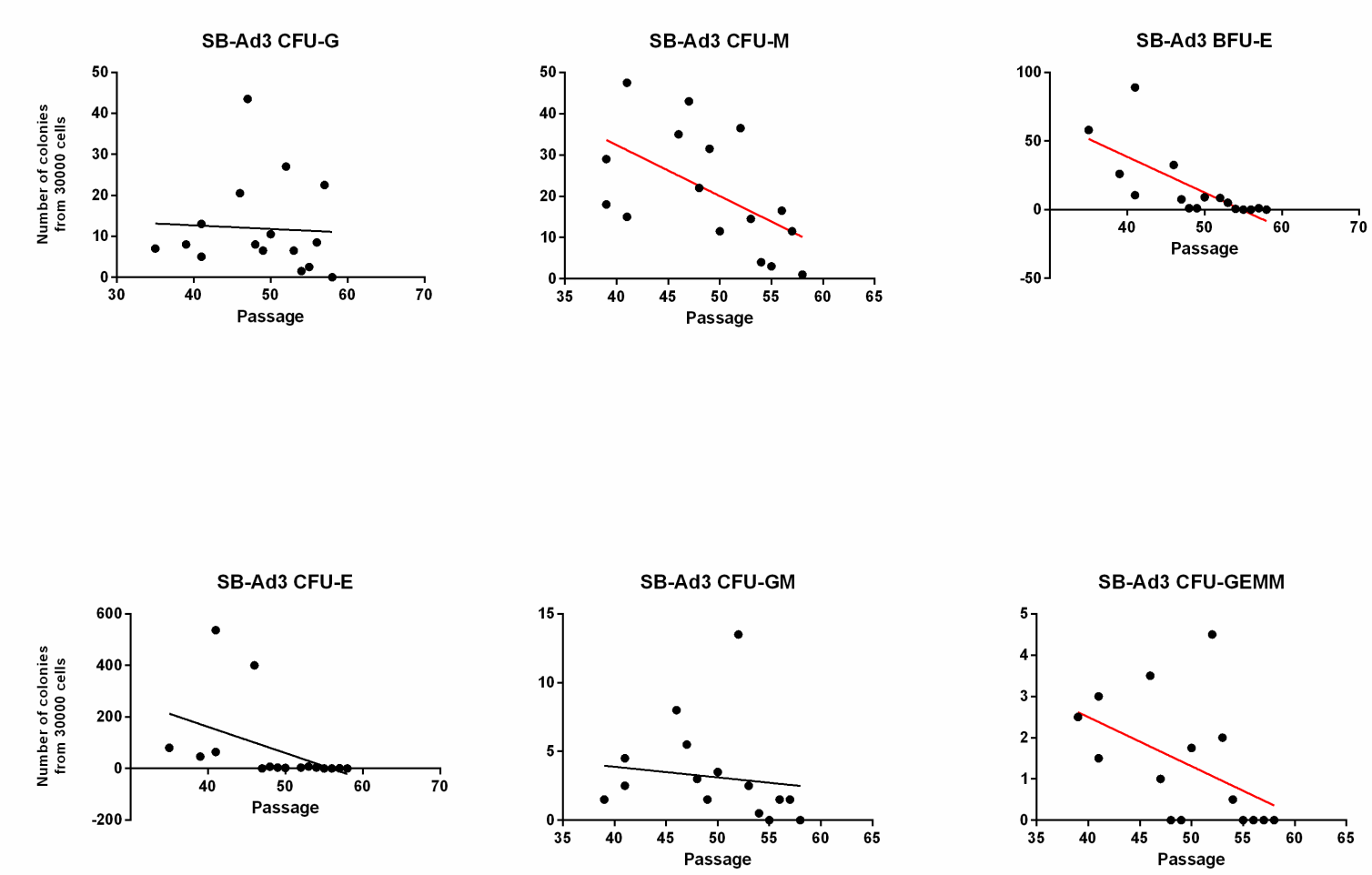


Figure 3.5: Scatterplots showing colony numbers of hematopoietic cell types (per 30000 live cells) vs. passage number for **a:** H9 cells and **b:** SB-Ad3 cells. Red lines indicate that the line of best fit deviates significantly from horizontal.

Flow cytometry assays: Having observed this pattern in the CFU data it became important to re-analyse the flow cytometry data which had been gathered throughout this project. The cells were analysed using the Kennedy 2012 markers (see **results section 3.2**), and using these were divided into Definitive hematopoietic cells, Megakaryocyte erythroid progenitors (MkEps), and multilineage myeloid progenitors (MMps).The data show a weaker trend than is seen in the CFU data (see **Figure 3.6**). There may be a small decrease in MkEps and a small increase in definitive cells at late passage, but the change is not significant.

Figure 3.6

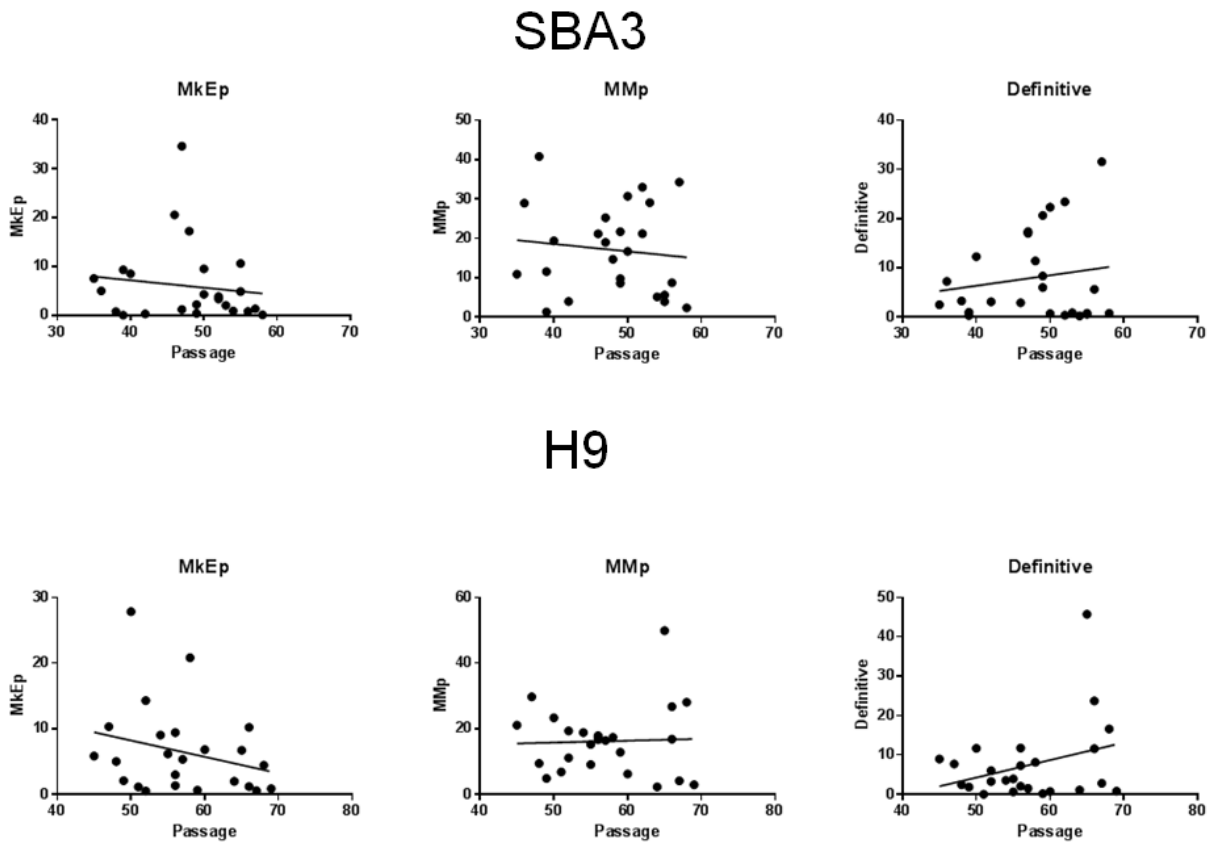


Figure 3.6: Scatterplots numbers of hematopoietic cell types (as a percentage of live cells) vs. passage number for H9 cells and SB-Ad3 cells. None of the results are statistically significant.

MkEp = Megakaryocyte erythroid progenitors CD43+CD34+CD235a+CD41a+CD45-,

MMp = multilineage myeloid progenitors CD43+CD34+CD235a-CD41a-CD45-

Definitive = CD43+CD34+/-CD235-CD41a-CD45+

Importance of passage number to the project: It is clear that there is a decrease in hematopoietic potential as passage number increases, and that this loss of potential does not affect all hematopoietic cell types equally. It is essential to recognise the importance of this discovery to the project, as failing to do so leads to misinterpretation of the results. For example it had appeared earlier in the project that the hiPSC line, SB-Ad3 had greater

hematopoietic potential than the H9 hESC line, however this apparent difference was due to the lower passage number of the more recently derived hiPSC line rather than a biological difference between ESCs and iPSCs. It also complicates the interpretation of results published by other groups, as some papers have reported better results with hiPSC lines than hESC lines [37], but different results between different cell lines may be due either to the biology of those lines or passage number. Information about passage number is almost never reported in these papers. Attempts to transplant PSC derived hematopoietic cells have almost always been done using well established and well characterised lines, for the same reasons as they have been used in this project, but this means that the cells used would likely not have been very early passage, which may be optimal for avoiding the loss of hematopoietic potential described here. Unfortunately it has not been possible to acquire very early passage (< 30) H9 or SB-Ad3 cells for analysis of their hematopoietic potential so it is not clear if hematopoietic potential would continue to improve at increasingly lower passages. It's important to note that although little has been published on the effects of very high passage number on differentiation potential of pluripotent stem cells it is well known in the field that yields of many different desired cell types can decrease at high passage number, and few of my colleagues continue to grow their cells after passage 60. The biological explanation for this is not definitively known, and my results suggest that the effect of passage number can be seen before the passage 60 cut off point, so this phenomenon is worth further study, both for this project and the field as a whole.

Identifying the unknown cell type: The flow cytometry results are given as a 'percentage of live cells', and the colony assay results are from a starting point of 30,000 live cells per well, so the differentiating cells are not disappearing or dying; they are becoming a non-hematopoietic cell type. Whatever the effect of passage on the cells it caused them to differentiate along another differentiation pathway in spite of the hematopoietic cytokines that they were being exposed to.

Flow cytometry of mesenchymal markers: The morphology of the cells is important in determining what cell type that these late passage cells become. Colonies of large adherent cells were seen in the colony assays at late passage, in contrast to the small round suspended hematopoietic cells produced in earlier passage experiments. These adherent cells are either polarised or have multiple processes (see **Figure 3.7a**). As it was not unusual to see occasional colonies of endothelial cells in CFU experiments from earlier passages the initial assumption was that these were also endothelial cells, which had been prevented by an

unknown mechanism from transitioning into hematopoietic cells. The cells seen at late passage do not form the 'spider web'-like structures which are typical of HUVEC (human umbilical vein endothelial cells) and which are seen in the endothelial colonies at early passages (see **Figure 3.7b**), however the colonies are more confluent so this might account for the differences in morphology. In order to confirm this, late and early passage cells were analysed by flow cytometry using a combination of markers designed by Vodyanik et al [172] to separate endothelial, mesenchymal and hematopoietic precursors made from hESCs. The results of this experiment confirm that the endothelial population is not increasing but do not prove that the mesenchymal population is increasing at late passage (**Figure 3.7c**).

Figure 3.7a

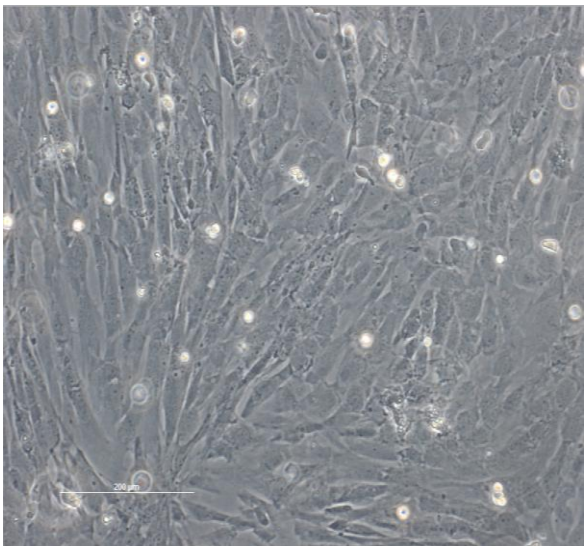


Figure 3.7b

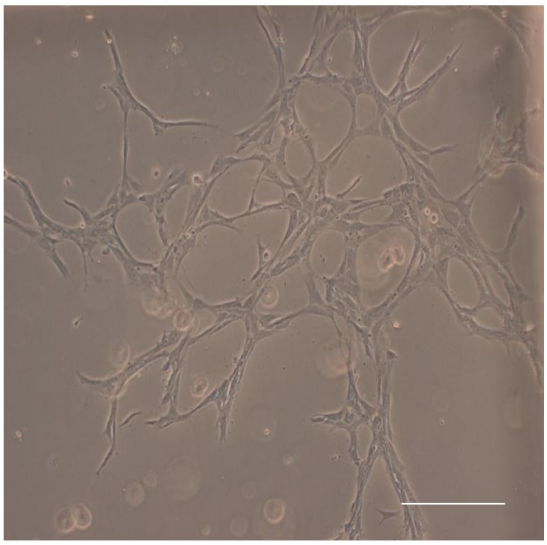


Figure 3.7c

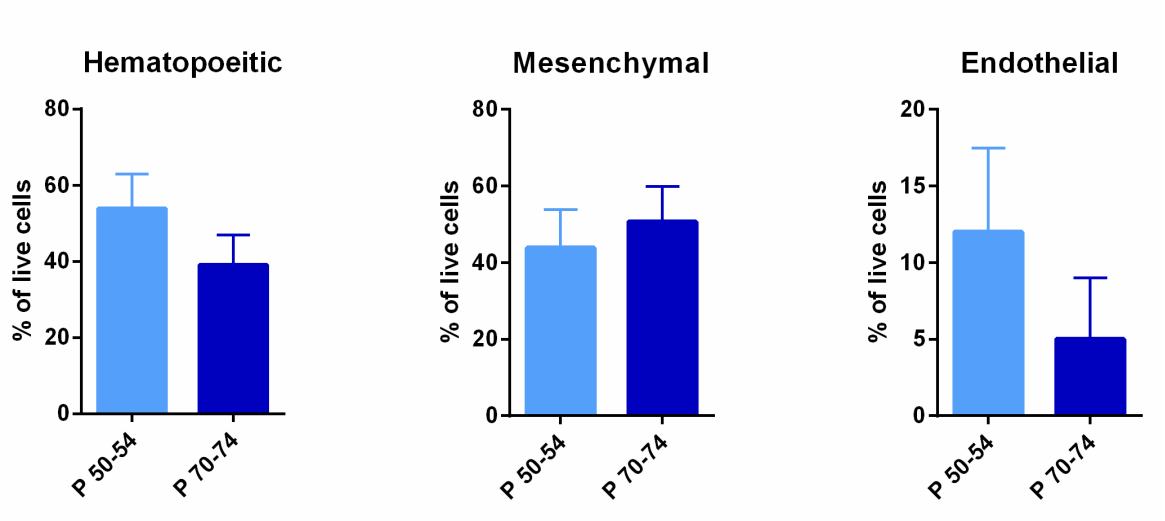


Figure 3.7d

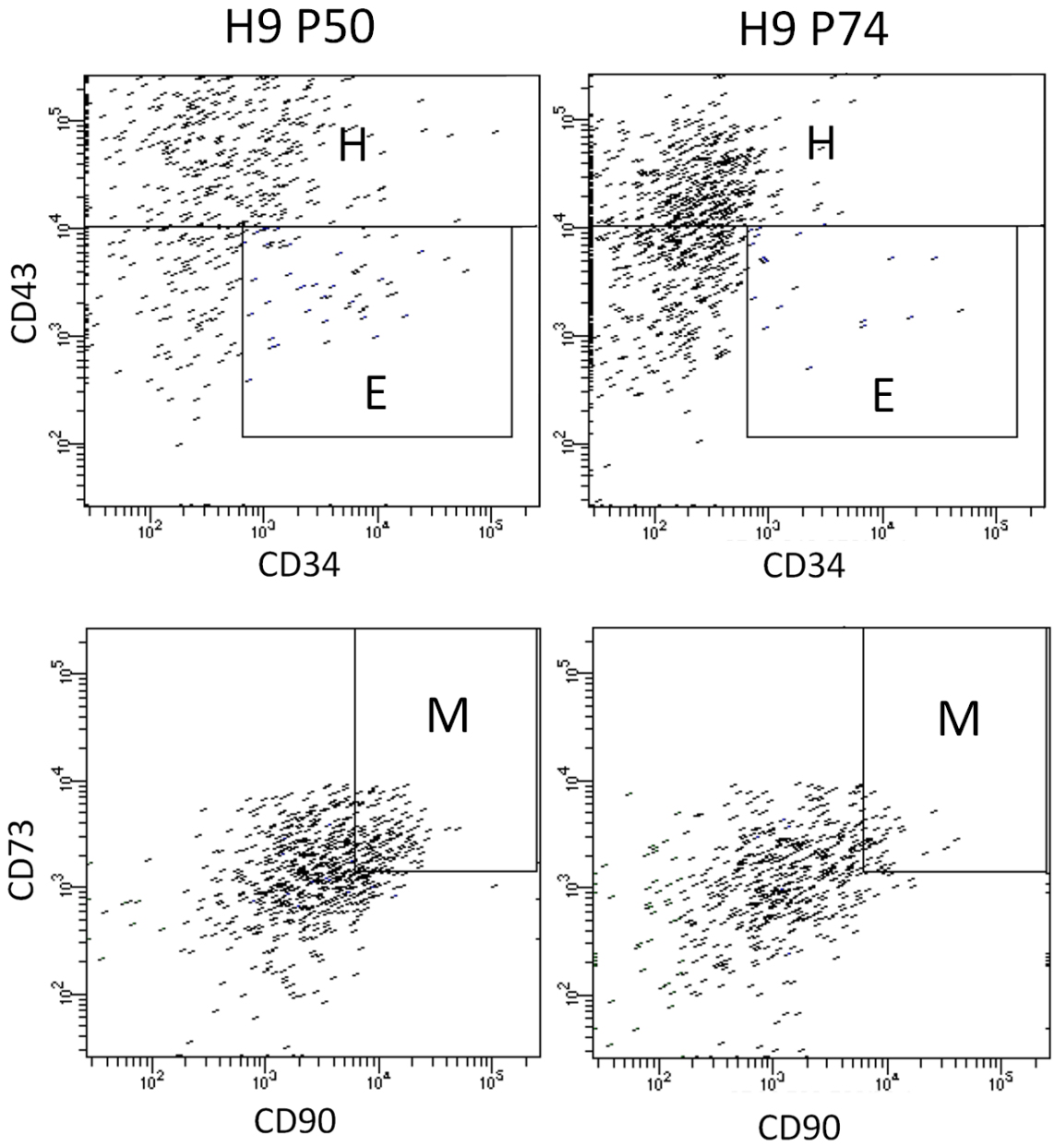


Figure 3.7a: Micrograph of cells found in late-passage CFU cultures. Large flat cells with multiple processes can be observed. Bar = 200 μm.

Figure 3.7b: Micrograph of adherent cells typically found in earlier-passage CFU cultures. Morphology is typical of foetal endothelial cells: 'lacy' or 'spiderweb' shaped colonies. Bar = 200 μm.

Figure 3.7c: Graph of flow cytometry data showing change in cell types found in day 12 SB-Ad3 cultures from early and late passage experiments. 'Hematopoetic cells' =CD43+CD34+/-, Mesenchymal cells= CD43-CD90+CD73+, Endothelial cells = CD34+CD43-.

Figure 3.7d: Representative flow cytometry plots for high and low passage differentiated cells showing **H:** hematopoietic cells CD34+/-CD43+, **E** endothelial cells CD34+CD43-, and **M**: mesenchymal cells CD43-CD90+CD73+

Gene expression results: In parallel to these experiments the change in gene expression from day 12 cells made at late passage compared to early passage was analysed. Since there is a possible change in the lineage bias, genes involved in lineage specification (*GATA1, PU.1, CEBPA, ERG*) were analysed as were genes associated with HSCs (*SCL, TGFB, GATA2, RUNX1a)* and genes involved in epigenetic changes (*EZH1 TET1, TET3*), as it is possible that there is some gradual epigenetic change which is responsible for the differences in lineage commitment at late passage (see **Figure 3.8**).

The qPCR results show very little change in most of the genes, the only gene which is significantly changed in both cell lines is *CEBPA*, which was analysed because it is a gene for myeloid lineage specification. However, it is also a gene which is highly expressed in MSCs [173], which is consistent with the morphology of the cells seen in CFU cultures at late passage but not with the flow cytometry results, although an increase in *CEBPA* could also explain the changes in which colonies are most frequent in the CFU assays as *CEBPA* pushes hematopoietic progenitors towards the monocyte lineage and away from the erythrocyte lineage [153]. There is little change in the expression of the epigenetic regulator genes but it is possible that epigenetic markers could shift slowly over time in culture without showing a major change in the regulatory genes which would fit with the slow and gradual change in hematopoietic potential, so this result does not conclusively rule out an epigenetic cause.

Figure 3.8a: Genes associated with HSCs

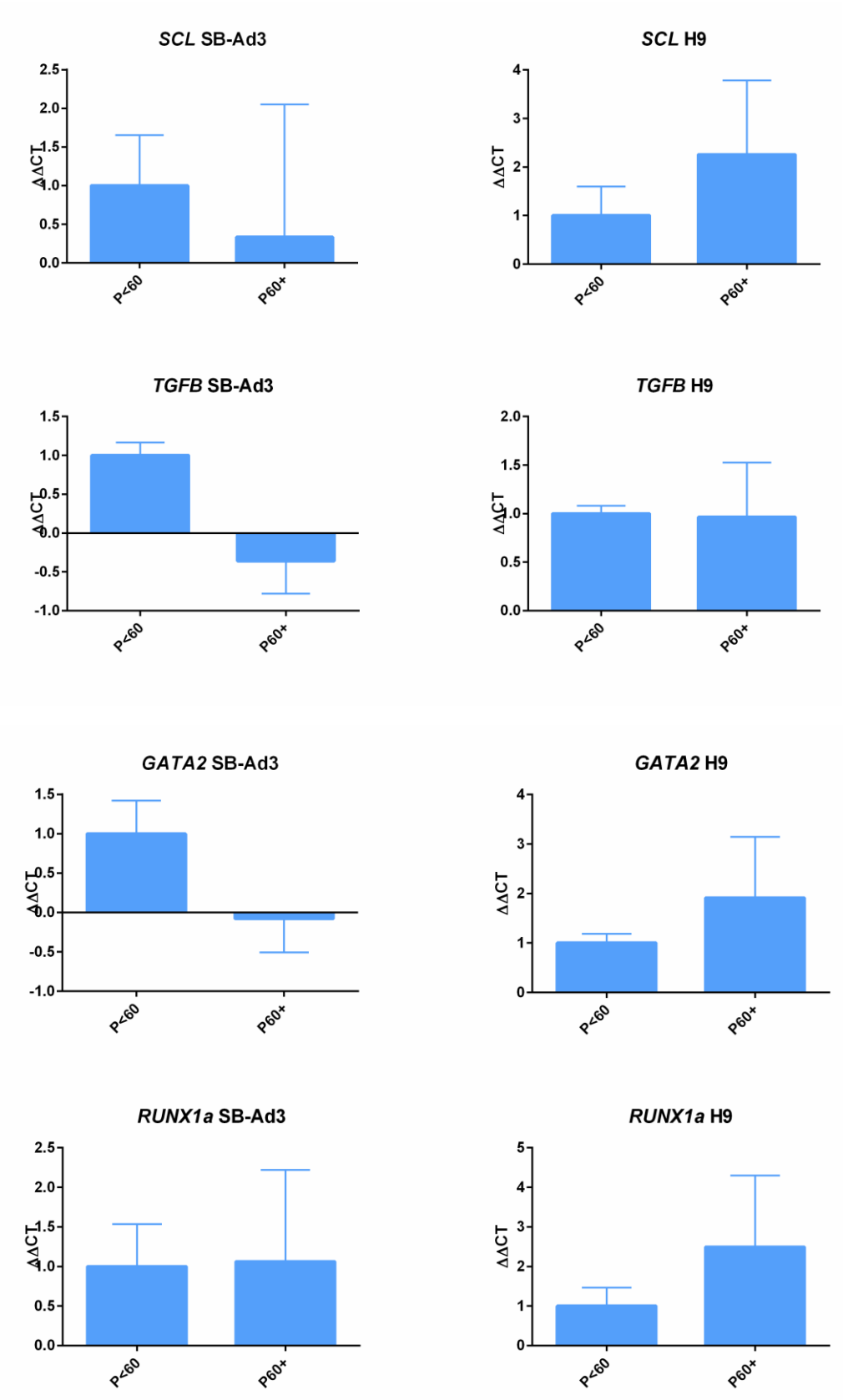


Figure 3.8b: Genes associated with hematopoietic lineage commitment

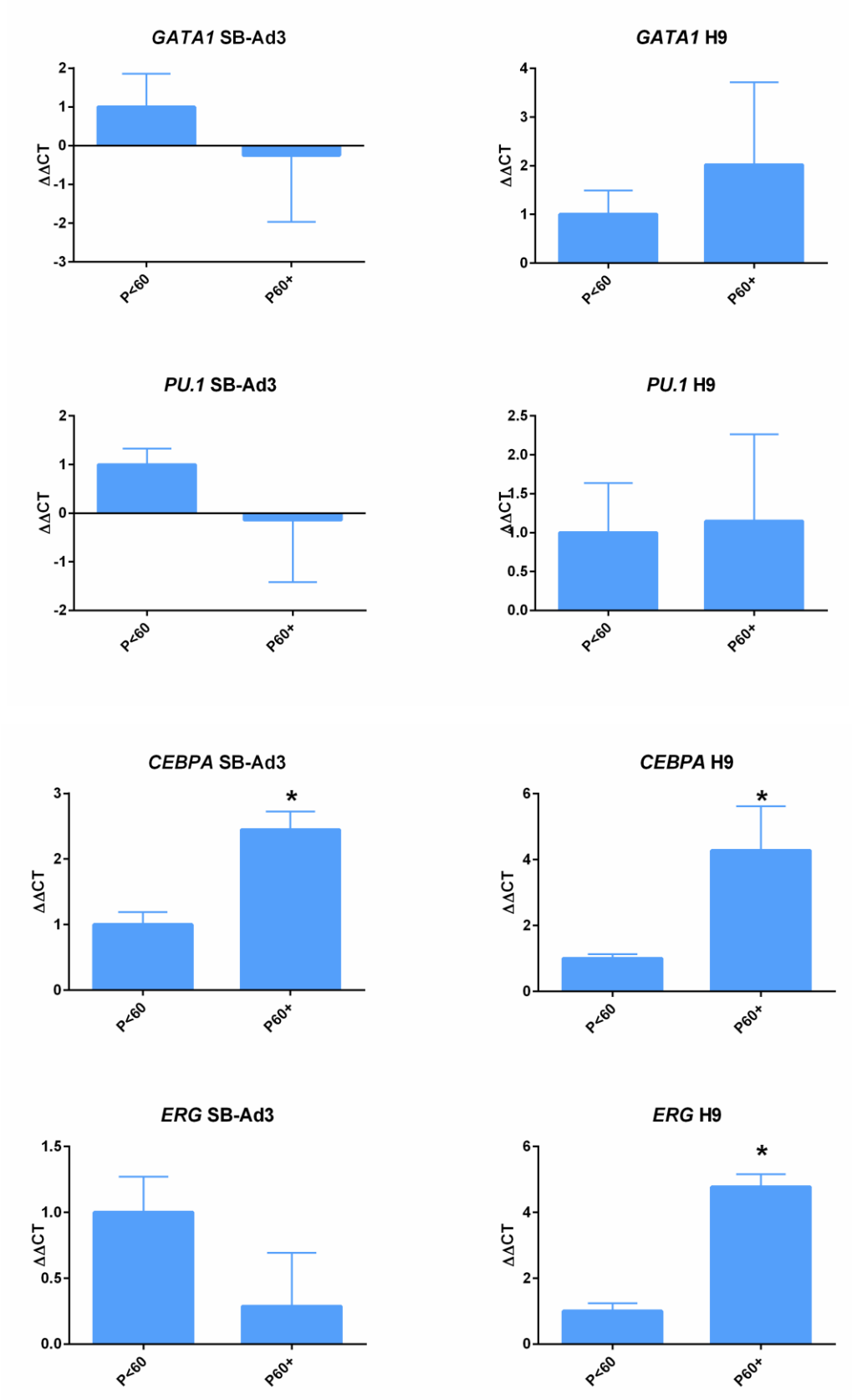


Figure 3.8c: Genes controlling epigenetic changes

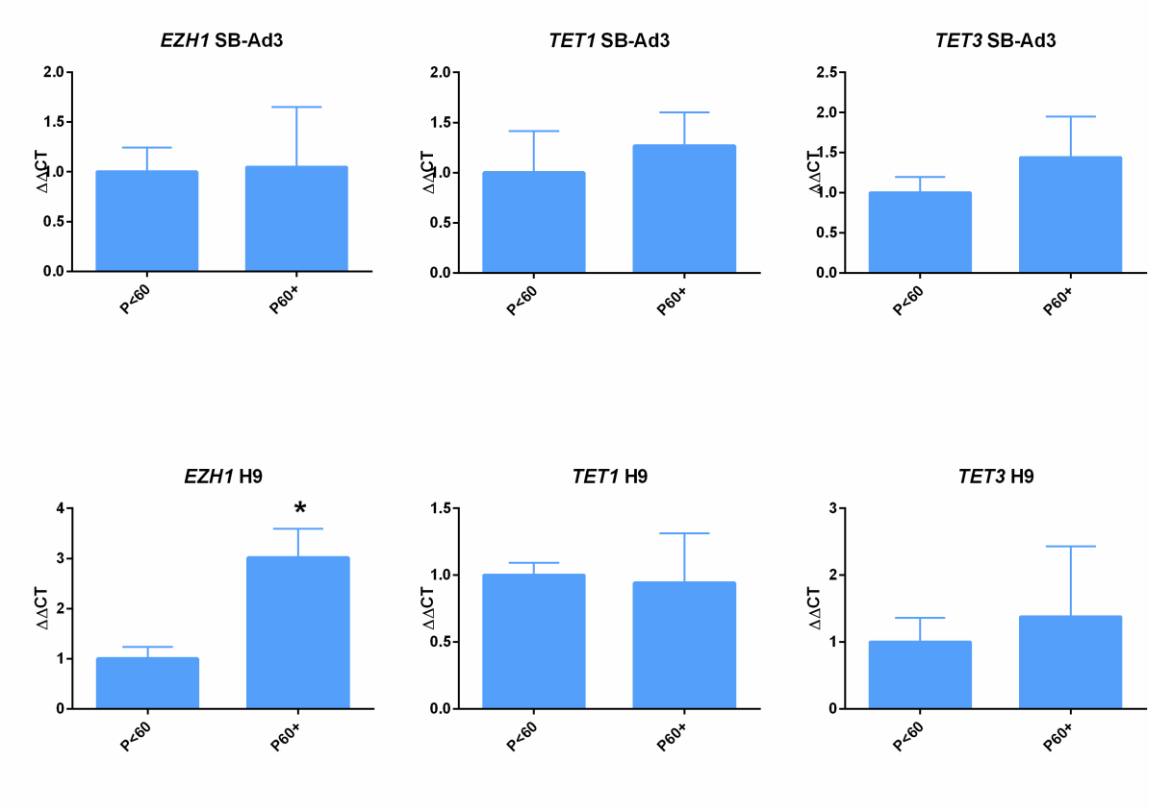


Figure 3.8: Graphs of qPCR data for H9 and SB-Ad3 cells showing ΔΔCT of high and low passage cells at day 12 of hematopoietic differentiation. **a:** genes associated with HSCs, **b:** genes associated with differentiation towards specific lineages, **c:** genes associated with epigenetic changes. Graphs show mean**±**SEM, n=3, significant results ($p<0.05$) are shown with a *. Housekeeping gene = *GAPDH*

Changes in miRNA expression: miRNA mis-expression has been suggested as the cause of the failure of P-HPC to thrive *in vivo* (see **section 1.4.4**). This mis-expression could be due to the use of later-passage, more established PSC lines so miRNA identified as mis-expressed in **results section 4.2** were analysed at late and early passage derived hematopoietic progenitors. The expression of several key miRNAs was analysed at late and early passage, only a few of the changes are significant and there is little in the way of a clear pattern which runs across both cell lines. However the down-regulation of miR-148 is consistent with the results showing fewer hematopoietic cells as this miRNA is hematopoietic specific. The increase in the other miRNAs at late passage in SB-Ad3 derived cells (see **Figure 3.9**) is consistent with an increased adhesion phenotype as these miRNAs are EMT suppressors (see **results section 4.2**).

Figure 3.9

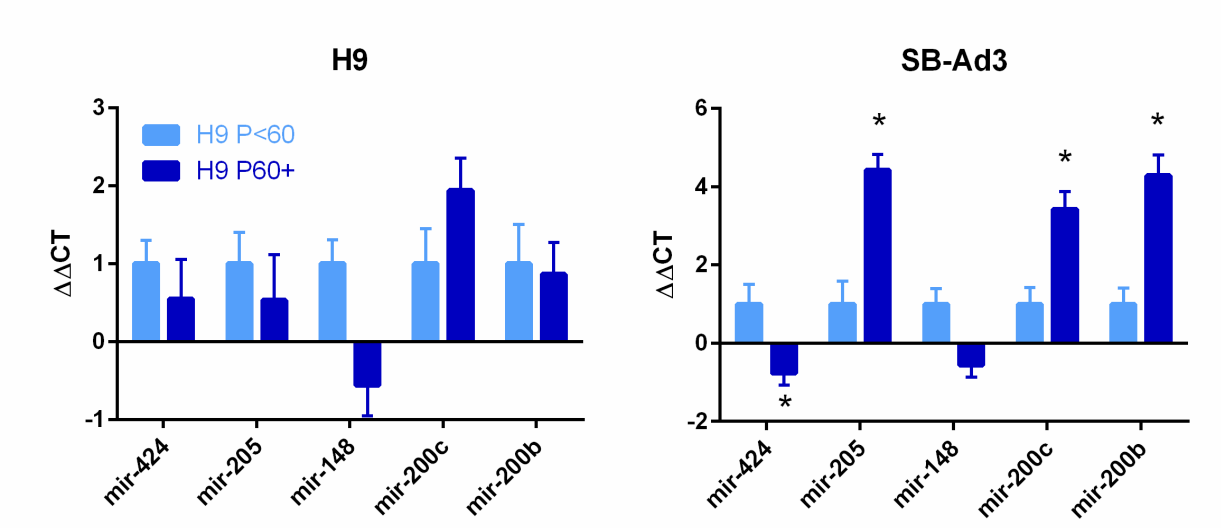


Figure 3.9: Graph showing qPCR data of key miRNAs at high and low passages at day 12 of differentiation. Calculated using ΔΔCT method. P = Passage number. Data is shown as mean±SEM, n=3, significant results ($p<0.05$) are shown with a *.

CytoSNP results: established stem cell lines are expected to be genomically stable, however the medium that is used for this project, STEMPRO is not typical and is not as commonly used as feeders or mTESR. The protocol has been tried by my colleague Dr Jarmila Spegarova using mTESR rather than STEMPRO but this was found to be ineffective. Both mediums use mercaptoethanol which protects against mutations caused by free radicals. CytoSNP was used to analyse both cell lines and to compare earlier passage cells of each line to later ones. The cytoSNP results are striking, showing duplications in chromosome 12 in both cell lines at later passage (see **Figure 3.10**). The SB-Ad3 cell line also shows a duplication of a large section of chromosome 1 at late passage and a duplication of part of chromosome 9 at early passage which does not appear in the later passage cells.

The literature about chromosomal abnormalities in pluripotent stem cells mostly focuses on the mutations which arise during reprogramming or early passages, but there are a few papers which focus on the effect of extended passaging [174-177].

Figure 3.10a SB-Ad3 early passage

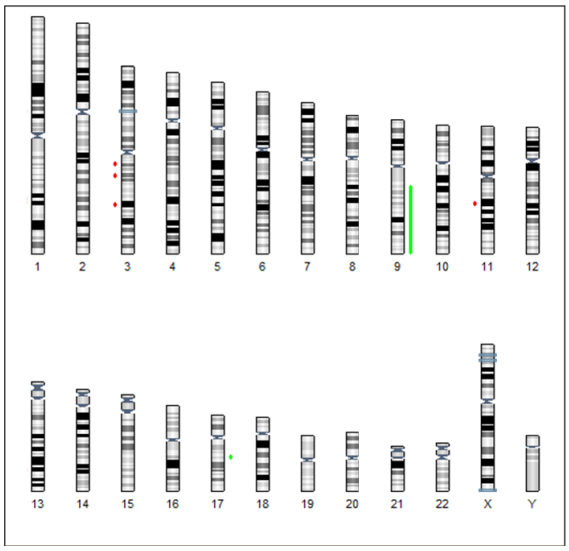


Figure 3.10b SB-Ad3 late passage

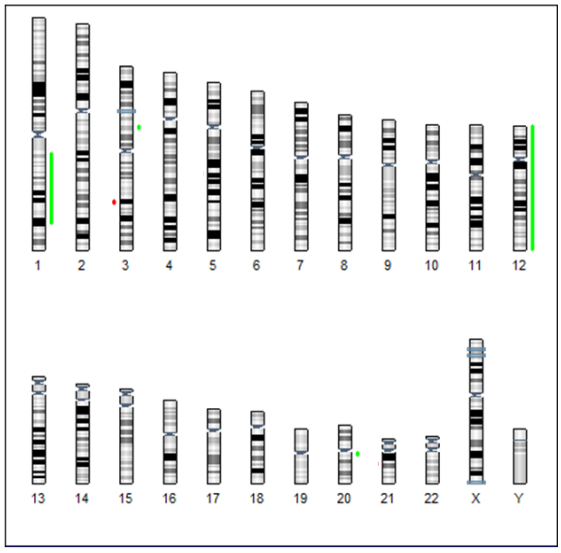


Figure 3.10c H9 early passage

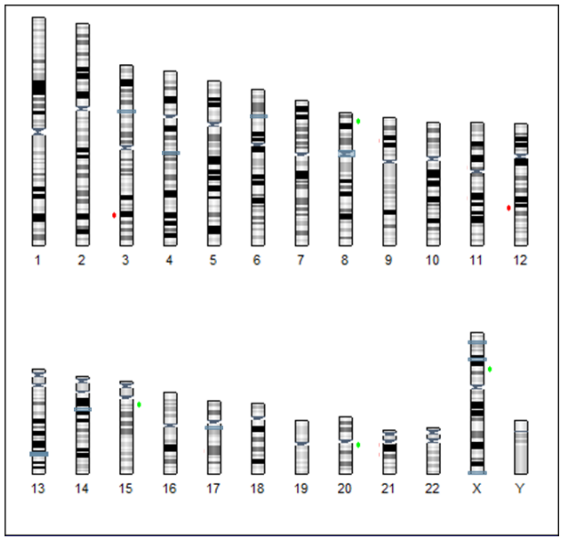


Figure 3.10d H9 late passage

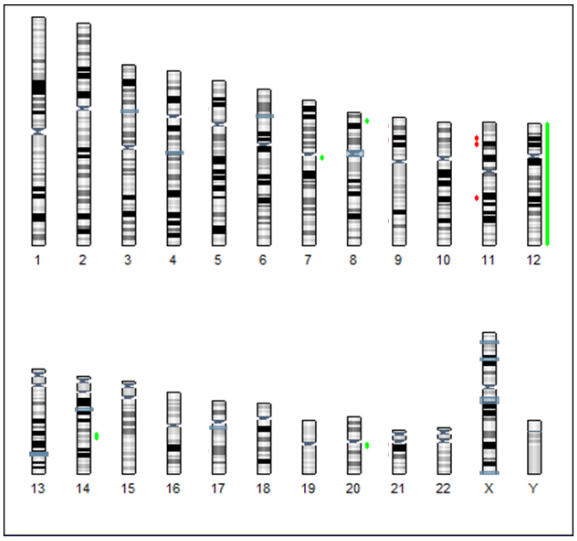


Figure 3.10: Schematic of CytoSNP data comparing mutations in late and early passage cells. Green = duplication, Red = deletion. **3.10a** = passage 45, **3.10b** = passage 64, **3.10c** = passage 47, **3.10d** = passage 75.

3.4.1 Mutations in PSC lines

Origin of mutations: Mutations found in iPSCs can originate in several ways. Some are amplified from mutations found at low levels in the original tissue from which they are derived [178], some are caused by the stress of reprogramming [179], and others arise spontaneously during long term culture of the cells [174]. Most of these mutations are caused by oxidative stress, which is a particularly acute problem in pluripotent stem cells because of their constant proliferation. Pluripotent stem cells compensate for this problem by repairing

their DNA using homologous recombination or NHEJ, although they are more likely to use the more accurate homologous recombination, which means they have a base rate of mutation around 10 times lower than somatic cells in culture [174, 180]. They are also sensitive to DNA damage which they can't repair and will activate the G1/S checkpoint and stop proliferating [181]. However, in the long term, and with hundreds of thousands of cells dividing constantly, some mutations are eventually inevitable.

Selection for advantageous mutations: All pluripotent stem cells which have been studied appear to have at least a low level of mosaicism within the culture. According to Laurent et al [182] this mosaicism undergoes 'dynamic shifts', with populations of a specific mutation growing and shrinking over time due to a drift effect, which explains the duplication in chromosome 9 in the early passage SB-Ad3 cells which does not appear at later passage. However there are a few mutations which are selected for in pluripotent stem cell culture. Deletion of chromosome 17 and duplications of all or part of chromosome 20 and chromosome 12 are the most common mutations found in PSC culture [175-177]. All three mutations have the effect of maintaining stemness and preventing apoptosis. The region of chromosome 12 which is commonly duplicated contains the gene *NANOG*, a pluripotency factor. High expression of this gene protects the cells from apoptosis via the *p53* pathway [183]. Assuming the chromosome 12 duplication impairs hematopoiesis, the results can be explained by a duplication arising in each of the H9 and SB-Ad3 cultures and gradually coming to form a larger percentage of the culture due to the survival advantage it confers.

Mutations which maintain stemness impair hematopoietic differentiation: Chromosome 12 contains many other genes aside from *NANOG*, including a *HOX* gene cluster, but it does not contain *CEBPA*, so the increased expression of *CEBPA* cannot be directly explained by the duplication although it may be indirectly controlled by any of the transcription factors on chromosome 12. Many of the genes on chromosome 12 might have a negative impact on hematopoiesis if incorrectly expressed, however a study of a chromosome 20 duplication in another iPSC line showed a similar phenotype upon differentiation in EBs: no hematopoietic cells were formed, and the EBs were made of mostly immature mesenchymal cells [184]. This suggests that it is not any particular gene on chromosome 12 which impairs hematopoiesis, but rather an effect of the high expression of pluripotency factors. Some of my colleagues have observed a similar effect in late passage cells on the differentiation of other highly specialised cell types such as RPE. In EBs which have these mutations *OCT4* and other pluripotency markers are expressed for longer [184, 185]. It seems that these mutations have the effect of slowing or stopping the specialisation process, leaving the cells in an immature

or undifferentiated state. This may explain the greater changes seen in the CFU results compared to the flow cytometry data: the colony forming cells have been cultured for a further 14 days to a terminally differentiated state, the mutations may be blocking this final stage of differentiation rather than the earlier stages. No studies have specifically analysed hematopoietic differentiation in these mutated cells as they are more concerned with the potential tumorgenicity of the mutated cells.

The flow cytometry results show a much weaker downward trend compared to the CFU assays. This is unexpected as a loss of hematopoietic potential should lead to fewer cells expressing hematopoietic markers. However the cells are analysed by flow at day 12 of differentiation and the antibodies used are for markers of hematopoietic progenitors. On the other hand the colonies are assessed 14 days after that and are testing for terminally differentiated hematopoietic cells so it is consistent with the theory that the duplication of the pluripotency gene, in this case *NANOG*, prevents the cells from fully maturing.

It may be impossible to entirely avoid this problem, however culture conditions which reduce the amount of stress on the cells, such as passaging them mechanically rather than enzymatically have been shown to reduce the number of mutations which arise [183, 186]. Although the tissue culture methods used in this project involved mechanical passaging and mercaptoethanol in the medium to reduce the number of free radicals, the cells were also cultured in feeder free conditions, which has been shown to have a higher rate of mutations than cells grown on feeders [175, 183]. This is a problem that will need to be overcome before stem cells can be widely used in a clinical setting.

3.5 Statistics

The results shown in this chapter were gathered and analysed at a late stage in the project, from controls which had been done for experiments on miRNA analysis and inhibition (see **results sections 4 and 5**). As this trend did not become obvious early on, many of the experiments done on this project have been repeated at very different passage numbers, which caused them to have a large amount of inter-repeat variability, making effective statistical analysis difficult. The original intention for these experiments was to perform ANOVA to assess the significance of the difference between the controls and the various miRNA inhibited cells, but this will not give meaningful results as there is too much variation. The most useful solution was to use ANCOVA instead, a variation of ANOVA which takes into account a confounding variable, in this case passage number. See **methods section 2.10** for details of how this analysis was performed. Passage number variability also creates a

challenge in visualising the data: bar charts can't take into account the covariate and scatter plots quickly become unreadable when more than one experimental condition is plotted. Fortunately the ANCOVA analysis can provide a solution, in the form of 'Estimated Marginal Means' (EMMs) which, as the name suggests, are estimates of the mean adjusted for the covariate, allowing us to plot, for example, estimates of the number of CFU-G colonies for all experimental conditions, if all the experiments had been done at passage 50. The CFU and flow cytometry results in later chapters are given in this form. Although some of the results in this chapter are from cells as late as passage 70+, all of the data shown in the later chapters was calculated from cells at passage <56, which allowed more meaningful results to be obtained.

4. Results: miRNA expression analysis

4.1 Methods for expression analysis

Novel miRNAs are discovered via cloning, and northern blot is used to confirm their existence [187]. miRNAs can also be predicted by algorithms which search the genome for conserved sequences with structure similar to known miRNAs.

Since their discovery, various techniques have been developed which can allow us to analyse the localisation and quantity of miRNA transcripts being expressed in a particular cell line, tissue or organism. There are several challenges in determining miRNA expression: it is difficult to design specific probes or primers for miRNAS as their GC content varies widely leading to very different melting temperatures, and they are small and have high levels of homology within miRNA families. This is further complicated by the multiple stages of processing miRNAs go through; probes and primers must be specific to the mature miRNA and not one of the earlier stages, as pri- and pre- miRNAs are often expressed at drastically different levels than the corresponding mature miRNA. The mechanisms which control the amount of precursor molecules which are processed into active miRNAs are not well understood but they produce important functional effects [188]. The various methods which have been used to overcome these technical challenges are discussed below.

Microarrays are a useful way of quickly assessing hundreds of miRNAs: A slide is printed with 1000s of oligos homologous to miRNAs and the RNA from the sample is conjugated to a fluorescent dye and allowed to hybridise to the oligos on the slide.

The accuracy of microarray analysis is limited because of the short length and varying melting temperatures of the miRNA but this problem can be ameliorated in several ways. Altering the length of the probes can solve the problem of the differences in melting temperature [189], but it can further reduce the specificity of probes which are already very short. Another solution is to chemically alter the probes to increase hybridisation stability, for example using locked-nucleic acids (LNAs) which allow hybridisation over a wider range of temperatures [190].

Probes are also printed multiple times in different places on the slide as this allows the researchers to take an average which is more accurate, and reduces the problems caused if the slide gets scratched or contaminated with dust.

In spite of these efforts, microarrays are still limited in their accuracy and are often poor at distinguishing between mature miRNAs and their precursors. However they are still frequently used as a first step in studying the role of miRNAs in disease, because they allow rapid screening of thousands of candidates. In these experiments a panel of thousands of miRNAs is used to compare diseased and healthy tissue and this comparison is used to suggest which miRNAs should be validated by other methods and further studied for their role in pathogenesis.

qPCR can accurately analyse the expression level of a miRNA: qPCR is often used to validate specific miRNAs which have been identified as being of interest by microarray data.

In qPCR the RNA in the lysed cells is converted to cDNA, PCR is then performed on this in the presence of a fluorescent DNA dye. The fluorescence level is measured during every PCR cycle which allows precise quantification of the target. A value is assigned to the sample based on the number of cycles it takes for the fluorescence to reach a particular level. This is known as the Ct value. qPCR can be used to assess the absolute or relative quantity of the target miRNA, in absolute quantification the Ct value is measured against a serial dilution of RNA of a known quantity and in relative quantification the Ct values of two samples are compared using a housekeeping small-RNA as an internal control using the ΔΔCT equation:

$$\Delta\Delta Ct = \Delta Ct,sample - \Delta Ct,reference$$

When performing qPCR on the mRNA for a gene, reverse transcription to create cDNA is performed using random short primers and/or oligo(dT) primers. However miRNA transcripts don't have polyA tails and are much too short for the random oligo hexamers to bind to. The first widely used solution found was to create specific primers for reverse transcription of a particular target miRNA [10]. In order to work specifically in spite of the short transcript the primer is designed to form a stem loop, with the homologous region overhanging (see **Figure 4.1**). This solution is effective and allows specific amplification but it requires cDNA to be made separately for each miRNA studied. This means that more sample must be used for each test, and reduces accuracy by increasing the chance of pipetting errors as well as being more time consuming for the researcher.

Figure 4.1

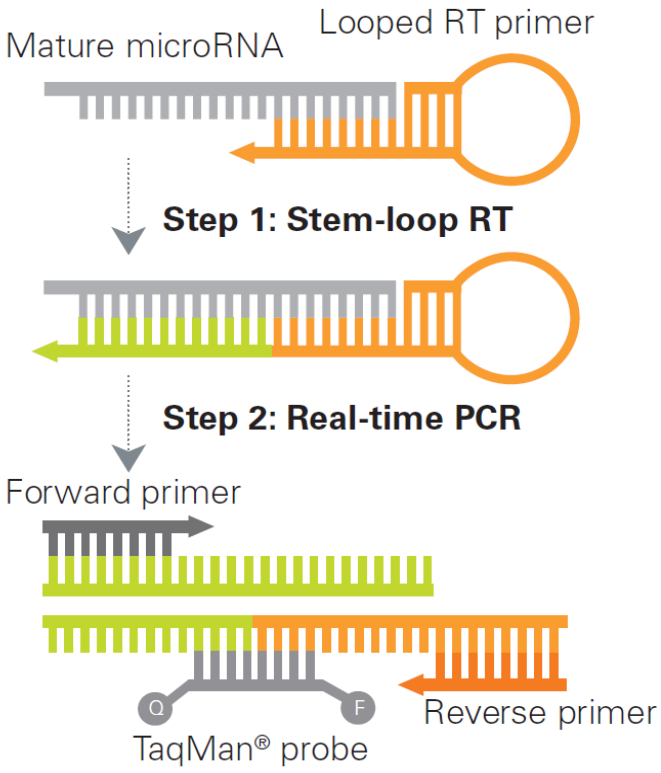


Figure 4.1: Schematic showing how the two-step stem-loop Taqman miRNA RT-PCR system works. [10]

A more recent innovation [191, 192], allows a polyA tail to be enzymatically conjugated to the miRNA before reverse transcription. cDNA can then be made by reverse transcription can then be done using an oligo(dT) primer. qPCR remains the most accurate and specific method of quantifying the expression of an individual miRNA.

Other methods for miRNA analysis: In addition to these methods levels of miRNA expression can be estimated using *in situ* hybridisation, which shows localisation in cells and tissues. The role of miRNAs in development has been studied using genetic modification to tie expression of a specific miRNA to a fluorescent marker, for example and cells which constitutively express Luciferase or GFP with 3' complimentary sequence to miRNA of interest and a bioluminescent or fluorescent protein of another colour under the same promoter but without the miRNA binding region acting as an internal control [193, 194].

These methods are good for information on the localisation of miRNAs but are not quantitative. Electrochemical biosensors for detection of miRNA are being developed as well [195], but recent advancements in RNA-Seq technology mean that it is now potentially viable to replace other methods such as microarray and qPCR [196].

4.2 Microarray analysis comparing hematopoietic progenitors derived from hESCs or bone marrow

Microarray analysis comparing miRNA expression in hematopoietic progenitors to hESC derived progenitors was done by Dr David Blesa (CIPF, Valencia, Spain) before the start of the project. The cells analysed were two hESC (H9) replicates, day 4 P-HPCs and CD34+ human bone marrow cells. This allowed a comparison between the expression of over 800 miRNAs in bone marrow derived HSCs, P-HPCs, and the pluripotent cells they were derived from. The expression of the miRNAs in these cell types can be divided into six broad patterns, as shown in **Figure 4.2**. The expression is plotted in the order hESC1, hESC2, P-HPCs and bone marrow, from left to right; so the patterns are named, for example, flat-down for the ones which are expressed at similar levels in hESC and P-HPC and at lower levels in bone marrow HSCs, or flat-up for the ones which are expressed at similar levels in hESC and P-HPC and at higher levels in bone marrow. The number of miRNAs in each group is given under the example graph; the miRNAs in each group are shown in **appendix table A**. Details of the analysis and how it was carried out are in **methods section 2.2**.

Microarray analysis identified mis-expressed miRNAs, which can be grouped by expression pattern: The 57 miRNAs in the flat-up group and the 32 miRNAs in the flat-down group are the most interesting for this project as they are the ones which are 'mis-expressed' i.e. they are expressed in P-HPCs at a more similar level to hESCs than to real HSCs. The intent in this project was to correct the expression of these miRNAs by inhibiting those in the flat-down group or ectopically expressing those in the flat-up group in the hope that it would result in some functional improvement in the hematopoietic potential of the P-HPCs.

During my mRES project I researched the functions of miRNAs in the flat-up and flat-down groups using published literature and TargetScan database [118] which computationally predicts the potential targets of miRNAs. Software can compare sequences of known miRNAs to 3’UTR from the genome sequence and predict likely mRNA targets of miRNAs by looking for conservation of binding sites across species. Algorithms predict targets based on complimentarity, especially strong base pairing in the 5’ 2-8 nucleotides and on structural availability of the surrounding sequence.

However this method will not necessarily predict all miRNA-mRNA interactions because miRNAs can target parts of the mRNA other than the 3’UTR and can target mRNA even

when there is imperfect matching. Target prediction using this kind of software can be very useful but different algorithms that are used are shown to give a substantial number of false positives and often come up with radically different predictions, so all predicted targets must be validated. The results of this research are summarised in **Table 4.1** and **4.2.**

Mis-expressed miRNAs fall into several broad functional categories: Several broad patterns emerged from analysis of the microarray data, which are informative about the differences between HSC and P-HPC and about the causes of the problems in transplantation experiments. In the flat-down group there are miRNAs which control various cellular functions, such as pluripotency, cell cycle, EMT and hematopoiesis (see **results section 4.2.2**). The flat-up group contains several miRNAs which target genes essential for hematopoiesis (see **results section 4.2.2**). Since microarray data is not reliable enough to be considered conclusive several key miRNAs were chosen from the flat-up and flat-down groups for further analysis by qPCR.

Figure 4.2

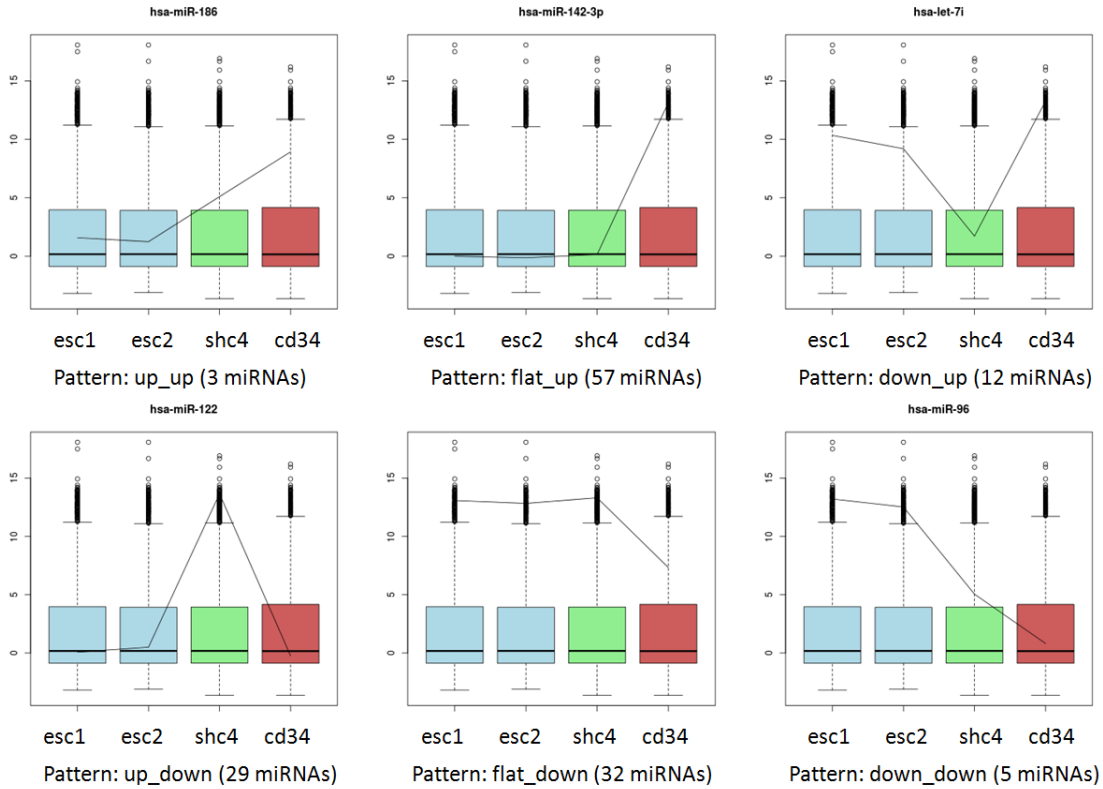


Figure 4.2: Graphs showing representative examples of estimated differential gene expression from microarray data in different cell types.

Left to right: esc1=hESC replica 1, esc2=hESC replica 2, shc4=hESCs differentiated to day 4 sorted for CD34+ CD31+KDR+CD45-(haemangioblast markers), cd34=CD34+cells from bone marrow.

The distribution of normalised intensity values for each sample is displayed in the box plots, and the relative fluorescence of the individual miRNA is shown as a line graph. miRNAs have been sorted according to the shape of the graph e.g. flat-down, flat-up etc. For example the flat down group miRNAs are expressed at similar levels in the hESC lines and in the day 4 differentiated hESC cells but at a lower level in the CD34+ bone marrow cells.

4.2.1 Flat-down group miRNAs are involved in pluripotency, hematopoiesis, EMT and the cell cycle:

Pluripotency: One of the most striking results from this microarray data is the high expression of the miR-302 cluster (miR-302a, miR-302b, miR-302c, miR-302d and miR-367) in P-HPCs. This is surprising because this group of miRNAs has been exclusively associated with pluripotency, and ectopic expression is even able to reprogram cells to pluripotency [21, 197]. The miR-302 cluster is always be down-regulated immediately upon exit from the

pluripotent state [198], so the continued expression of this cluster, which is expressed as a polycistronic precursor, is aberrant, and is likely to be disruptive to the function of the P-HPCs. Expression of the miR-302 cluster in cells after they have left pluripotency is so unlikely that it is reasonable to be sceptical of this result but the fact that so many members of the group show the same pattern of expression does suggest that the result is real. Several members of the miR-302 group (miR-302a, miR-302a*, and miR-367) were selected for validation by qPCR.

Hematopoiesis: Unsurprisingly several of the misregulated miRNAs from the flat-down group target genes associated with haematopoiesis or hematopoietic differentiation (see **Table 4.1**). Although these all target hematopoietic genes they seem to target different ones at different stages in the hematopoietic process, so it seems likely that it is an effect rather than a cause of the functional deficiencies of P-HPCs. In any case miR-148a, miR-150 and miR-150*, miR-18b, miR-34a, miR-424 and miR-9 were chosen for analysis by qPCR for their roles in hematopoietic differentiation.

EMT: Another unexpected pattern in these results is the very high number of flat-down group miRNAs which are associated with suppression of EMT; many of them are described in the literature as inhibitors of metastasis and angiogenesis (see **Table 4.1**). 12 of the 32 miRNAs in the flat-down group have some role in suppressing EMT. EMT is the process which cells lose their adherent epithelial phenotype and become mesenchymal, it is characterised by loss of E-cadherin, cytoskeletal rearrangement, and increased motility. It is heavily involved in both normal developmental processes and in malignancy/metastasis.

EMT suppressors are expressed at high levels in pluripotent cells, which are epithelial in nature and at low levels in hematopoietic cells which are mesodermal. Therefore the pHSCs should express low levels of these miRNAs, however they maintain the high expression found in pluripotent stem cells. This failure to down-regulate these miRNAs indicates a likely problem in the *in vitro* differentiation. Hematopoietic cells' most important transition is the one in which the cells change from an endothelial cell type to a hematopoietic cell type (see **introduction section 1.3.2**). Although this is called the EHT rather than the EMT the processes share most molecular and functional characteristics. Inhibitors of EMT are likely detrimental to EHT and would almost certainly make the cells less motile. It is plausible that this miRNA expression is responsible for the failure (described **in introduction section 1.4.4**) of P-HPCs to home to the bone marrow niche. Although we know that EHT does occur in the *in vitro* system, since endothelial colonies can be seen giving rise to hematopoietic clusters

which eventually detach from the basement layer of cells, the microarray results indicate that this EHT process may be impaired or incomplete. If the cells retain some of the characteristics of the endothelial/adherent phenotype they may have some difficulty attaining the level of motility needed for chemotaxis to the bone marrow niche. miRNAs from the miR-200 family (miR-200b, miR-200c and miR-205) were chosen for qPCR validation as they are well known EMT inhibitors (see **Table 4.1**).

Cell cycle: Finally, many of the miRNAs in the flat-down group are involved in regulating the cell cycle. PSC and HSC have very different cell cycle protein profiles as HSCs are typically quiescent the vast majority of the time but PSC are characterised by their constant proliferation. In spite of this, the microarray results show that the p-HPCs express miRNAs which target cell cycle genes such as cyclin D, which indicates that the cell cycle of these cells may be more similar to that of pluripotent stem cells than HSCs. This could have negative effects such as causing any HSCs which do arise in the cultures to differentiate more rapidly. However the first HSCs to arise in the foetal aorta and foetal liver are known to be more proliferative, and only become quiescent later, in the bone marrow, so it is possible that the expression of miRNAs pertaining to the cell cycle merely reflect the foetal characteristics typical of pluripotent-derived cells. Nevertheless the expression of miR-148a, miR-200c and miR-200b, the miR-302 cluster, miR-34a, miR-424 and miR-9 were analysed by qPCR because of their role in cell cycle control.

4.2.2 Flat-up miRNAs regulate pluripotency and hematopoiesis

The flat-up miRNAs also show an interesting pattern, many of the let-7 group are under-expressed in p-HPCs compared to HSCs from bone marrow, which is consistent with the data showing high expression of the miR-302 group (see above, **section 4.2.1.**), since these two groups of miRNAs reciprocally inhibit each other. However it is cause for concern for the same reason; it indicates that the cells are more pluripotent in character than would be expected.

For many miRNAs in the flat-up group the significance in hematopoiesis is not clear, however miR-142 is known to be essential for hematopoiesis to occur at all in zebrafish, miR-146 is hematopoietic stem cell specific, miR-181 is known to play a critical role in lymphoid cell development, which is of particular interest to us due to the difficulty in directing PSCs into lymphoid cells, and miR-223 is an important regulator of hematopoietic development (see **Table 4.2**). The relative lack of any one of these miRNAs might be enough to damage the

hematopoietic potential of the cells so their expression was analysed further by qPCR.

miR-150 shows differential expression between strands: miR-150 showed an interesting pattern in that the active molecule (miR-150 or miR-150-5p) was in the flat-down group whereas miR-150* (mir-150-3p) was in the flat-up group, showing the opposite expression. This is unexpected since the two molecules are made from the same transcript, but it is not completely unbelievable because miR-150* is highly expressed in ESCs but not processed into 150 until the cells differentiate [97]. This means the high miR-150* expression is consistent with the general pattern of aberrantly maintained pluripotency associated miRNA expression. miR-150 is known to target hematopoietic transcription factor c-MYB [115, 199, 200] as well as EMT transcription factor ZEB1 [201] and has a role in hematopoietic lineage commitment, so both miR-150 and miR-150* were selected for qPCR validation. All of the key miRNAs chosen for further analysis are highlighted in **bold** in **Table 4.1.**

Table 4.1: FLAT_ DOWN	**Pluripotency associated**	**Epithelial-mesenchymal transition**	**Hematopoiesis and angiogenesis associated**	**Cell cycle and apoptosis**	**Other**
hsa-miR-151-3p		Regulates EMT via RhoGDIA [202, 203] and *TWIST1* [204]	Associated with foetal haemoglobin switch [205, 206]	Targets cyclin E1[207]	
hsa-miR-432				Promotes proliferation via WNT pathway [208]	Biomarker for schizophrenia [209, 210]
hsa-miR-598					Involved in DNA damage response pathway[211]
hsa-miR-1181					Possible role as a tumour suppressor [212, 213]
hsa-miR-1224-5p					Possible role in inflammation [214]
hsa-miR-134	**Regulates pluripotency via *C-MYC, NANOG, OCT4, SOX2, LRH1*[215, 216]**	**Supresses EMT via *FOXM1*[217, 218]**	**Enhances erythroid differentiation [219]**		**Pushes mESCS towards the ectodermal lineage [216]**
hsa-miR-148a			**Role in innate immunity via NF-KB pathway [220]**	**Targets p53, mTOR [221, 222]**	**Mir-148a is a tumour suppressor; promotes adipogenesis and osteoclastogenesis [223], suppresses mesenchymal lineage via *EPAS1*,** **Targets *DNMT1* [224]**

hsa-miR-18b		**Modulates cell migration [225]**	**Down-regulated during erythroid differentiation, targets *STAT3, HIF-1α, VEGF* [226]**	**Targets the MDM2-p53 pathway [227]**	**Promotes epithelial differentiation [228]**
hsa-miR-193b		Suppresses metastasis via targeting uPA, and *NF1* [229, 230]	Targets hematopoietic transcription factors *C-KIT* [231], *c-MYB* [232]	Targets Cyclin D1 [233, 234]	
hsa-miR-200b		**Targets critical EMT regulators *ZEB1, ZEB2, BMI1, RND3, SNAIL, ETS1* [201, 235, 236]**	**Regulates angiogenesis via *ZEB1* [201], targets immune system pathways TLR4 and NF-KB [237]**		
hsa-miR-200c	**Used to enhance reprogramming [21]**	**Inhibits EMT via *BIN1, ZEB1, RND3, LHFP, TGFb* [237, 238]**	**Targets *VEGF* [239], *ETS1* [240] and *cMYB* [241]**		
hsa-miR-205		**Targets *ZEB1*and *ZEB2* [235]**		***E2F1, PTEN*, (p73/DNp73-miR-205)**	**Complex role in tumour formation [242]**
hsa-miR-210					Key hypoxia associated miRNA [243, 244]
hsa-miR-214			Targets *VEGF* [245]	Targets p53 [246]	Biomarker for cancer [247, 248], role in osteoblast formation [249, 250]
hsa-miR-218			Regulates vascular patterning via *SLIT-ROBO* signalling [251]		Tumour suppressor [229]

hsa-miR-301a		Targets E-cadherin [252]	Regulates NF-κB signalling [253, 254]		
hsa-miR-302a, b, c, d, hsa-miR-367	**Used to enhance reprogramming, [21, 197, 198]**			**Targets cyclin E-CDK2 and cyclin D-CDK4/6 pathways [255]**	
hsa-miR-34a		**Targets *MYC* [256]**	***NOTCH1, FOXP1*(B cell differentiation) [111],** **Targets *CEBPA* (myeloid differentiation [257]**		**Very important tumour suppressor [229, 258]**
hsa-miR-365				Targets cyclinD [259]	Adipocyte differentiation [260]
hsa-miR-424			**Hematopoietic genes *PU.1* [101], *CEBPA[261], VEGF, VEGFR2, FGF2, FGF1, FGFR2[262]***	**CyclinE1, CDC25A, MEK[263]**	**Controls myeloid and monocyte differentiation [101], inhibits angiogenesis [264], cell cycle regulator**
hsa-miR-455-3p		Targets *ZEB1* [265]			Regulates chondrogenesis [266] and adipogenesis [267]
hsa-miR-630		Inhibits EMT via SLUG-TGFB pathway [268-270]			
hsa-miR-7		Inflammation and innate immunity [271]			Role in organogenesis, particularly the brain [272]

hsa-miR-9	**LIN28 [273]**	***E-Cadherin* [274]**	**Hematopoiesis, myeloid differentiation via *FOX01* and *FOX03* [275]**	**Cyclin D1, *ETS1* [276]**	**Promotes pluripotency [273], tumour suppressor and oncogene, involved in inflammatory response. In hematopoietic cells it promotes terminal differentiation and apoptosis [275]**

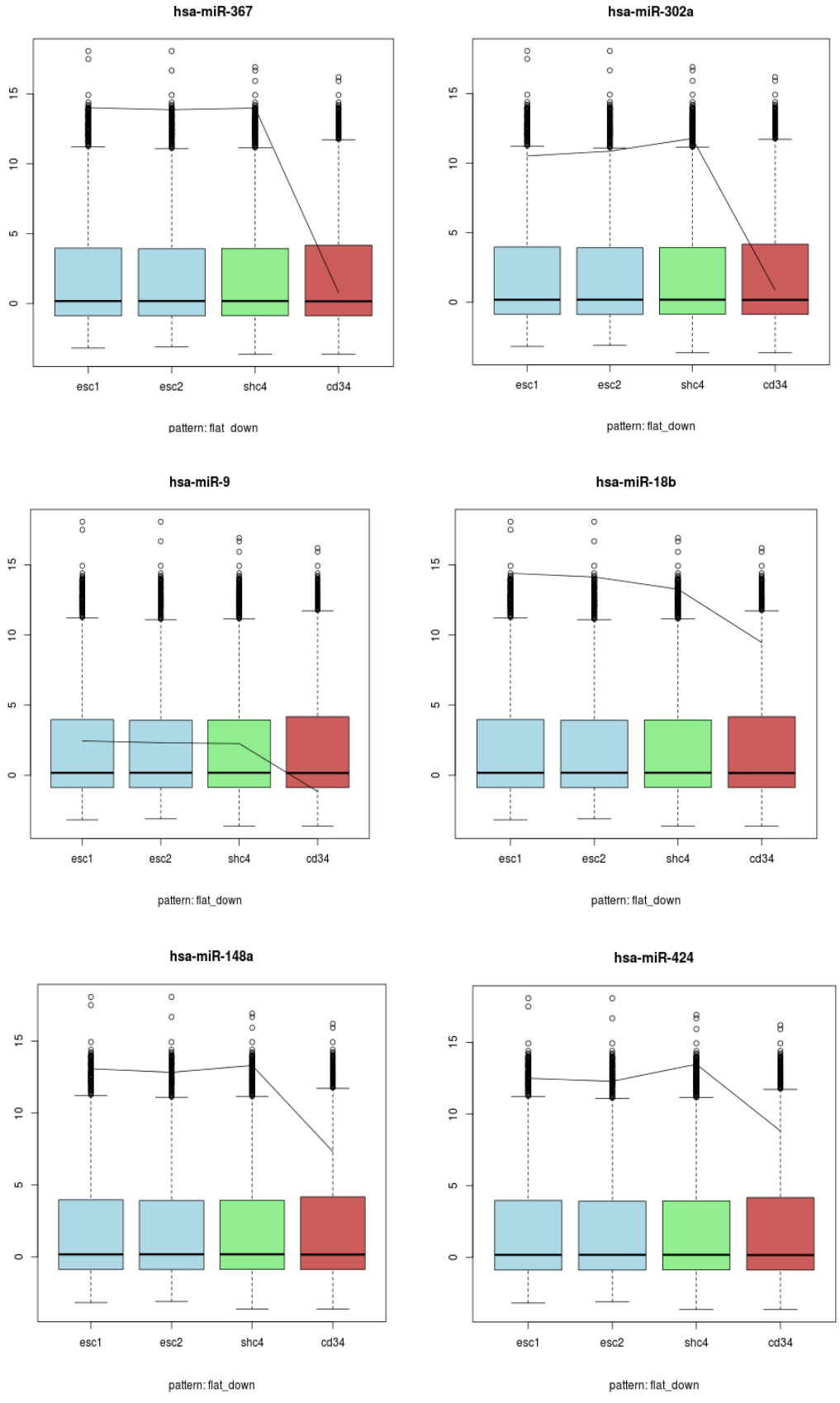
Table 4.2: FLAT_UP	**Pluripotency**	**Inflammatory pathway**	**Hematopoiesis**	**Cell cycle and proliferation**	**Other**
hsa-let-7b, 7b*, 7c, 7d, 7f-1*, 7g, miR-98	Negative regulator of pluripotency via LIN28, C-MYC, SALL4 [273]			Targets Cyclin D1, A2, CDK8, RAS, HGMA2[277]	
mir-7-1*	Targets KLF4 [278]			Regulates mTOR, PI3K/AKT pathway [279]	
hsa-miR-10a*					
hsa-miR-1225-3p					
hsa-miR-1237					
hsa-miR-1238					
hsa-miR-127-3p					
hsa-miR-138-2*					
hsa-miR-142-3p, 5p			**Essential for HSC formation by targeting IRF7 , essential**		

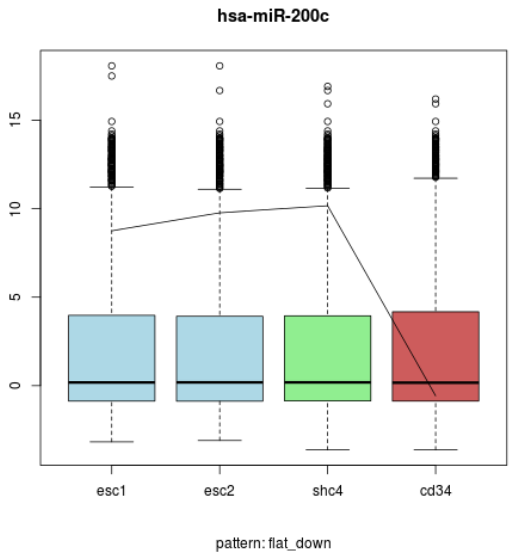
			for hematopoiesis in zebrafish, controls myeloid differentiation [280-282]		
hsa-miR-146a, 146b-5p			**Involved in the differentiation of primitive HSCs and T lymphocytes[93, 280, 283, 284]**	**TRAF6, IRAK1, CARD10, COPS8 (NF-κB pathway)[253]**	
hsa-miR-1470					
hsa-miR-150			**MS4A3, AGA, PTPRR (erythroid), C-MYB, NOTCH3, promotes megakaryocyte development at the expense of erythrocytes Regulates HSC migration [285], targets c*MYB*[286], promotes T-lymphoid lineage[287]**	**Inhibits cell division via CyclinD1, BCL2, MYB [199, 200, 286]**	
hsa-miR-1539					
hsa-miR-155		PI3K-AKT, IFN[253]	SOCS1 (encourages T cell differentiation) [288], myeloid differentiation [289]		
hsa-miR-16-2*					
hsa-miR-181a*, 181a-2*, 181b, 181c		**C-Fos and PCAF(supress inflammatory response)[290]**	**Encourages B-lymphoid lineage [291], T-cell development [98, 292]**	**HTR-A1, TIMP3, BCL2 [258, 293]**	
hsa-miR-191*					
hsa-miR-1913					
hsa-miR-202					

hsa-miR-212		IRAK4, STAT4(inflammatory) [294]	Regulates B-cell development via SOX4 [295]		Mir-212 is an EMT suppressor [296]
hsa-miR-221*					
hsa-miR-223, 223*		**Inflammation associated [297, 298]**	**Regulates granulopoiesis, erythropoiesis and hematopoetic proliferation via LMO2 and CEBPA [103, 299-302]**	**Regulates the cell cycle [303]**	
hsa-miR-23a*					
hsa-miR-338-3p, 5p				Targets cyclin D1 [304]	Contributes to the formation of basolateral polarity in epithelial cells [305]
hsa-miR-361-3p					Biomarker for many cancers
hsa-miR-376c				Inhibits proliferation by targeting BMI1 and LRH [306, 307]	
hsa-miR-378, 378*			Targets RUNX1 [308], promotes macrophage proliferation [309]		Involved in adipogenesis and angiogenesis [310, 311]
hsa-miR-431*					
hsa-miR-486-5p					Diagnostic for many types of cancer
hsa-miR-564					
hsa-miR-634					
hsa-miR-650		Promotes IL6 expression [312]			

hsa-miR-665					EMT suppressor [313]
hsa-miR-766		Upregulated in inflamed tissue [314]			
hsa-miR-767-3p					DNA methylation via TET1 [315]
hsa-miR-770-5p					
hsa-miR-933					
hsa-miR-1306					
hsa-miR-324-5p					
hsa-miR-487b			Negative regulator of macrophage activation [316]		Promotes angiogenesis [317]
hsa-miR-92b	Controls G1/S checkpoint in ESCs [318]				EMT promoter [319, 320]

Figure 4.3a


hsa-miR-367
hsa-miR-302a
hsa-miR-9
hsa-miR-18b
hsa-miR-148a
hsa-miR-424
esc1
esc2
shc4
cd34
pattern: flat_down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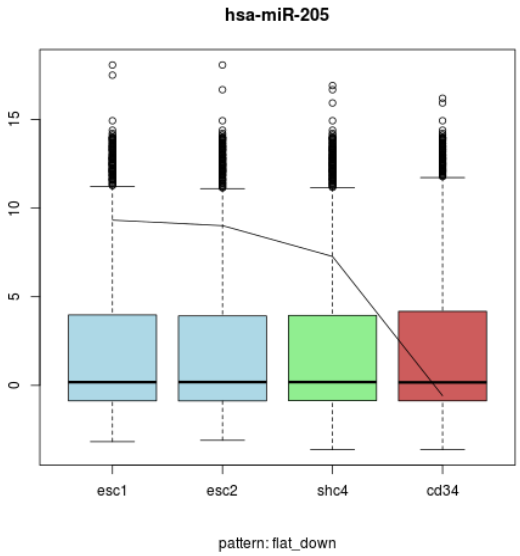
5
10
15
esc1
esc2
shc4
cd34
pattern: flat_down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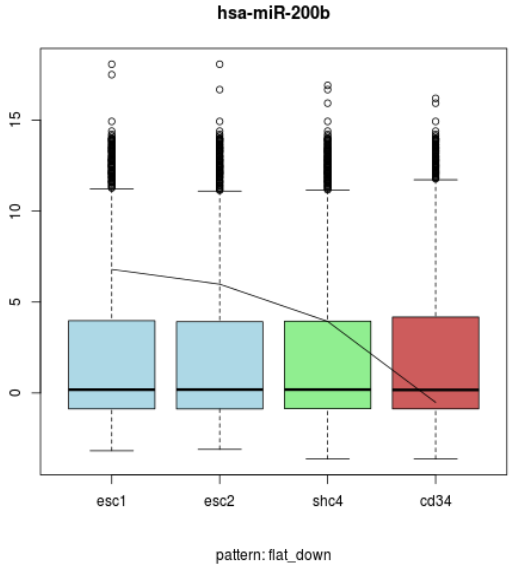
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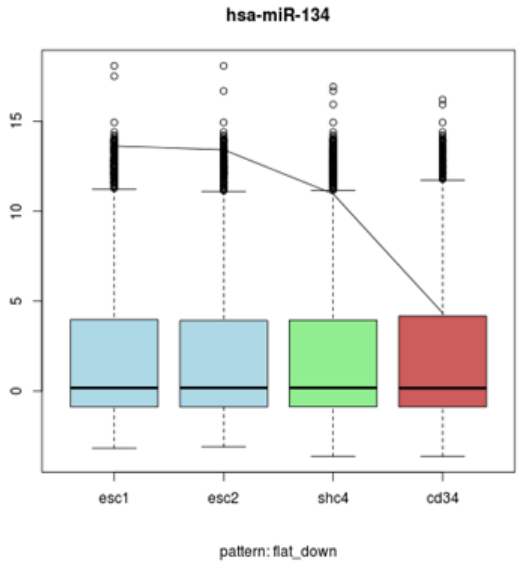
10
15
esc1
esc2
shc4
cd34
pattern: flat_down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10
15
esc1
esc2
shc4
cd34
pattern: flat_down

Figure 4.3b

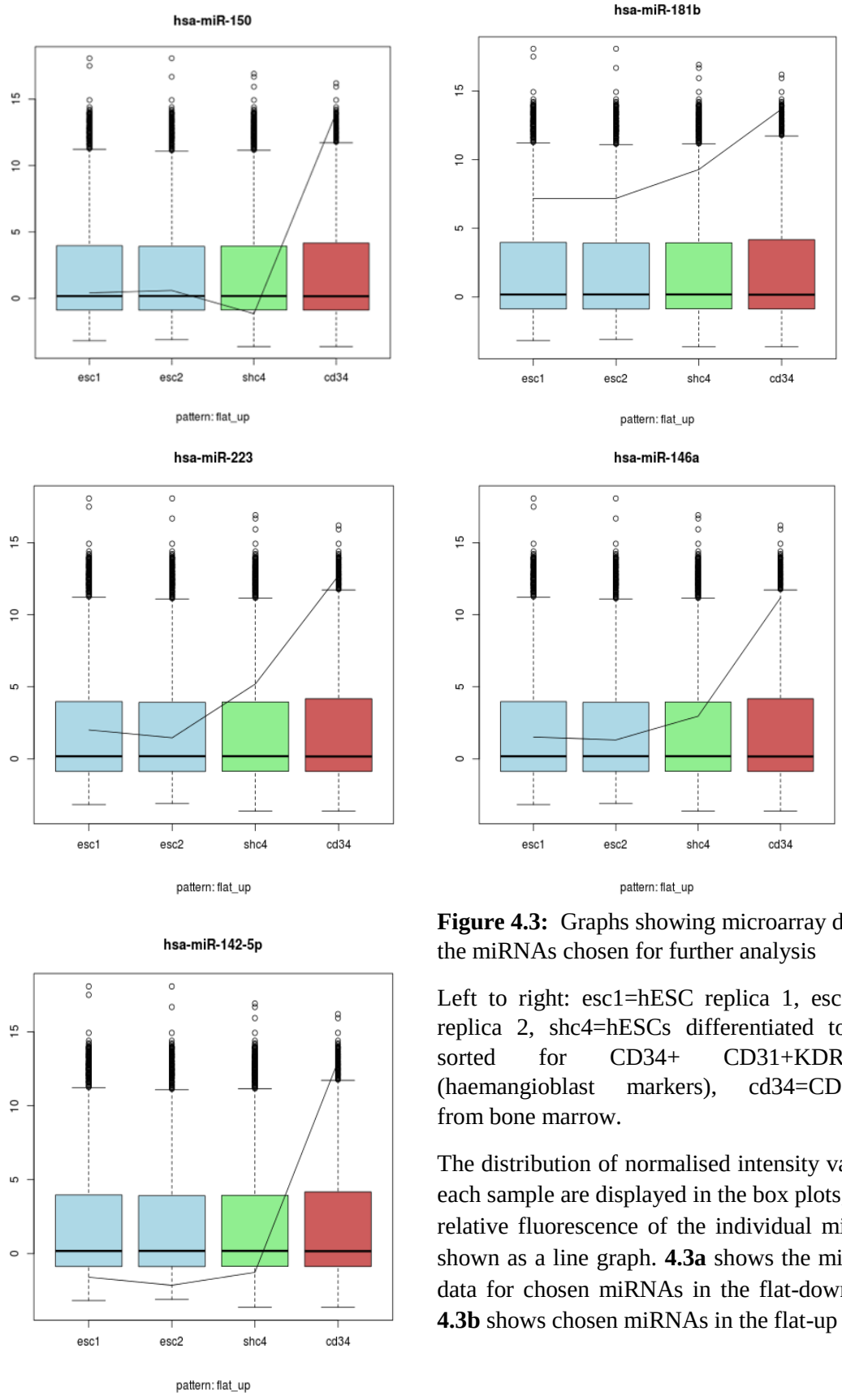


Figure 4.3: Graphs showing microarray data of the miRNAs chosen for further analysis

Left to right: esc1=hESC replica 1, esc2=hESC replica 2, shc4=hESCs differentiated to day 4 sorted for CD34+ CD31+KDR+CD45- (haemangioblast markers), cd34=CD34+cells from bone marrow.

The distribution of normalised intensity values for each sample are displayed in the box plots, and the relative fluorescence of the individual miRNA is shown as a line graph. **4.3a** shows the microarray data for chosen miRNAs in the flat-down group, **4.3b** shows chosen miRNAs in the flat-up group.

4.3 qPCR validation of miRNA candidates selected from microarray analysis

The candidates from the microarray analysis must be validated: microarray analysis is not considered accurate enough to be conclusive (see **results section 4.1**), and the protocol used for this project is more advanced and produces hematopoietic cells more similar to the HSC cell type than the one which was used for the microarray analysis. The method used for qPCR was the Taqman two-step system (see **methods section 2.5.1**), as this was state of the art at the time the project started.

miRNA expression was analysed in several hematopoietic progenitor populations: Expression of the chosen miRNAs was analysed at several time-points during development and in various subpopulations of cells sorted by FACS using cell surface markers for hematopoietic progenitors. PSC derived cultures of hematopoietic stem cells contain many different cell types which arise at different time-points. Several hematopoietic populations were sorted and analysed in order to get a clearer picture of when the key miRNAs are up or down regulated and in what cell types, in order to give an idea of the progression of hematopoietic development in different hematopoietic progenitors. The cell types chosen for analysis were hemogenic endothelium at day four (CD34+KDR+CD31+ [64, 321]) primitive hematopoietic cells (CD41a+CD235a+ CD43+CD34low/- CD45- [8, 65, 322]) which arise in large numbers at day 7 and definitive cells which arise in detectable numbers at day 12 (CD43+CD34low/- CD41a-CD235a-CD45+ [8]), as well as analysing the primitive cells at the same time-point. Non-hematopoietic populations (negative for all hematopoietic markers, referred to as 'negative' in this chapter) were also analysed so as to provide a comparison for the expression of miRNAs which are known to be hematopoietic specific. Details of the cell sorting process and the markers used are given in **methods section 2.4**. These populations were compared to three other cell types: Undifferentiated pluripotent stem cells, bone marrow derived CD34+ cells (as in the microarray data) and cord blood derived CD34+ cells as it is thought that pluripotent derived hematopoietic cells have more in common with foetal/neonatal cells.

4.3.1 qPCR analysis for flat-up miRNAs

Expression levels of miRNAs in PSC derived hematopoietic progenitors were compared to bone marrow and cord blood CD34+ cells. Several of the miRNAs (miR-142, miR-223) are not expressed in the ‘negative’ non-hematopoietic populations, but highly expressed in all of the hematopoietic populations, which supports the literature saying they are hematopoietic specific. miR-146 on the other hand was shown to be expressed at equivalent or higher levels than bone marrow and cord blood in all the differentiated populations, and miR-181 is expressed at high levels

in the negative population at day 12, so these miRNAs are probably less useful as markers for hematopoiesis in PSC derived cultures (**Figure 4.4a**).

The 'flat-up' group miRNAs are mostly hematopoietic specific miRNAs and were shown by the microarray data to be expressed at too low a level in P-HPCs. The results of the qPCR analysis show a different pattern; in definitive hematopoietic progenitors (which are the ones that this project is most interested in) the miRNAs are either not expressed at significantly different levels to bone marrow and cord blood or in some cases are expressed at significantly higher levels than either bone marrow or cord blood progenitors (**Figure 4.4a**). Although these results contradict the microarray data the high expression of hematopoietic-specific miRNAs is encouraging as it suggests that the differentiation protocol which was used for the qPCR analysis promotes more effective hematopoiesis than the earlier protocol used for the microarray data (see **methods section 2.1.2**). The fact that the expression of some of these miRNAs is higher than that in cord blood or bone marrow reflects the more-differentiated nature of the sorted cell population, as these miRNAs are expressed in CD34+ progenitors but increase in expression as the cells differentiate towards specific blood lineages. All of these miRNAs can be ruled out as targets for manipulation of expression as they seem to be expressed correctly in the definitive population.

qPCR analysis for flat-down miRNAs: The results from the flat-down group can be divided into two broad categories as shown in **Figure 4.4b**; group A, which has miRNAs that eventually down-regulate to a level not statistically significantly different from cord blood or bone marrow, and group B, in which the miRNAs maintain high expression throughout development in all of the sorted populations.

The group A results include all of the miRNAs from the miR-302 cluster as well as miR-18b. This data shows that the cells rapidly lose expression of the miR-302 cluster miRNAs upon exit form pluripotency, which is what would be expected based on the literature. Where miR-302a and miR-18b are different from bone marrow and cord blood they are significantly lower in expression, indicating that the cells have fully exited the pluripotent state. miR-302a* is expressed at significantly higher levels in the definitive population than in bone marrow in both H9 and SB-Ad3 populations, but it is not significantly different from the expression in cord blood, which may be due to the more foetal nature of cord blood. miR-302a* is also the inactive strand, and is present in quantities which are at the limits of detection, which reduces the accuracy of the results.

It is not clear why so many of the miR-302 cluster fell into the flat-down group in the microarray data, it is possible that the early stage that the cells were tested as well as the lesser effectiveness of the protocol that was used meant that the cells were still expressing some molecular markers for

pluripotency. Group A miRNAs can be ruled out as candidates for inhibition, as they appear to be expressed at the correct level in definitive hematopoietic cells when the Glasgow protocol is used.

The group B miRNAs (miR-9, miR-134, miR-148a, miR-150, miR-150*, miR-200b, miR-200c, miR-205, miR-424) are consistently highly expressed throughout development from pluripotent stem cells, in all the sorted populations, both hematopoietic and non-hematopoietic. Although for the definitive population the expression of some miRNAs such as miR-200b and miR-205 the difference is not statistically significant this is due to the large amount of variation and is a technical problem caused by the low numbers of this population reducing the accuracy of the results. The qPCR data for this group is consistent with the microarray data. Failure to down-regulate all of these 9 miRNAs could impair the function of hematopoietic cells. Their expression indicates problems in the regulation of the cell cycle, adhesion/motility and hematopoiesis specifically. All these miRNAs are therefore possible candidates for inhibition.

Figure 4.4a

Flat-up

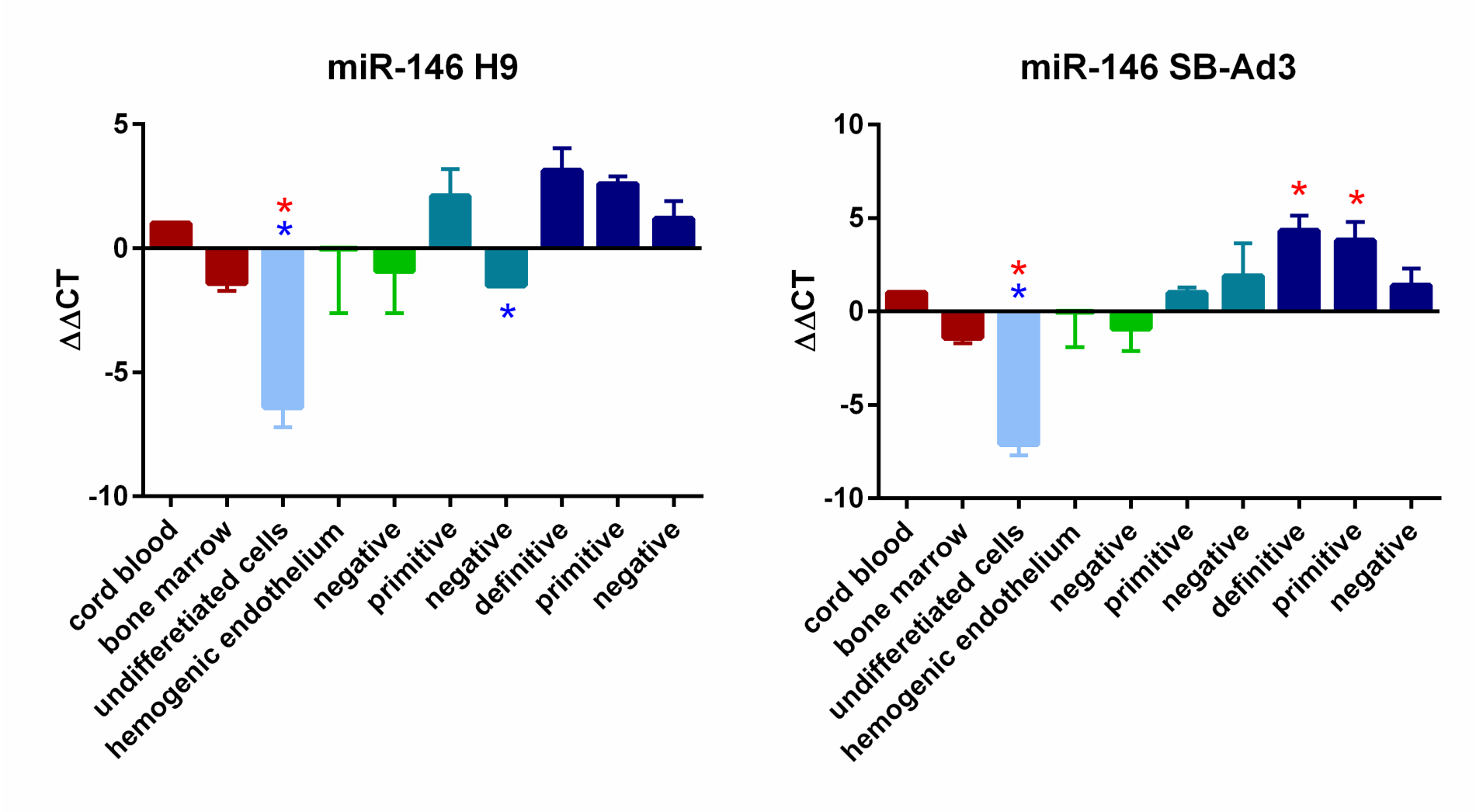


Figure 4.4a

Flat-up

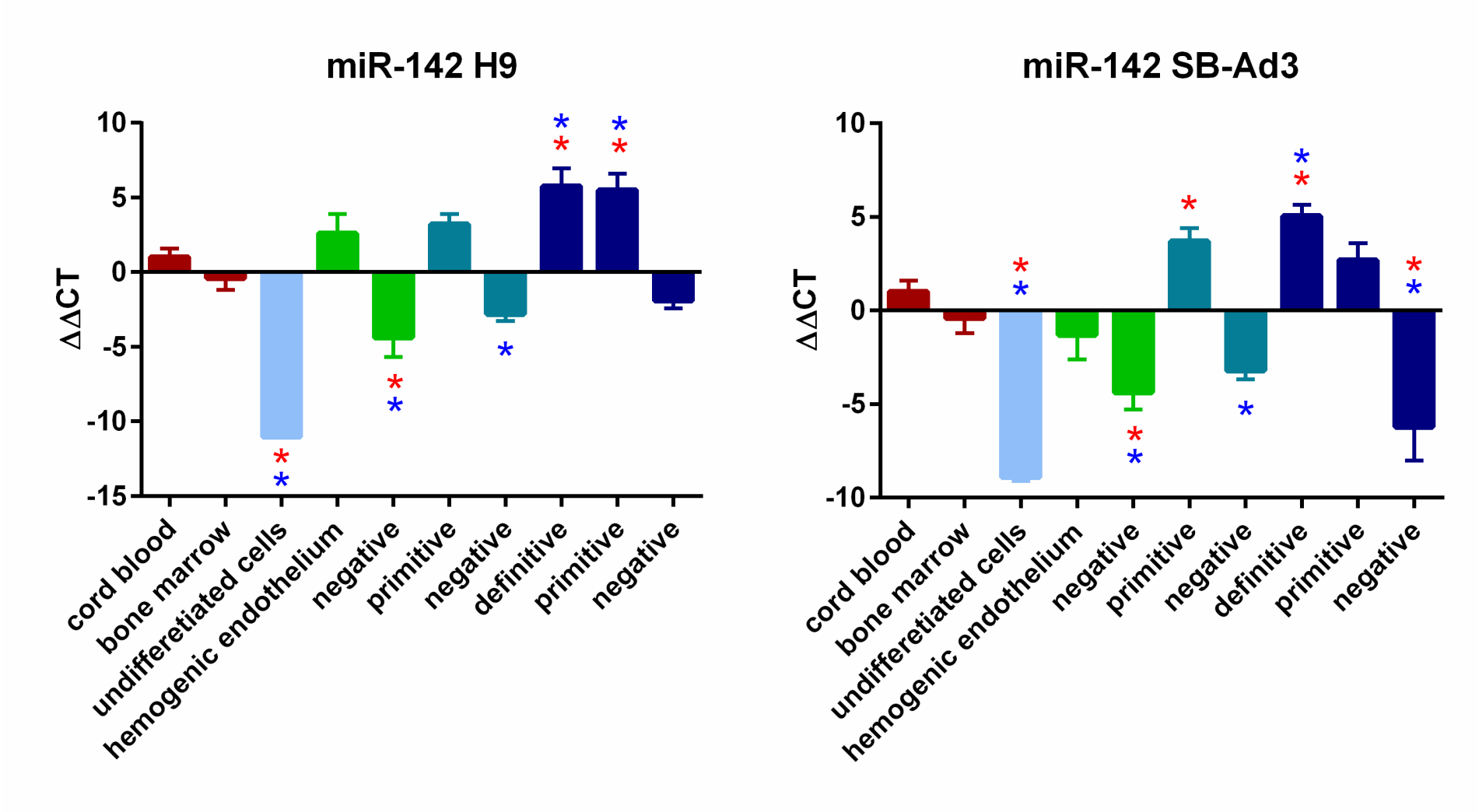


Figure 4.4a

Flat-up

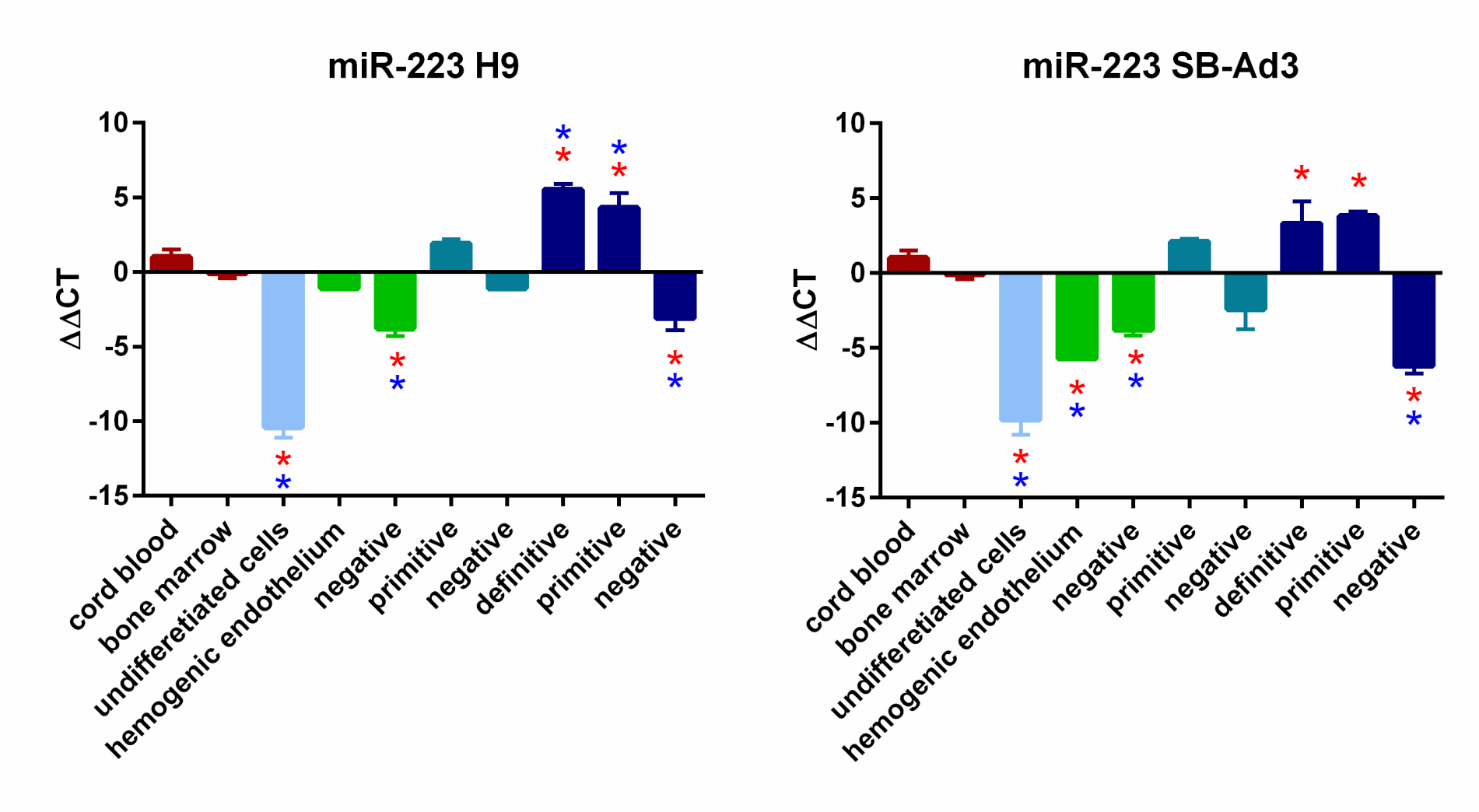


Figure 4.4a

Flat-up

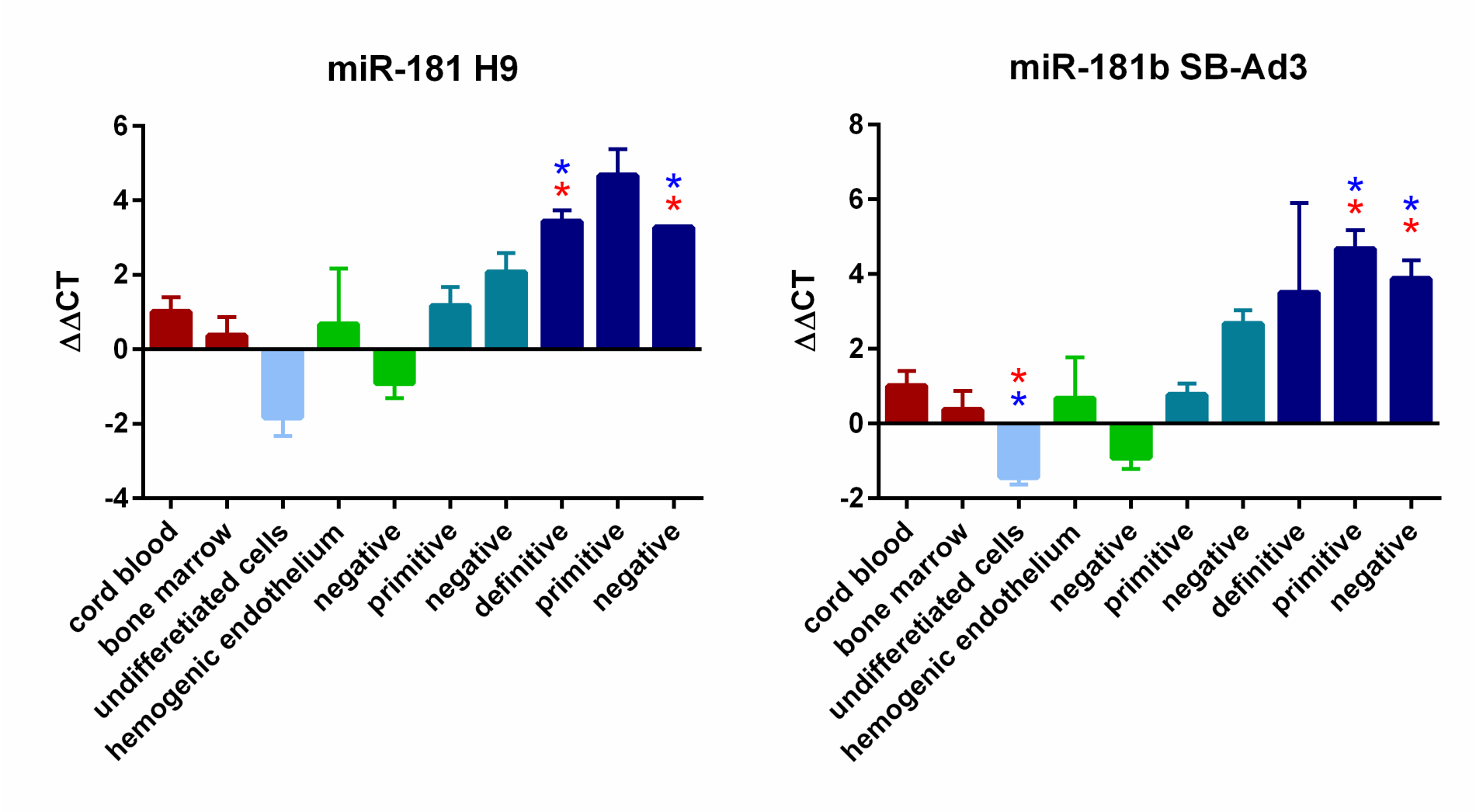


Figure 4.4b

Group A

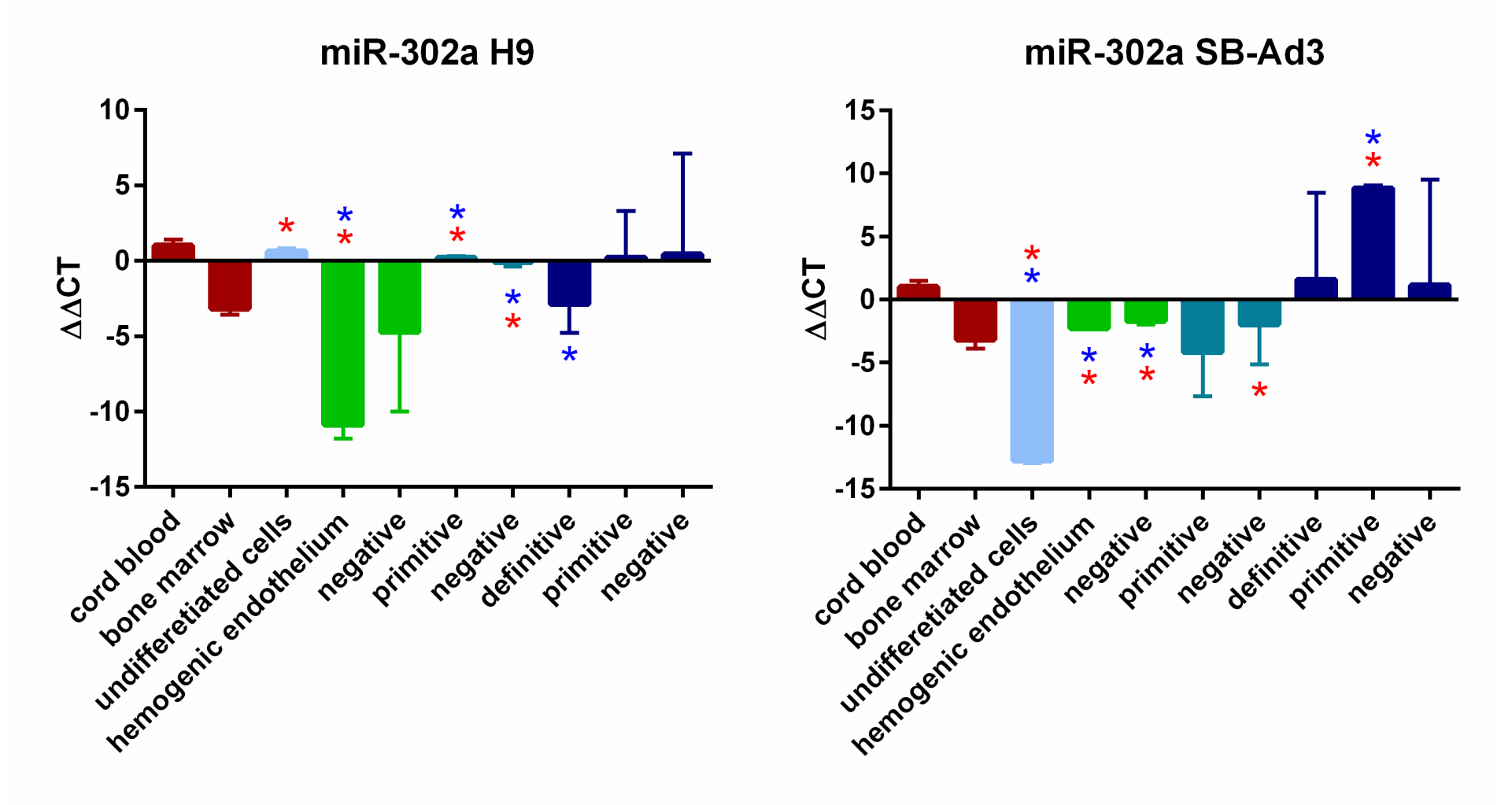


Figure 4.4b

Group A

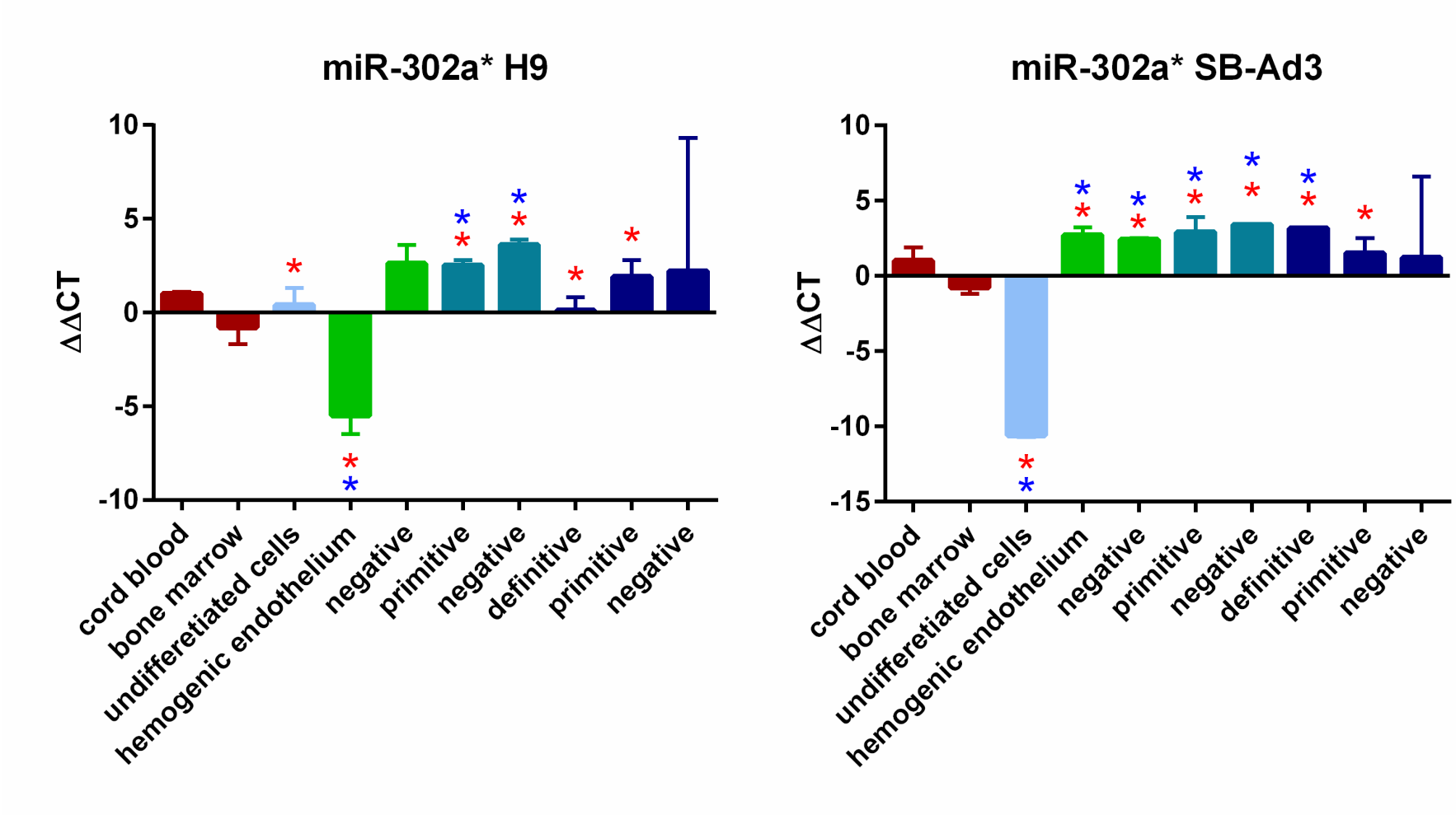


Figure 4.4b

Group A

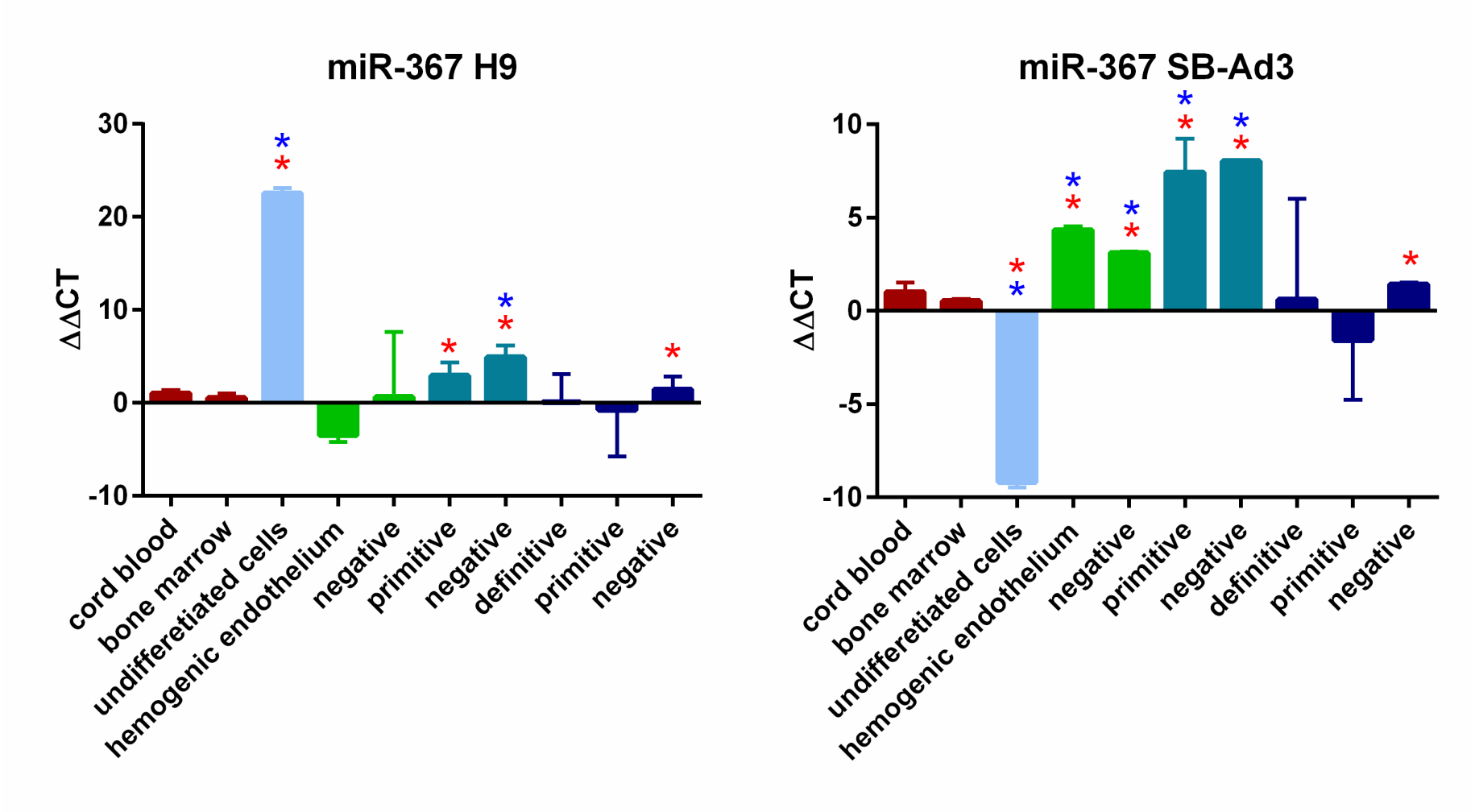


Figure 4.4b

Group A

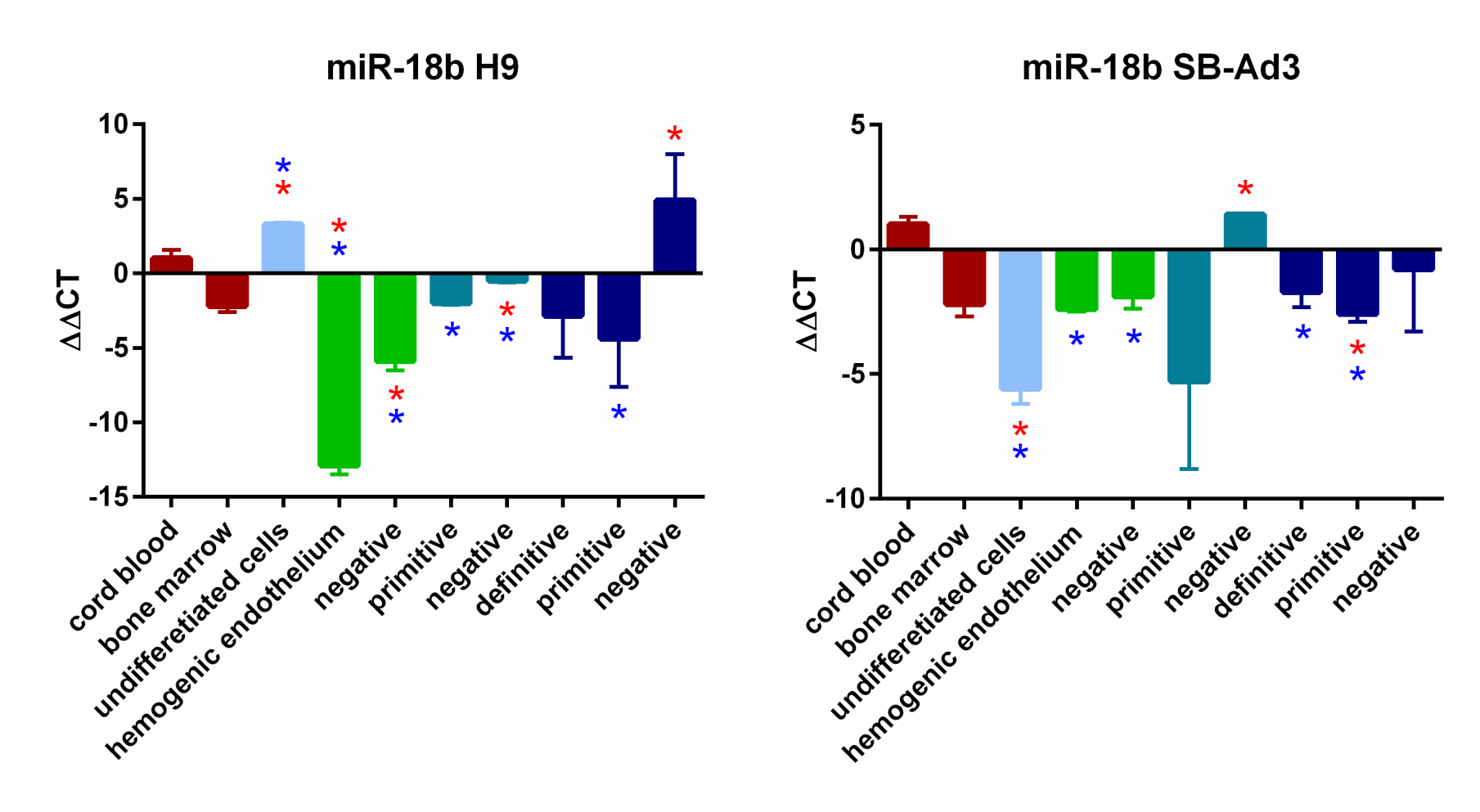


Figure 4.4b

Group B

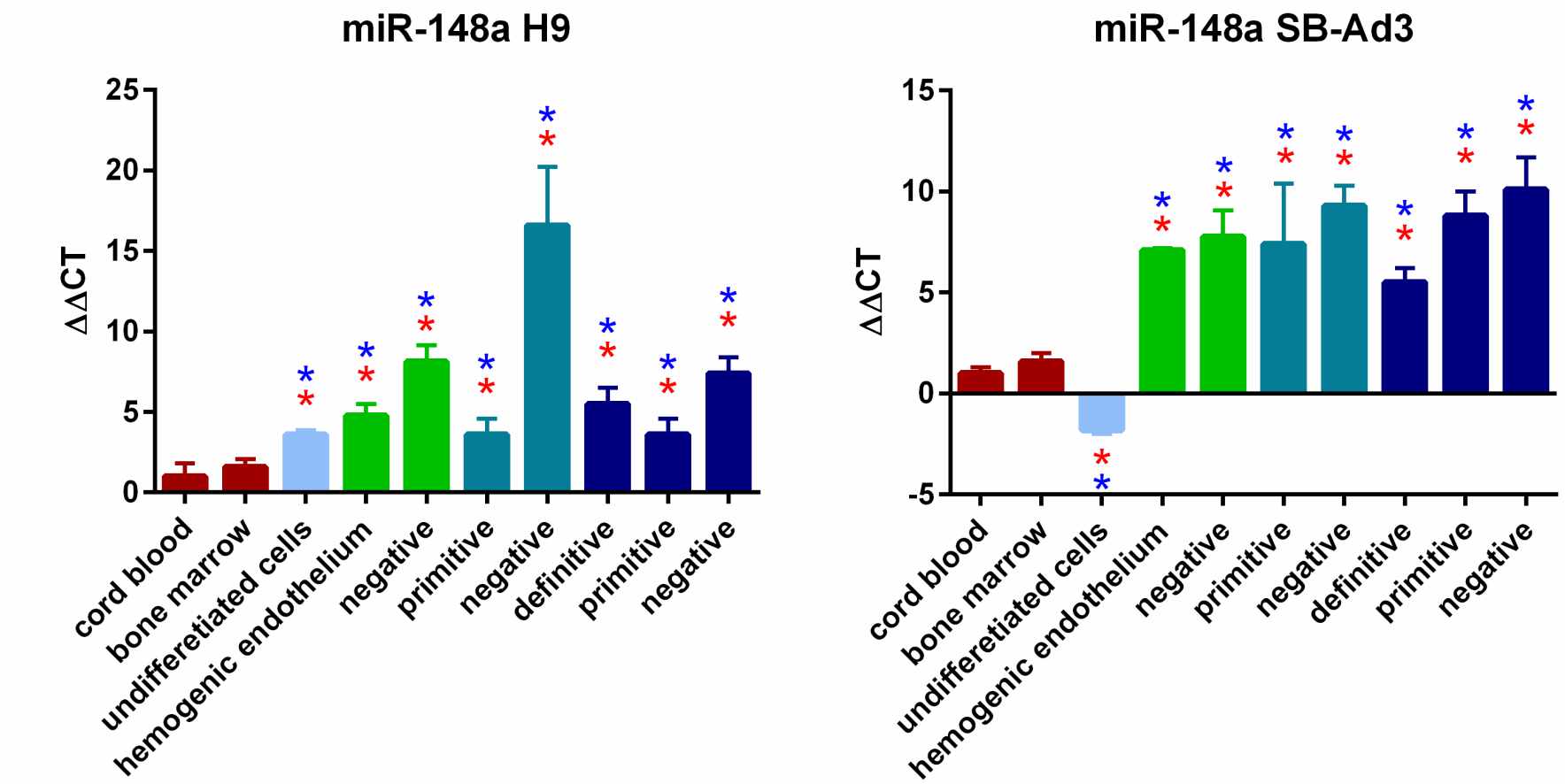


Figure 4.4b

Group B

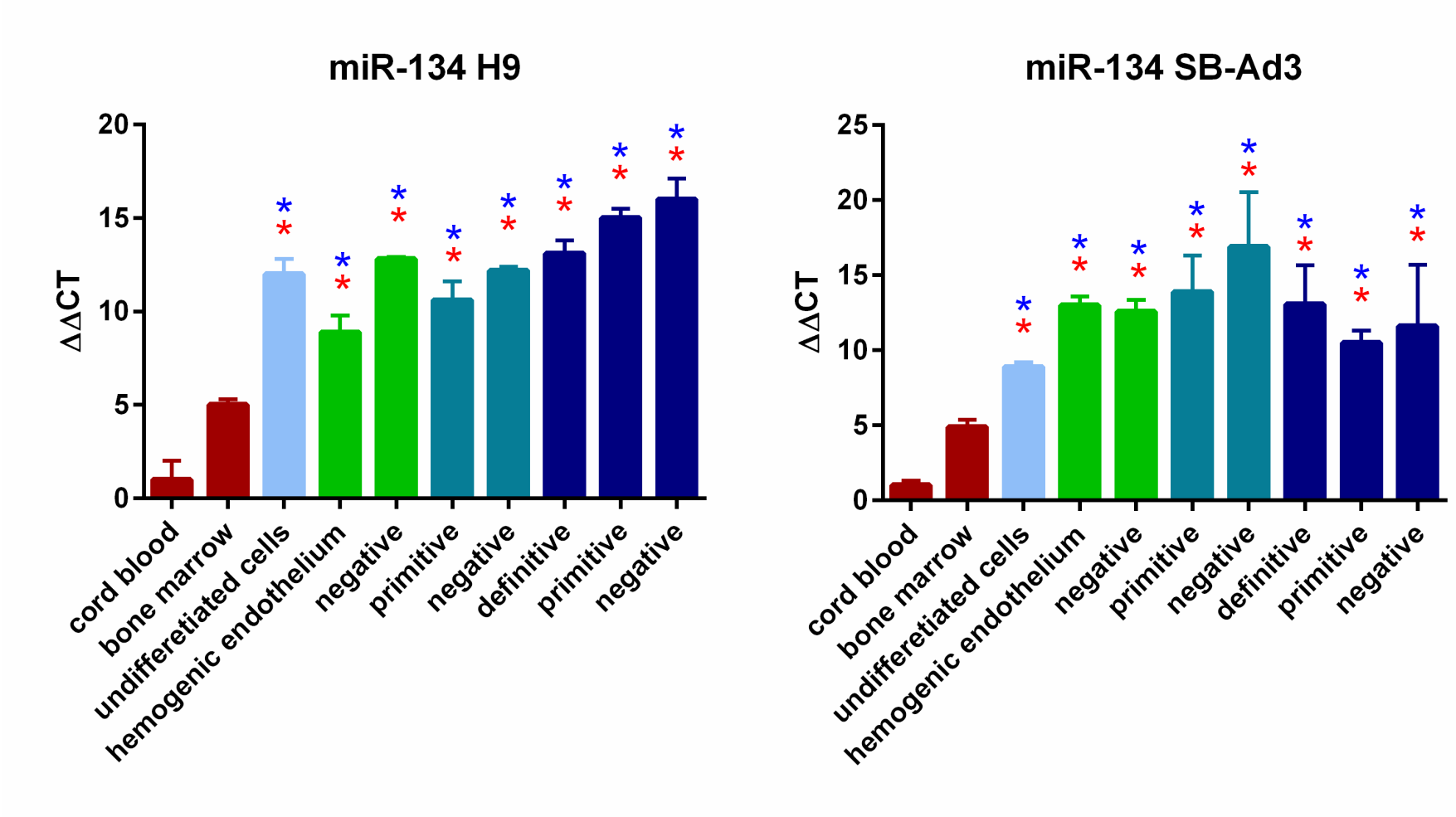


Figure 4.4b

Group B

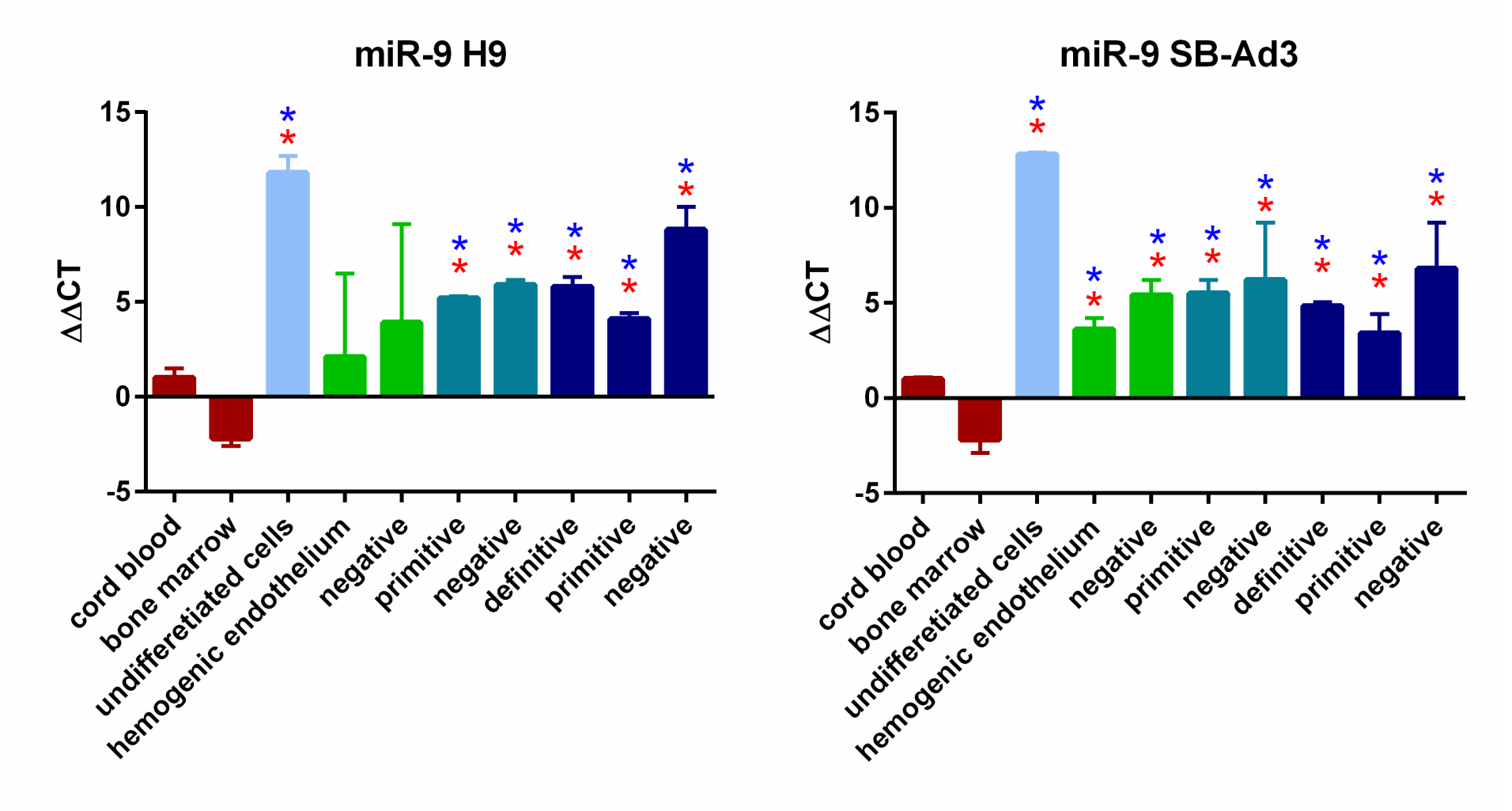


Figure 4.4b

Group B

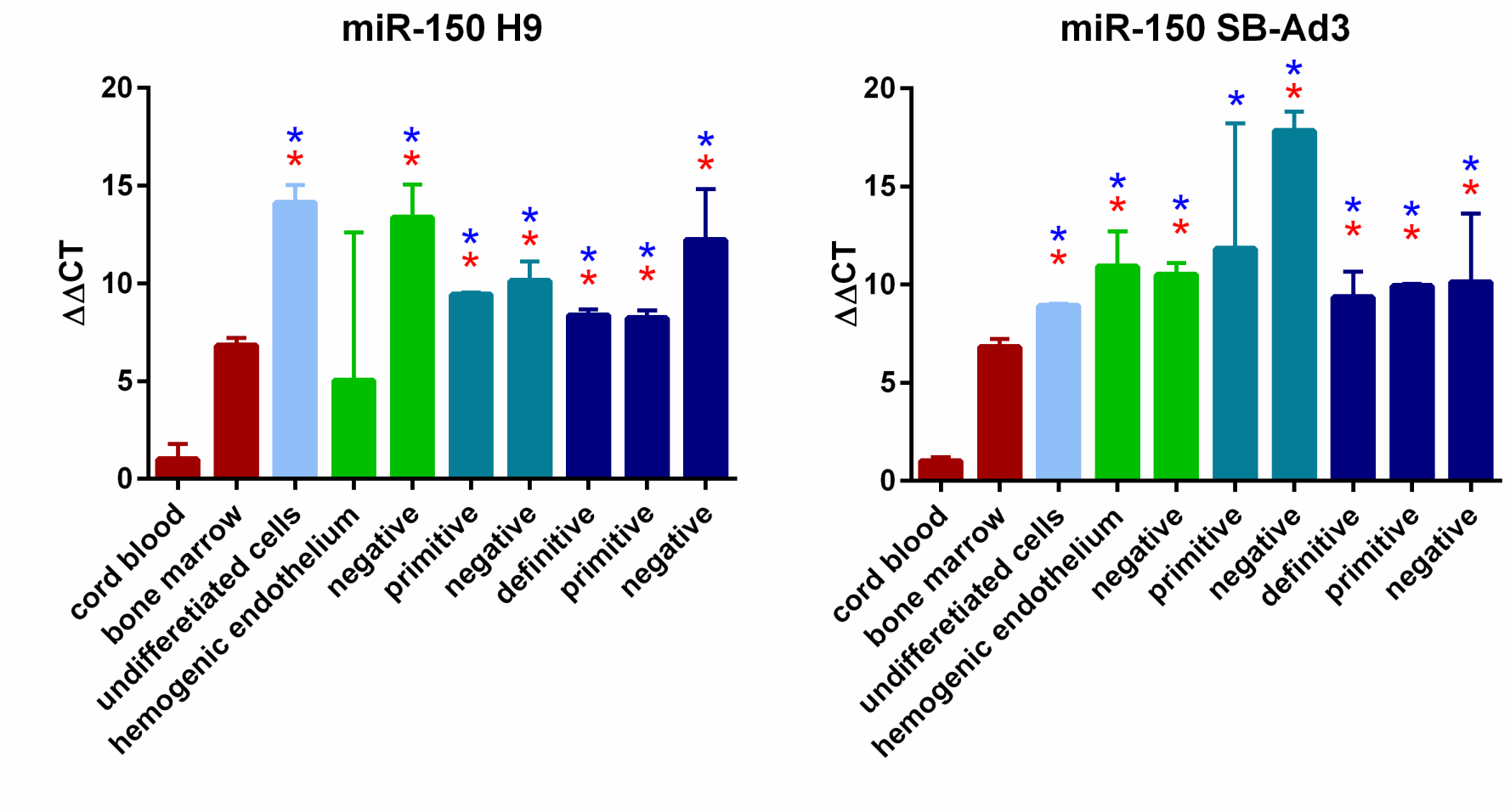


Figure 4.4b

Group B

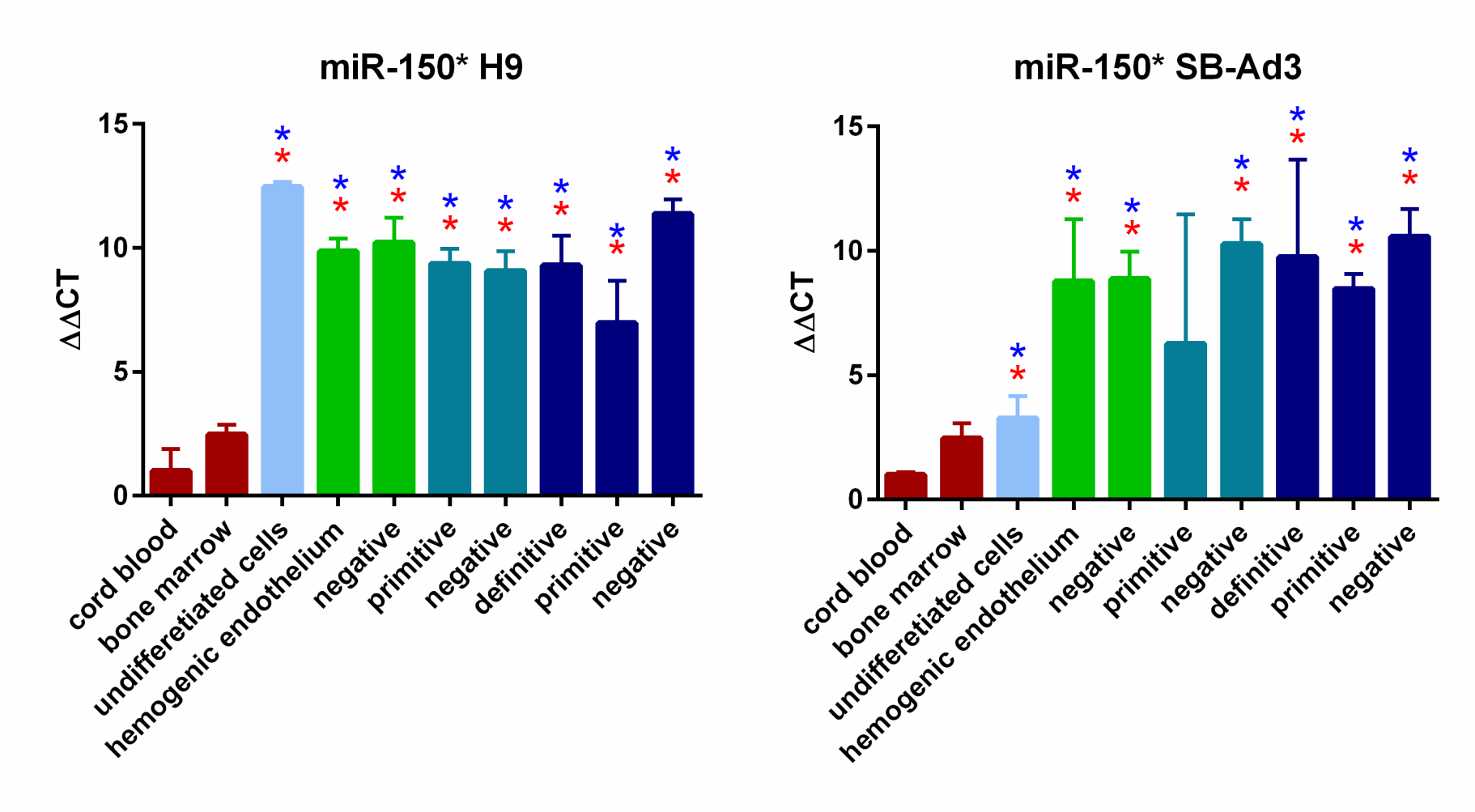


Figure 4.4b

Group B

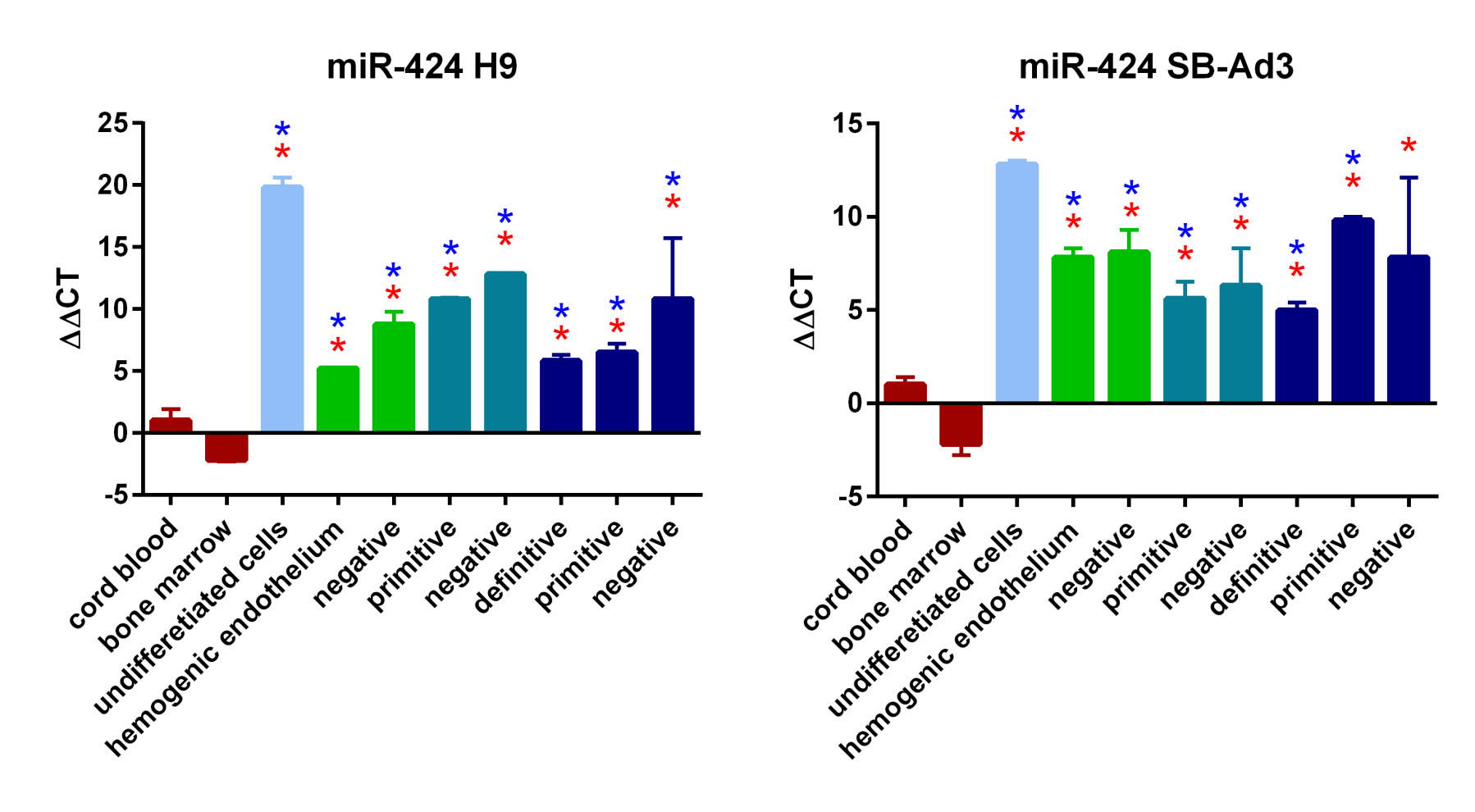


Figure 4.4b

Group B

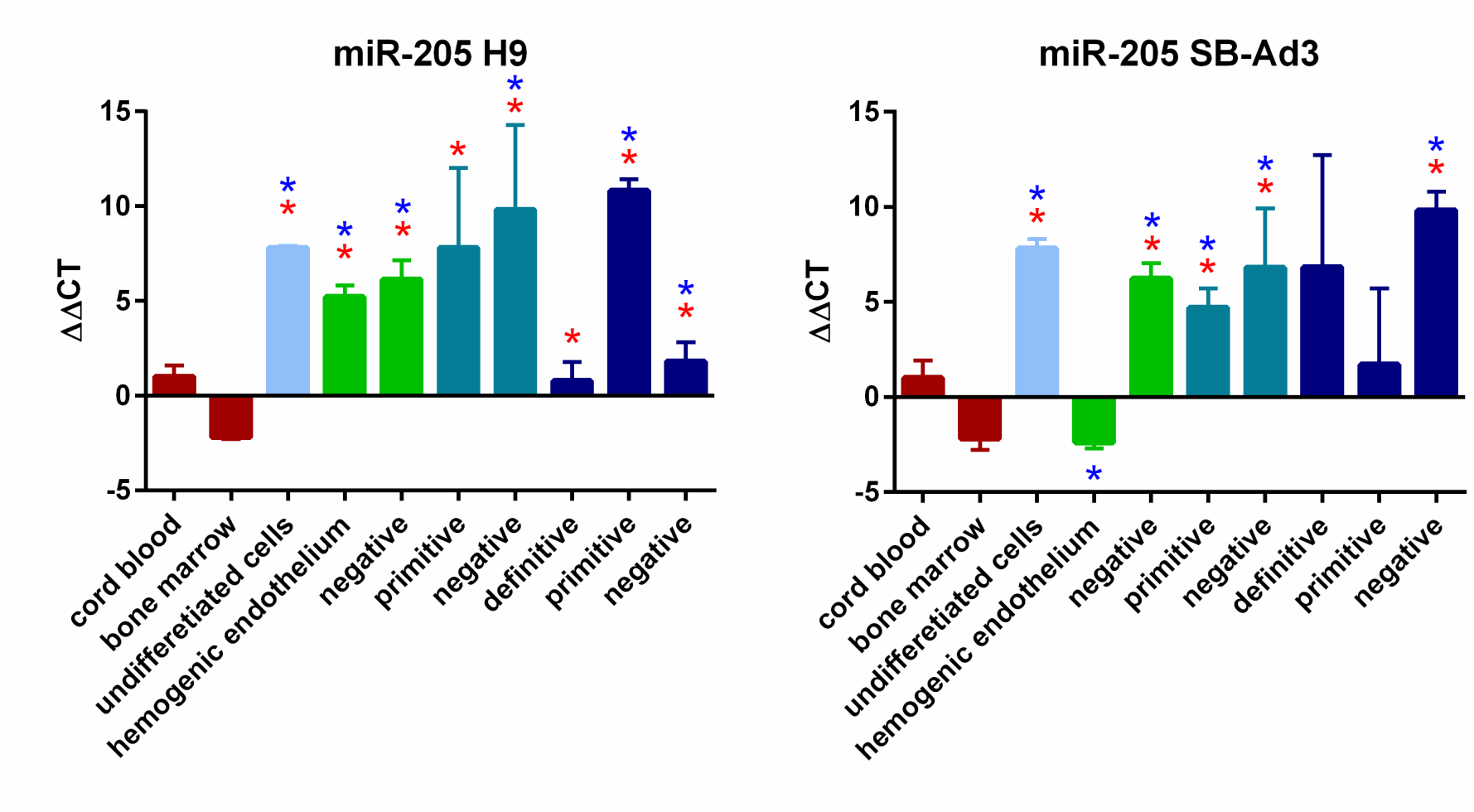


Figure 4.4b

Group B

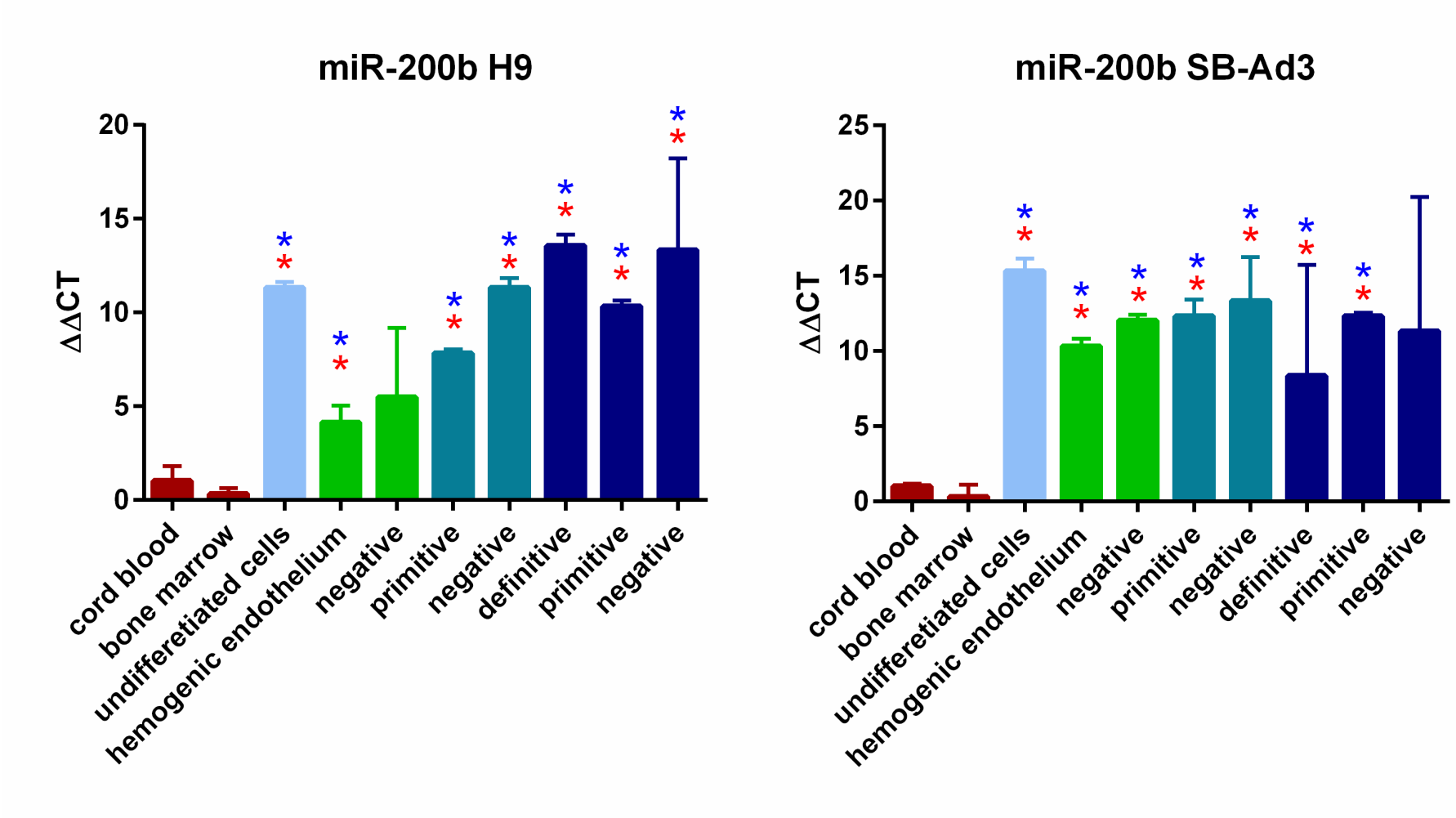


Figure 4.4b

Group B

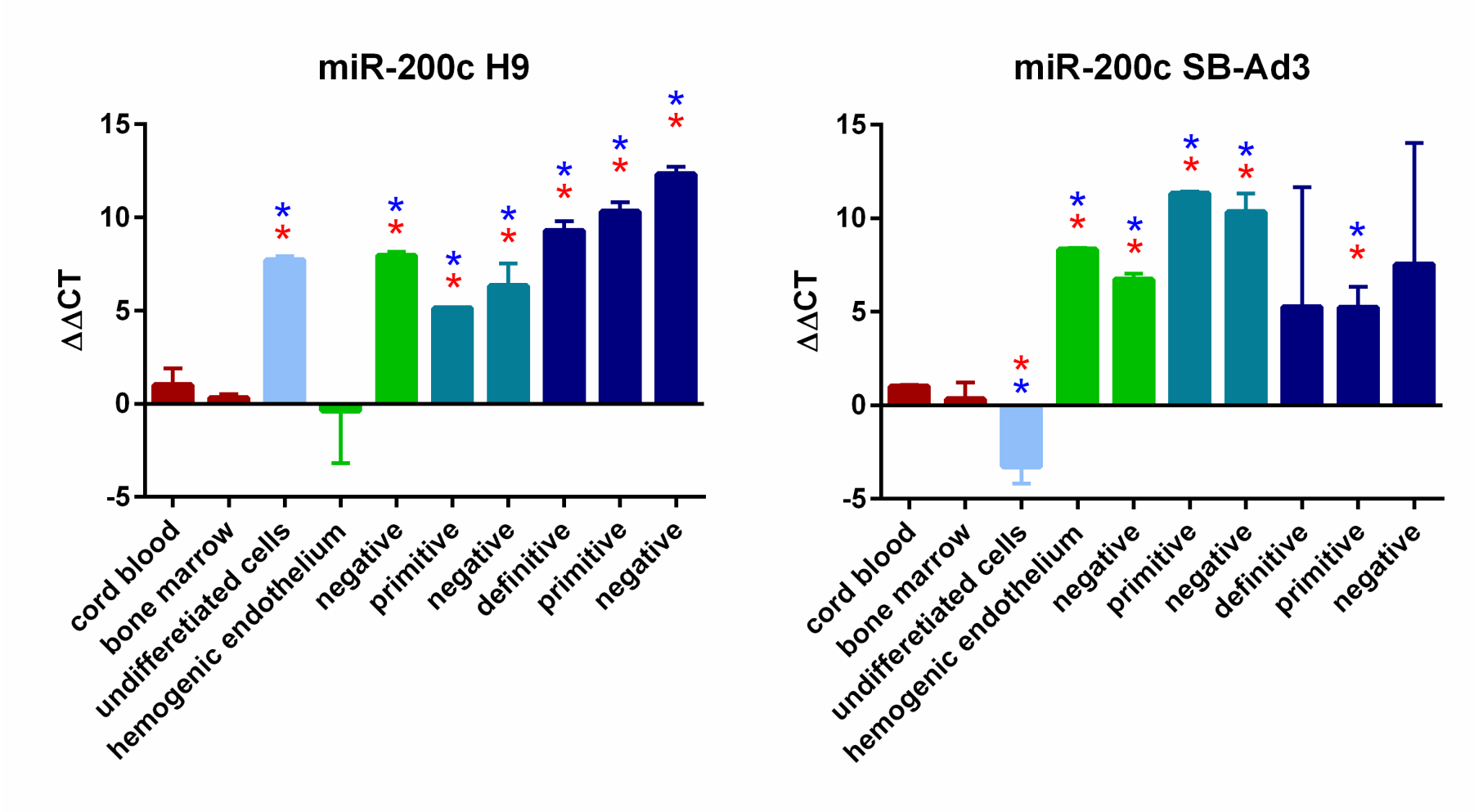


Figure 4.4: qPCR data for expression of miRNAs calculated using ΔΔCT method using RNU44 and RNU48 as endogenous controls; all values were calculated with respect to a reference value of expression of RNU44 and RNU48 in bone marrow. Data is presented as average +/- SEM. n=3. See methods **section 2.3.1** for experimental details.

P values were calculated using unpaired two tailed t-tests comparing the three replicates for each of the cell types for each miRNA against both bone marrow and cord blood.

Figure 4.4a: Flat-up miRNAs **Figure 4.4b**: Group A micro-RNAs (down regulated by day 12), Group B micro-RNAs (maintained high expression throughout differentiation).

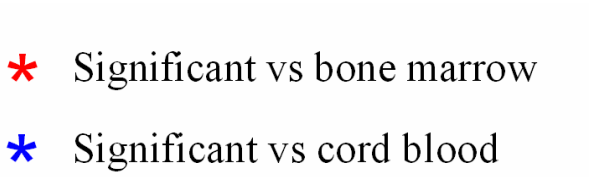
Columns are labelled according to their cell types as sorted in section 4.3

Hemogenic endothelium= CD31+ CD34+ KDR+ CD45-

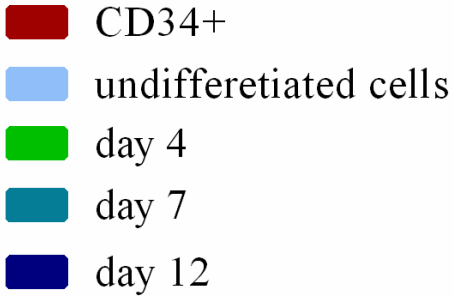
Primitive= CD34low/-CD41a+CD43+CD235a+CD45-

Definitive= CD34low/-CD41a-CD43+CD235a-CD45+

Negative= negative for all markers used in the sort



Different time-points are shown in different colours:



5. Results: Methods for manipulating miRNA expression

Methods for miRNA inhibition: Choosing an effective and appropriate means of inhibiting the target miRNA was essential for this project. miRNAs can be inhibited by introducing RNA molecules which are complimentary to the miRNA of interest; the inhibitor RNA binds to the target miRNA and either prevents translation or causes mRNA degradation (see introduction **section 1.4.1**). There are several types of miRNA inhibitor and several ways of introducing them into the cell.

Inhibition was one of the first techniques used to study the function of miRNAs [323, 324]. The earliest miRNA inhibitors used were based on the design of siRNAs which had been used to inhibit gene expression [325]. siRNA are short RNA molecules complimentary to a portion of a mRNA. They are introduced into the cell and processed by DICER, then they bind to the RISC complex and degrade or prevent translation of mRNA using the same mechanism as miRNA [326]. Since RNA is unstable, chemically modified RNA nucleotides (reviewed by Zhang et al. 2013 [192]) have been used to prevent enzymatic degradation and increase the siRNA's half-life within the cell. The most common chemical alterations are 'locked nucleic acids' (LNA) which have a cross link between 2'-O and 4'-C [327, 328] and 2'-OMe modified RNA [323] (see **Figure 5.1a**). These modifications can also improve the binding affinity for the target molecule and the stability of the RISC complex [329].

Lipofection allows RNA inhibitors to be introduced into cells: Introducing the inhibitor RNA to the cell is a challenge because nucleic acids are negatively charged and can't pass through the cell membrane. To overcome this, lipofection is used: cationic lipids are allowed to form complexes with the RNA, creating a neutral-charge hydrophobic complex which can more easily pass through the cell membrane (see **Figure 5.1b**). The exact chemical formulation of the cationic lipid can vary depending on the application, but achieving the correct ratio of lipid to RNA is essential and the complexes must be formed in serum free medium [330].

Figure 5.1a

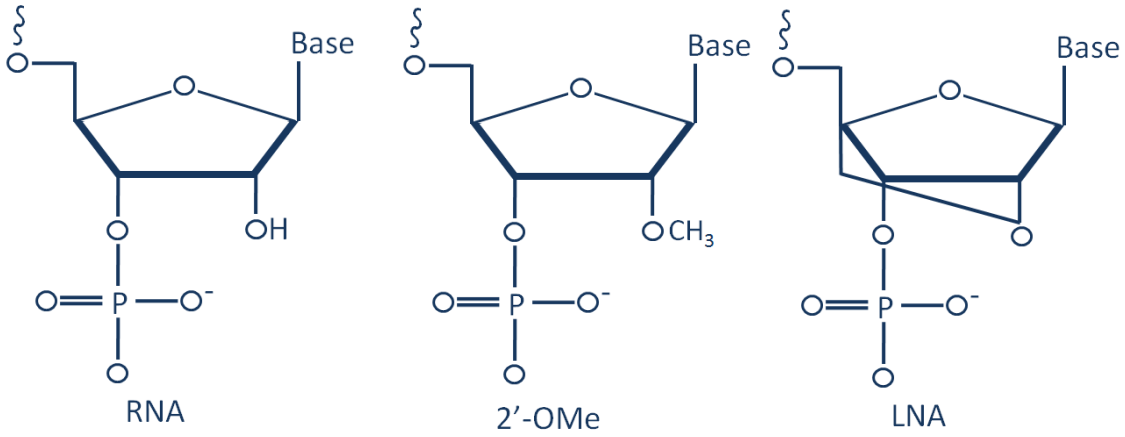


Figure 5.1b

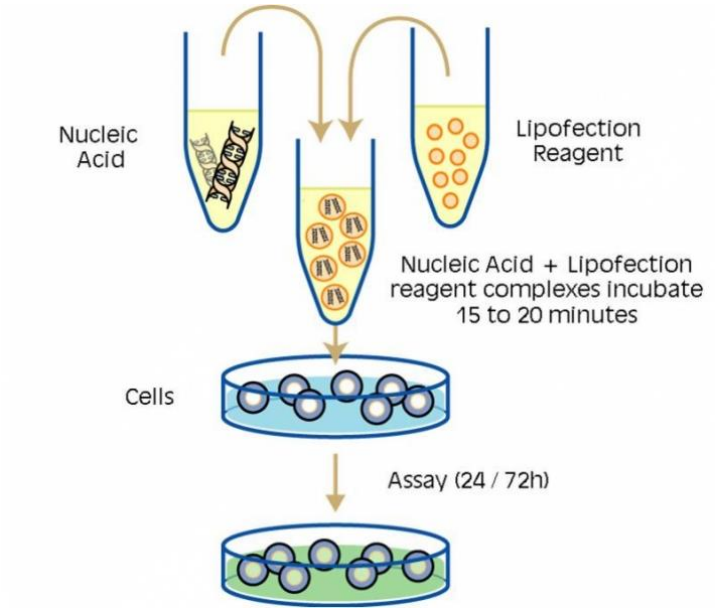


Figure 5.1a: Schematic showing chemical structure of RNA, 2'OMe modified RNA, and 'Locked nucleic acid' modified RNA.

Figure 5.1b: Schematic of Lipofection process [6].

Lipofection is a short-term solution: The most serious limitation of the lipofection technique is that the amount of inhibitor introduced to the cells is finite and it is eventually degraded, or diluted when the cells divide. Expression of a miRNA inhibitor in a line of cultured cells can be achieved by introducing a plasmid with the complimentary miRNA inhibitor sequence under a constitutively active promoter. The plasmid can be integrated into the genome, allowing long term inhibition, but the promoters on plasmids are usually silenced

over time. The time can be extended by using antibiotics to select for cells still expressing the genes on the plasmid.

Inhibitor RNAs can be designed with secondary structures to increase effectiveness: The inhibitor on the plasmid can be made more robust by introducing a longer RNA strand with secondary structure either side of the miRNA binding site, which leaves the inhibitor sequence open and able to interact with the RISC complex but protects the inhibitor from degradation [331]. The most common secondary structure is a short hairpin loop, which is known as a 'tough decoy' inhibitor [332]. Insertion of a 1-3 nucleotide region of non-complimentary RNA between bases 8 and 9 from the 3' end of the inhibitor sequence allows the RISC complex to form but prevents Ago2 from degrading the inhibitor, increasing the efficiency of down-regulation [332].

An alternative type of inhibitor is the RNA sponge, which is also cloned into a vector and transfected into the cell. miRNA sponges have multiple miRNA binding sites separated by short spacer sequences, allowing them to sequester many miRNA molecules. They can be efficiently generated and can be used to inhibit several different types of miRNA from the same family [333-335].

It is also possible to study the effects of loss of miRNA expression by knocking down a gene in a cell by using CRISPR [336-341] or TALENs [342, 343], however these methods are challenging as all copies of the miRNA-gene must be mutated without causing off site mutations, and the short length of these genes limits the possible target sites.

5. 1 Lentiviral transfections

miR-200c was chosen as the first target for down-regulation as it is a well-studied regulator of EMT. The first method chosen for miRNA inhibition was an inhibitor based on the tough decoy design, inserted into the cells using a lentiviral vector. Inhibitor plasmids are commercially available pre-packaged in lentiviruses with reporter genes and antibiotic resistance genes, allowing a rapid start to the experiments and immediate assessments of the efficiency of transfection. Initially a lentivirus with a miR-200c inhibitor, a Puromycin resistance gene and an mCherry reporter was purchased from Genecopoeia. Unfortunately transfection efficiency was low, even at extremely high multiplicity of infection (MOI) (see **Table 5.1**) in both H9 cells and the easier to transfect normal human dermal fibroblasts (NHDFs). MOI, polybrene (hexadimethrine bromide) concentration, percentage-confluence and time between passages were also altered in an attempt to increase the efficiency but at no

point was it possible to achieve a transfection efficiency of greater than 0.4% (see **Figures 5.2b and 5.2c**). Cells were expanded after selection with Puromycin but rapidly lost mCherry expression. Lentiviral transfection is specifically used for its high efficiency but there are several problems which can occur; cells must be healthy to survive infection, the virus itself must be competent and the plasmid, once inside the cell must have the opportunity to express its genes. Since it was never possible to get this virus to transfect efficiently, trouble-shooting was difficult, but it was possible to achieve efficient transfection with another lentivirus (see below), and neither the miR-200c inhibitor virus or the scramble control virus were effective, so it is more likely that the cause of the low tranfection efficiency was either the virus or the plasmid itself, rather than experimental technique or the properties of the cells. Lentiviruses are not stable if stored above -80^{o}C and are known to be extremely sensitive to freeze-thaw cycles [344] so it is possible that the viruses were damaged at some point when they were in storage. Alternatively, the cells may have rapidly silenced the promoters, which is known to happen in some cases with newly introduced plasmids [345, 346]. Either of these explanations is plausible.

Figure 5.2a

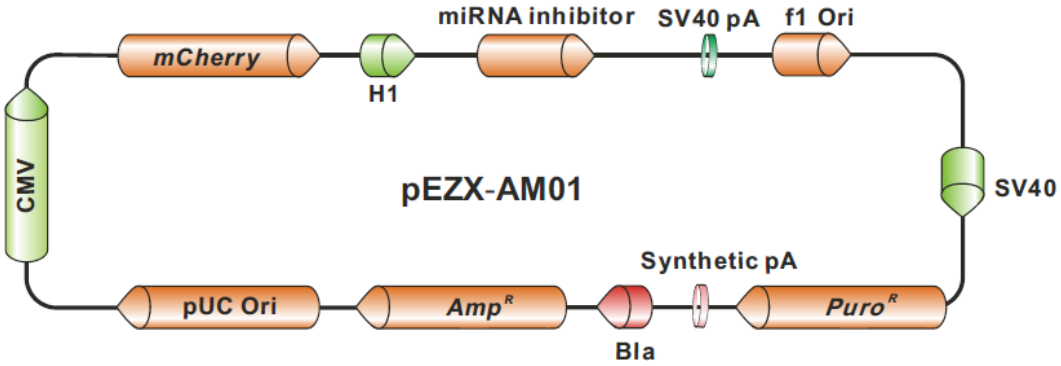


Figure 5.2b

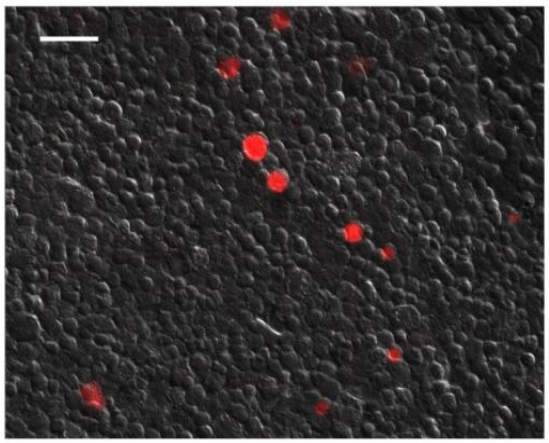


Figure 5.2c

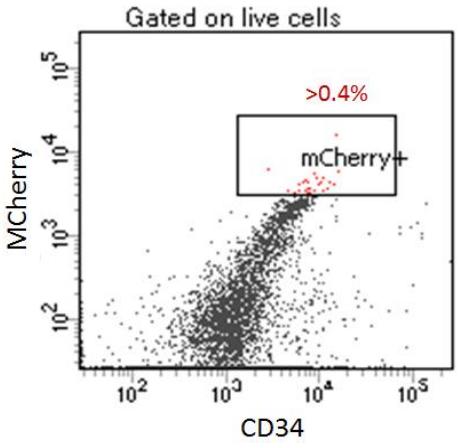


Table 5.1: Efficiency of transfection		
Polybrene (ug/ml)	MOI	% mCherry
5	100	0.0065
	200	0.0005
	300	0.034
8	100	0.034
	200	0.0415
	300	0.124
10	100	0.046
	200	0.0005
	300	0.026

Figure 5.2a: Schematic representation of genes and promoters on the Genecopoiea lentiviral plasmid (provided by the manufacturer)

Figure 5.2b: Representative image of NHDF fibroblasts transduced with lentivirus (MOI 300, 8µg polybrene/ml) showing mCherry reporter 48hr after transduction. Bar = 200µm

Figure 5.2c: Flow cytometry data showing mCherry expression in hematopoietic progenitors 48hr after transduction. MOI = 460, polybrene concentration = 2µg/ml

A second attempt was made with a SigmaAldrich MISSION^{TM} miR-200c tough decoy inhibitor lentivirus. This was more successful in that the transfection efficiency was much higher, based on Puromycin resistance after transfection. Up to 60% of cells survived, enabling qPCR analysis of miR-200c after transfection. However the expression was not significantly reduced when analysing expression 48hrs after transfection, even in cells which had been selected for with Puromycin (see **Figure 5.3a**). This means that although the cells had taken up the plasmid the inhibitor is either not being expressed or the design is not effective. The most likely explanation is that the promoter for the miRNA-inhibitor was silenced whereas the Puromycin resistance gene continued to be expressed. Most plasmid genes, including antibiotic resistance genes, are expressed under constitutively active, very strong RNA polymerase II promoters such as CMV or hPGK. However miRNA-inhibitors, due to their small size, are more effectively expressed under RNA polymerase III promoters such as U6 or H1 (see **Figures 5.2a, 5.3b**) [347]. Both types are promoters for constitutively expressed housekeeping genes, however genes on vectors introduced into hESCs are silenced in unpredictable ways depending on both the cell line transfected and the promoter which is used to drive gene expression [348]. It is also possible that the design of the inhibitor is flawed; the inhibitor is designed based on a proprietary algorithm.

Although lentiviral introduction of inhibitors is in theory an ideal method of inhibiting a miRNA's expression it did not appear to be effective in inhibiting our target miRNAs for technical or biological reasons so this approach was eventually abandoned.

Figure 5.3a

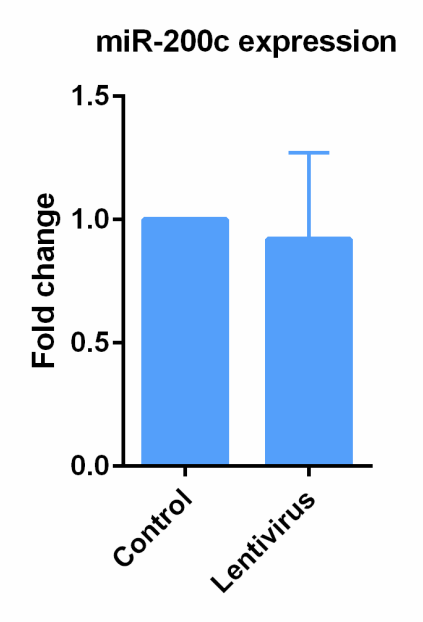


Figure 5.3b

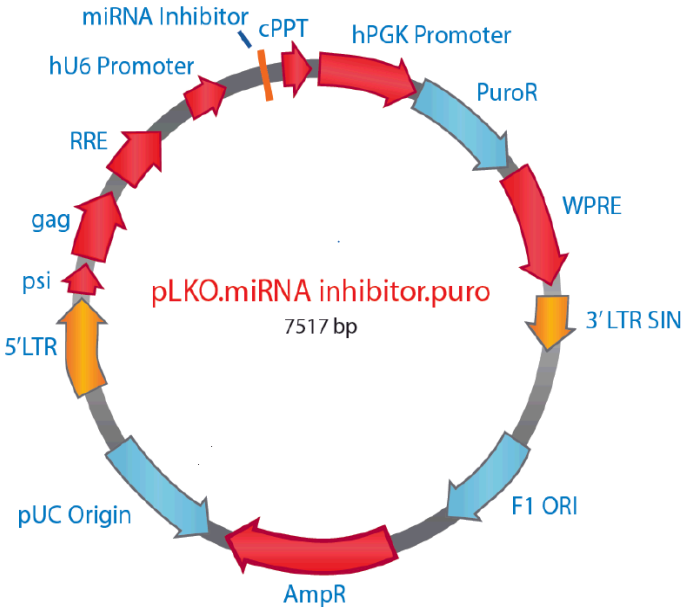


Figure 5.3a: qPCR expression analysis of miR-200c expression in puromycin selected day 12 SB-Ad3 cells which were transfected with a lentiviral mir-200c inhibitor at day 10. Calculated using ΔΔCT method, presented as a mean±SEM, n=3. Results with H9 cells and undifferentiated cells are comparable.
Figure 5.3b: Schematic representation of genes and promoters on the MISSION lentiviral plasmid, provided by the manufacturer.

5.2 Lipofection

Lipofection of small chemically modified miRNA inhibitors is one of the most well established techniques for studying the effects of miRNA down-regulation. The lipofection protocol (see **methods section 2.8**) was optimised on undifferentiated H9 cells by adjusting the ratio of cationic lipofectamine to negatively charged RNA. The half-life of the inhibition molecule *in vitro* is critical in lipofection, so after the ratio was optimised the length of time the inhibition lasted was analysed (see **Figures 5.4b, 5.4c**). In undifferentiated H9 cells the inhibition was very effective at reducing the expression of miR-200c for the first 48 hrs, after which it becomes less effective over time, and the effect is no longer significant after 96 hrs. The brief time-span of the effect meant that careful strategizing about the timing of inhibition was necessary. Although it is possible to perform multiple lipofections on the same cells at different times, long term exposure to lipofectamine can be toxic to the cells so this was avoided.

The results of the expression analysis of miRNAs suggested that the EHT is the critical period in differentiation of p-HSPCs, as so many of the miRNAs mis-regulated control EMT (see

section 4.2.1). Therefore miR-200c, a critical EMT regulator, was inhibited at a time point where the 48hr window of inhibition would have the maximum effect on the EHT process of definitive cells. Since definitive cells arise in small numbers starting at day 7 and reach maximum numbers at day 12 [8], inhibition was done at day 10 to allow the measurement of the effect at day 12.

At days 10-12 there are multiple distinct cell types in the culture, including committed endothelial cells, hemogenic endothelial cells, primitive progenitors, definitive progenitors and lineage committed hematopoietic progenitors (see flow cytometry results, **Section 5.3, Figure 5.7**). Some of these cells are in suspension, and some are adherent, which can affect the efficiency of transfection, and some may be proliferating at different rates, affecting the longevity of the effect. Since the main focus of this project is on the definitive hematopoietic lineage it was necessary to ensure that those cells in particular took up the inhibitor and maintained an acceptable level of inhibitor at the time-point when the cells were measured. A fluorescent-conjugated scramble-control (see **methods section 2.8**) was transfected into P-HPCs at day 10 and analysed by flow cytometry. Fortunately the FITC dye was present in 100% of cells after 24 hours and the effect holds in 96.8% of cells up to 48 hours (see **Figure 5.4a, 5.4b**) so we can analyse the effect of inhibition on the whole population of cells up to 48 hours after lipofection with confidence the inhibitor was introduced into the overwhelming majority of cells, including the definitive progenitors.

The effectiveness of the inhibitor was tested by measurement of miR-200c by qPCR after the inhibitor was transfected into undifferentiated pluripotent stem cells (**Figure 5.4c**). As with the fluorescent control, inhibition is strong at 48 hours but grows weaker over time.

Lipofection of mir-200c inhibitor at day 10 of differentiation was also successful, with qPCR analysis showing significant down-regulation in both H9 and SB-Ad3 cells at 48 hours after transfection, although most of the effect is lost by 96 hours (**Figures 5.4d-5.4g**), an effect equivalent to that in undifferentiated hESCs. There appears to be more variability between experimental repeats which are further from the time of transfection in all the different lipofection experiments which were done; it is not clear why this is but it may be due to cells in different experimental repeats proliferating at different rates.

This method was found to be effective and reliable so the rest of the project used the same method of lipofection with an inhibitor at day 10 and analysis of the cells 48 hours after.

Figure 5.4a

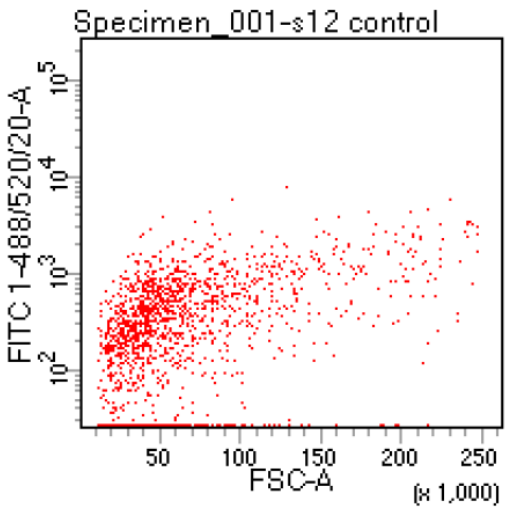


Figure 5.4b

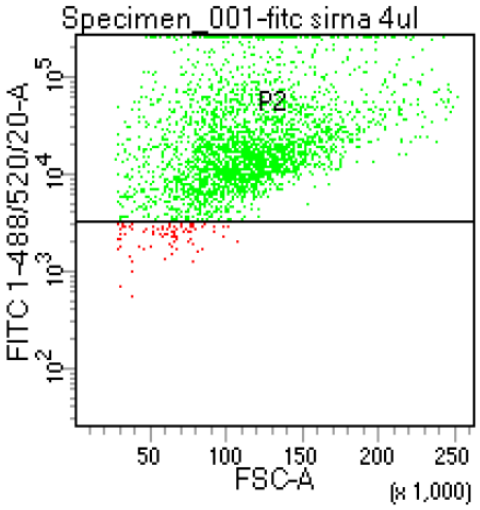


Figure 5.4c

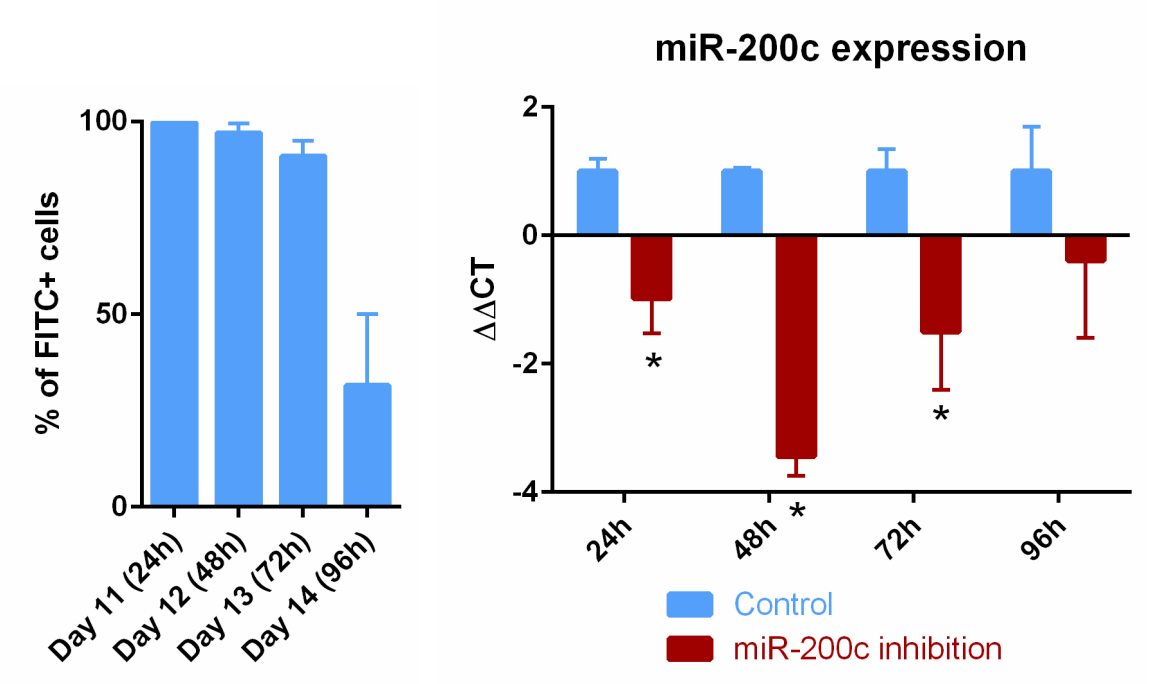


Figure 5.4a: Flow cytometry data showing FITC fluorescence in control and FITC transfected H9 day 12 cells. **5.4b:** Flow cytometry data showing % FITC fluorescent H9 cells at different timepoints after lipofection with FITC-conjugated control siRNA. Data is shown as mean±SEM, n=3. Day = day of differentiation, hour = hours post lipofection.

5.4c: qPCR expression analysis of mir-200c in undifferentated H9 cells at 24, 48, 72 and 96 hours after lipofection. Data is shown as mean±SEM, n=3.

Figure 5.4d

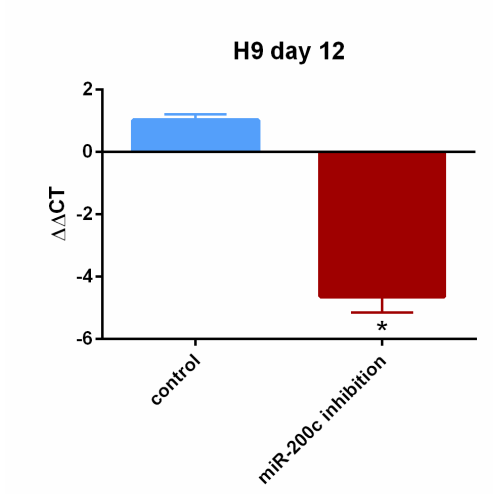


Figure 5.4e

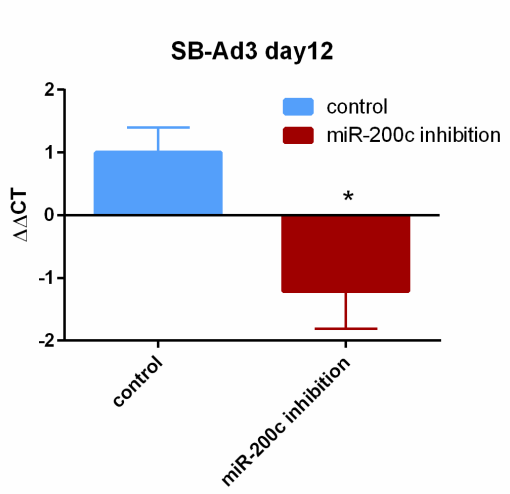


Figure 5.4f

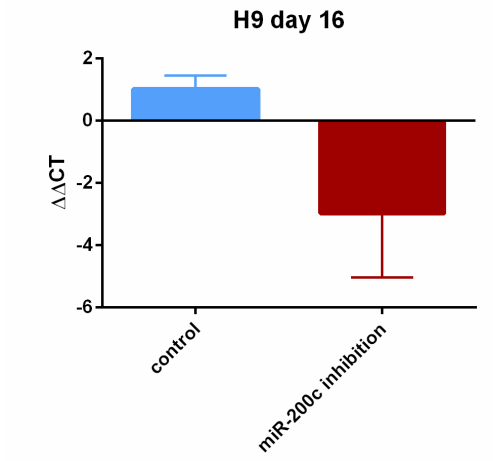
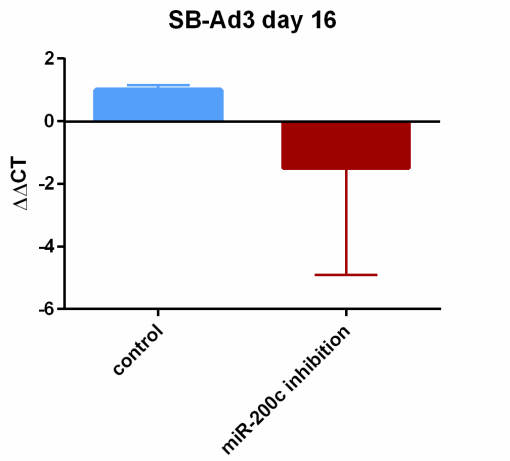


Figure 5.4g



5.4d-5.4g qPCR expression analysis of mir-200c in day 12 and day 16 differentiated H9 and SB adult 3 cells which were lipofected with a mir-200c inhibitor at day 10. Data is shown as mean±SEM, n=3.

5.3 Effects of miRNA inhibition

Once an effective method for inhibiting miR-200c in hematopoietic progenitors was established the same method could also be used for inhibiting other group B miRNAs which were chosen as potential targets in **results section 4**. The miRNAs chosen were miR-148a, miR-200b, miR-200c, miR-205 and miR-424. The other candidates for inhibition, miR-9, miR-134, miR-150, miR-150* were excluded as these miRNAs are less involved in EHT. The main focus of this project was on EHT-supressing miRNAs because as shown in **results section 4.2**, these miRNAs represent the largest group of mis-regulated miRNAs and therefore likely the most important mechanism preventing P-HPCs from functioning

effectively, as well as providing a clear mechanistic explanation for their failure to home to the niche.

The chosen miRNAs all have validated functional roles in the process of EMT, often inhibiting some of the same genes, and these overlapping functions mean that it is likely that there is some redundancy between these miRNAs, so miRNA inhibitions were carried out both individually and in combination with others. miR-200b and miR-200c inhibitors were introduced to the cells together due to their high level of sequence homology making them the most likely to have functional overlap. Experiments where all 5 miRNA inhibitors were introduced to the cells at once were used to determine if the effect of inhibition would be greater when all the inhibitors were combined.

miRNA inhibition: The effectiveness of the inhibitor molecules was analysed by measuring the quantity of their target miRNAs by qPCR (see **Figure 5.5**). This was done after both individual miRNA inhibition and combined inhibition, as there was some concern that attempting to inhibit multiple miRNAs at once would decrease the efficiency of inhibition. The inhibitors were all found to be very effective at reliably inhibiting their target miRNAs. Interestingly multiplex inhibitions don't down-regulate miRNAs to the same level as the individual inhibitions. miR-205 appears to be inhibited less efficiently when combined with other inhibitors, but the miR-200 group are even more down-regulated when combined with the other inhibitors. This suggests that there is some co-regulation of the inhibitors. Papers on the function and regulation of these miRNAs indicate that they are part of a complex regulatory network, with multiple feedback loops (see **Figure 5.9**). As many of these miRNAs regulate some of the same genes it appears that inhibiting one can have an impact on the expression of the others. Although the effect of an individual miRNAs expression on any one gene is usually small, effects of changing the expression levels of more than one can change the precise balance of gene and miRNA expression on a larger scale, which is positive in that it suggests that manipulation of miRNA expression may be a viable strategy for altering cell phenotype/fate/functionality, however it is also concerning as the effects of inhibition on the expression of other miRNAs are not immediately predictable.

Figure 5.5

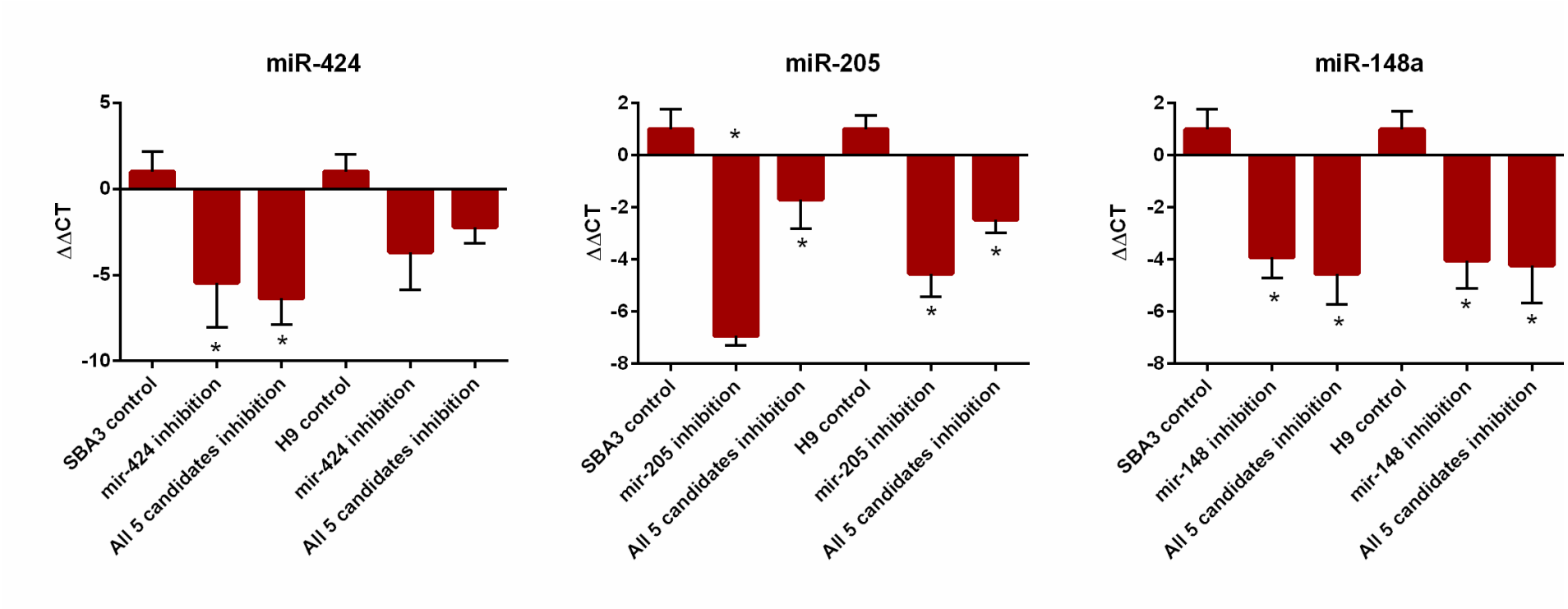

miR-424
ΔΔCT
5
0
-5
-10
*
*
SBA3 control
mir-424 inhibition
All 5 candidates inhibition
H9 control
mir-424 inhibition
All 5 candidates inhibition
miR-205
ΔΔCT
2
0
-2
-4
-6
-8
*
*
*
*
SBA3 control
mir-205 inhibition
All 5 candidates inhibition
H9 control
mir-205 inhibition
All 5 candidates inhibition
miR-148a
ΔΔCT
2
0
-2
-4
-6
-8
*
*
*
*
SBA3 control
mir-148 inhibition
All 5 candidates inhibition
H9 control
mir-148 inhibition
All 5 candidates inhibition

Figure 5.5

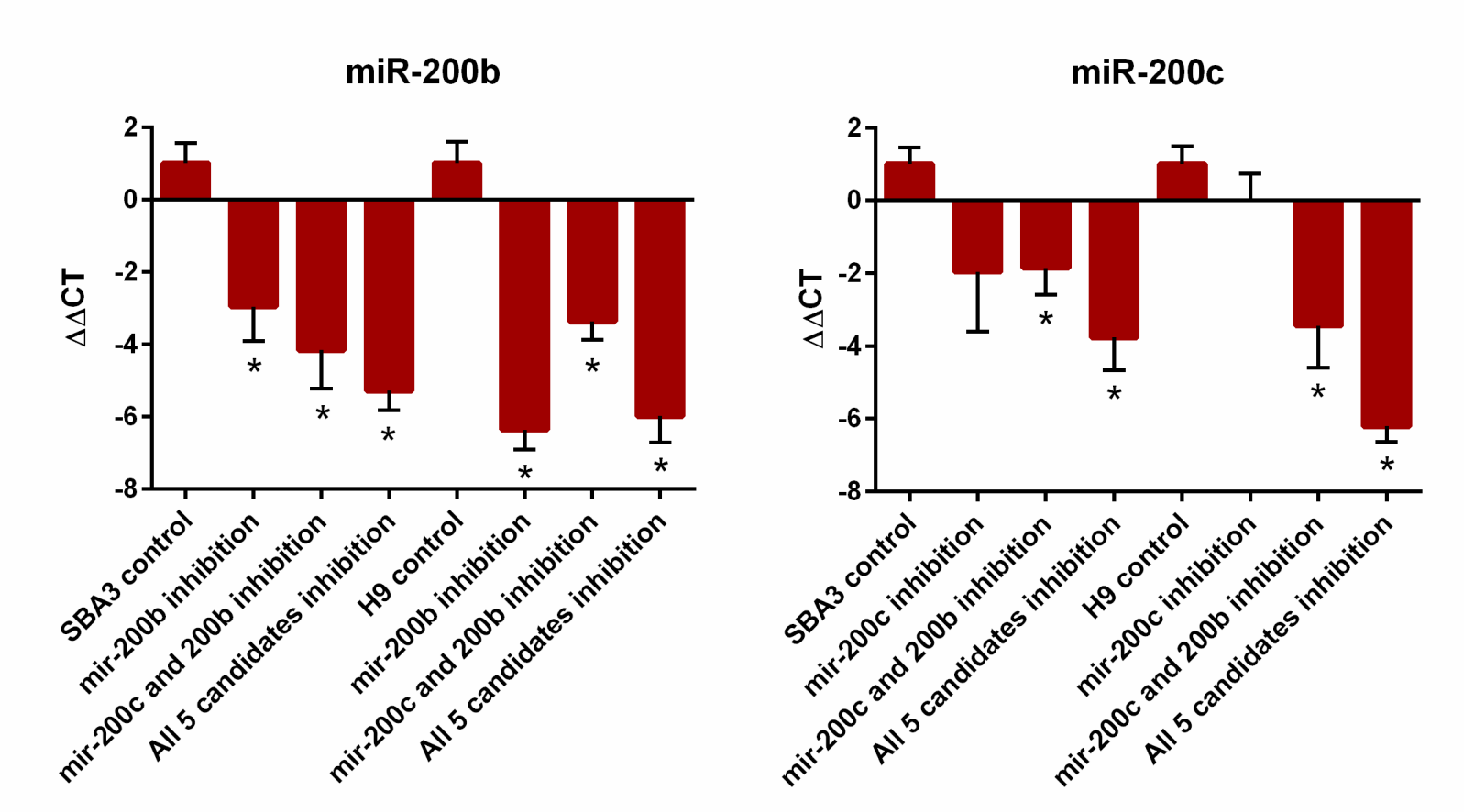


Figure 5.5: qPCR data showing miRNA expression in hiPSCs and hESCs at day 12, 48hr after inhibition. Data is shown as mean ΔΔCT values ±SEM, n=3.

The cells were analysed for changes in hematopoietic potential at day 12, 48 hours after inhibition of the selected miRNAs. Their hematopoietic colony forming potential, cell surface marker expression and gene expression were assessed.

Colony forming assays and flow cytometry: The results of the colony forming assays show no significant improvement in hematopoietic development (see **Figure 5.6**). Most of the inhibitions show no significant changes, although as would be expected, the largest change comes after inhibition of all 5 miRNAs in one population of cells. Few colony types are significantly different in cells inhibited with any of the miRNAs, in fact the only significant change which occurs in both cell lines is in CFU-GEMM potential, which is significantly decreased in both hESCs and hiPSCs. This is the opposite result to what was hoped for as it indicates a decrease in the fraction of multi-potent hematopoietic progenitors.
The results of these experiments have less statistical power than is ideal (see **results section 3.5**) so it is possible that the apparently statistically insignificant differences seen in the other colony types are real decreases, or that the inhibition of these miRNAs is having an effect on CFU-GEMMs in particular. This may be because the inhibited miRNAs are themselves inhibitors of hematopoietic differentiation (see **section 4.2**), causing the earliest progenitors to spontaneously differentiate, or it may be that the combined effect is preventing them from differentiating at all.
Flow cytometry shows no significant changes in either the megakaryocyte erythroid progenitors, multilineage myeloid progenitors or definitive hematopoietic progenitor populations after miRNA inhibition (see **Figure 5.7**), consistent with the CFU results.

Gene expression: Following miRNA inhibition the expression of known target genes was analysed by qPCR. A miRNA can have up to thousands of predicted target genes so several criteria were used to choose which genes to analyse: They had to be validated targets of one or more of the inhibited miRNAs, and to have known relevance to either EMT or EHT, as this is the key cellular process which was identified in **results section 4** as being regulated by the aberrantly expressed miRNAs. Given the robust down-regulation of the miRNAs you would expect to see some up-regulation of their target genes. Many of the genes show an increase (see **Figure 5.8**), but it is small and unfortunately there is considerably more variability between repeats of these qPCR experiments than the ones analysing miRNA expression, in spite of them both being derived from the same RNA samples in most cases. Few genes show an increase that is statistically significant, which explains the lack of dramatic changes in the cell surface markers and CFU assays.

Figure 5.6a SB–Ad3 cells

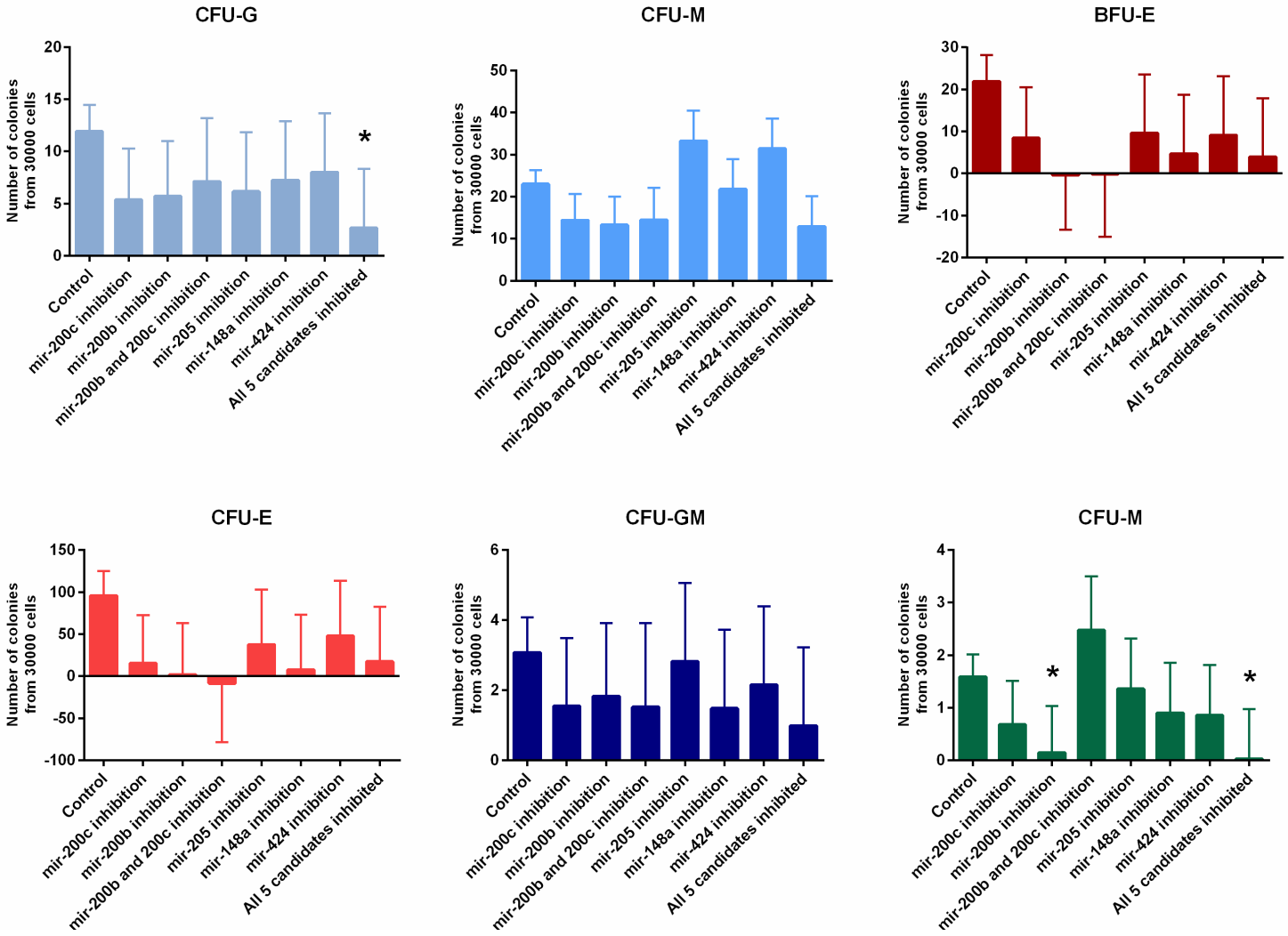


Figure 5.6b H9 cells

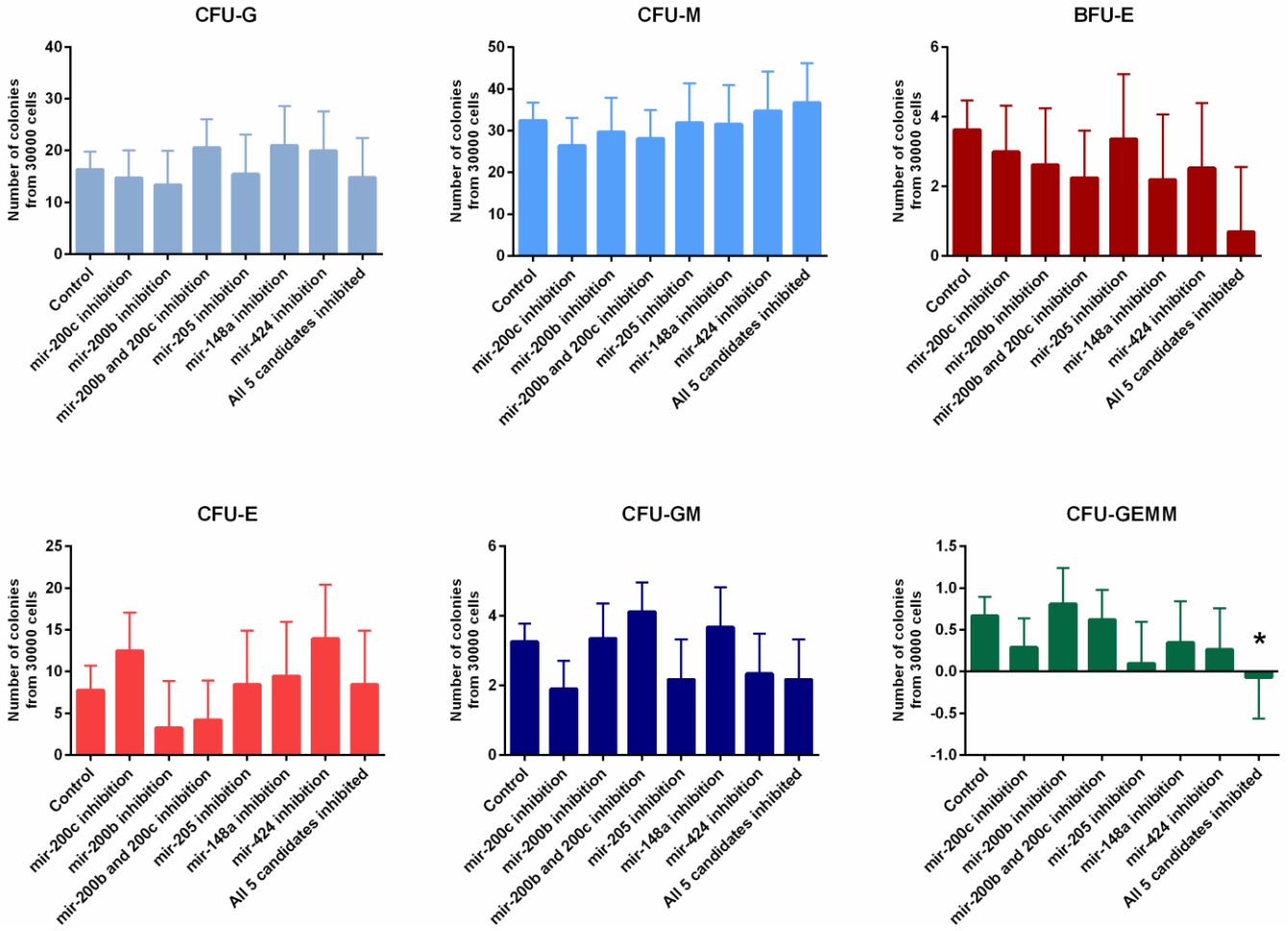


Figure 5.6: Estimated marginal means ±SEM for CFU made from day 12 pluripotent stem cell derived hematopoietic progenitors **a:** SB-Ad3 cells, **b:** H9 cells, n=3. See **methods section 2.10** for how EMMs are calculated.

Figure 5.7

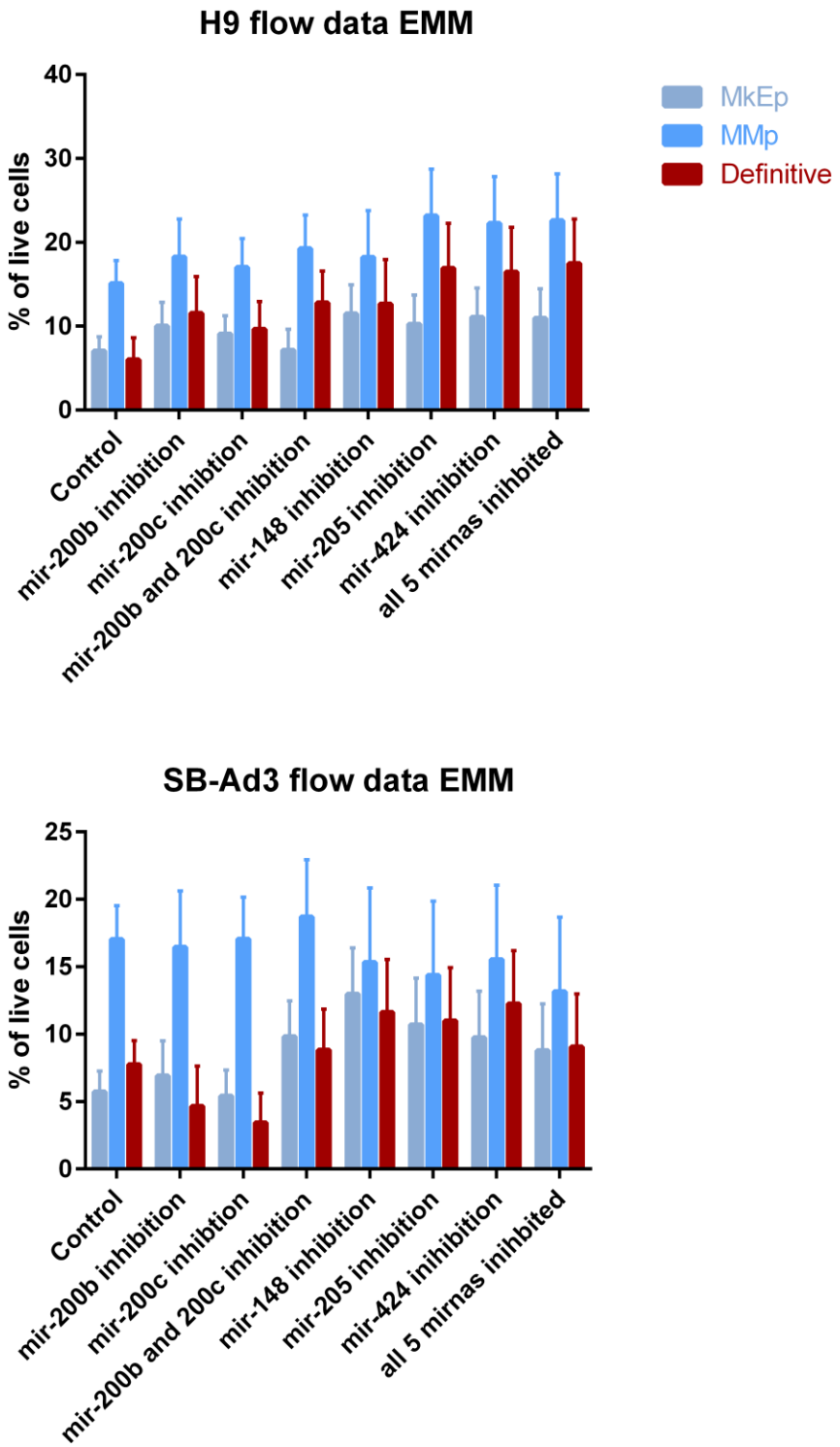
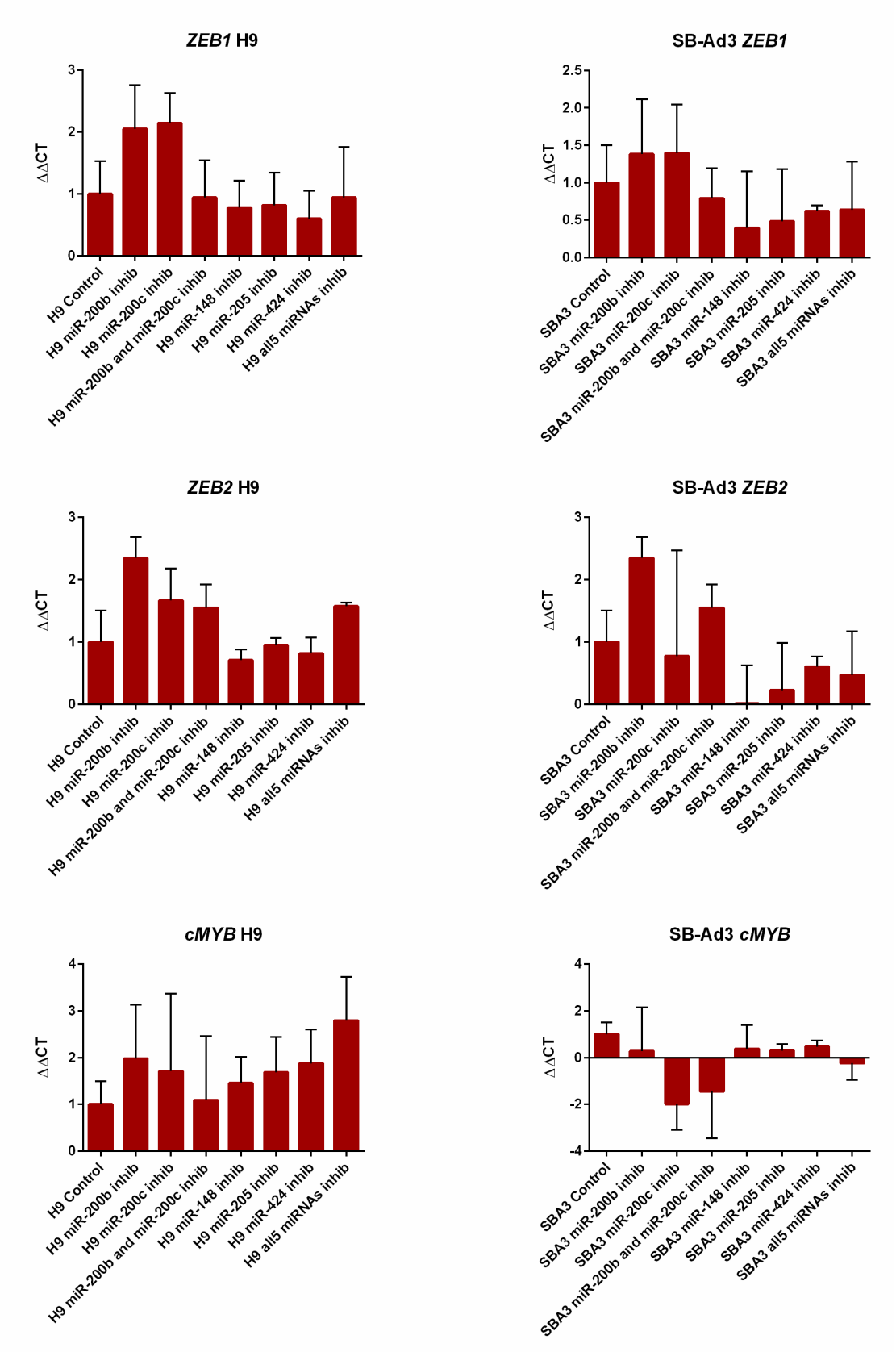
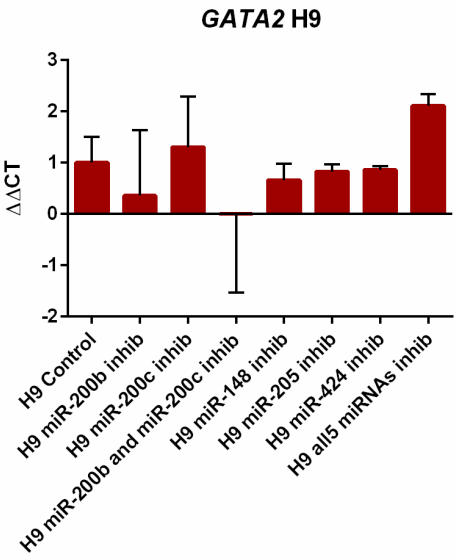
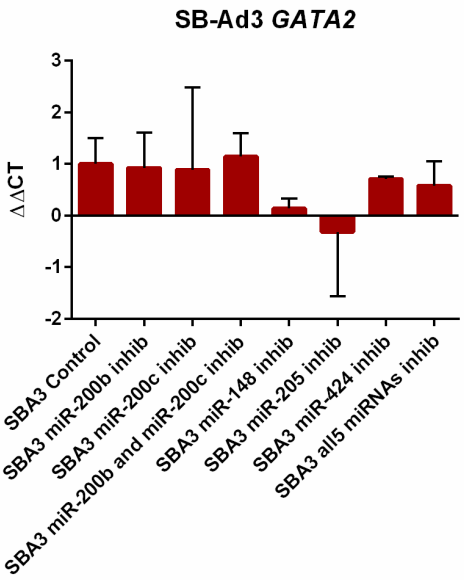


Figure 5.7: Graphs showing percentage of live cells expressing MkEP (megakaryocyte erythroid progenitors) = CD43+CD34+CD235+CD41a+CD45-; MMp (multilineage myeloid progenitors) = CD43+CD34+CD235-CD41a-CD45-; Definitive hematopoietic progenitors = CD43+CD34+/-CD235-CD41a-CD45+. Data is shown as estimated marginal means ±SEM , n=3.

Figure 5.8


ZEB1 H9
ΔΔCT
H9 Control
H9 miR-200b inhib
H9 miR-200c inhib
H9 miR-200b and miR-200c inhib
H9 miR-148 inhib
H9 miR-205 inhib
H9 miR-424 inhib
H9 all5 miRNAs inhib
SB-Ad3 *ZEB1*
SBA3 Control
SBA3 miR-200b inhib
SBA3 miR-200c inhib
SBA3 miR-200b and miR-200c inhib
SBA3 miR-148 inhib
SBA3 miR-205 inhib
SBA3 miR-424 inhib
SBA3 all5 miRNAs inhib
ZEB2 H9
SB-Ad3 *ZEB2*
cMYB H9
SB-Ad3 *cMYB*


GATA2 H9
ΔΔCT
3
2
1
0
-1
-2
H9 Control
H9 miR-200b inhib
H9 miR-200c inhib
H9 miR-200b and miR-200c inhib
H9 miR-148 inhib
H9 miR-205 inhib
H9 miR-424 inhib
H9 all5 miRNAs inhib


SB-Ad3 GATA2
ΔΔCT
3
2
1
0
-1
-2
SBA3 Control
SBA3 miR-200b inhib
SBA3 miR-200c inhib
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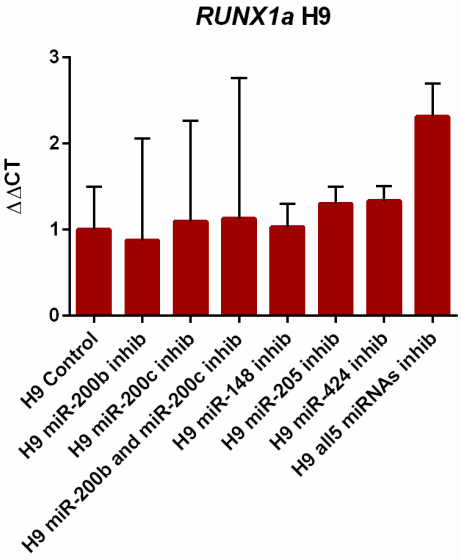
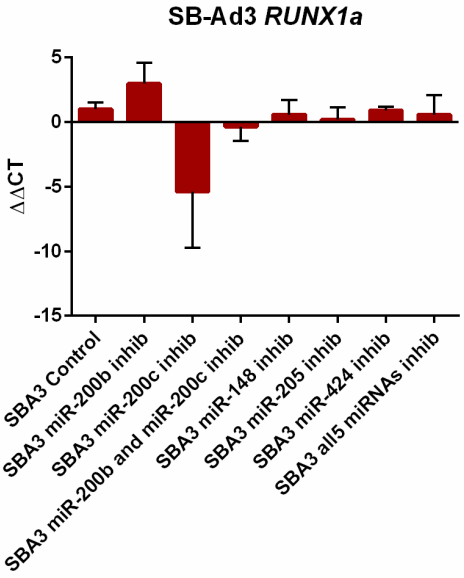
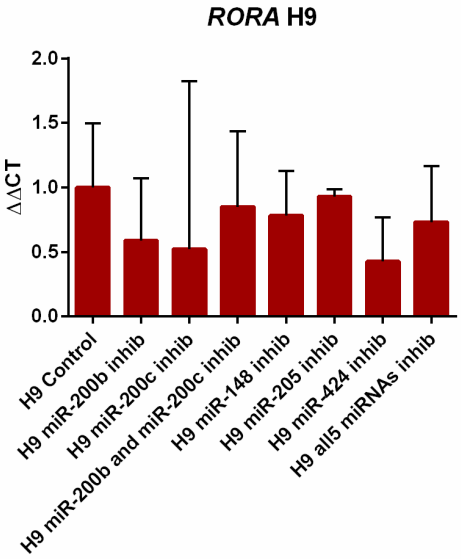
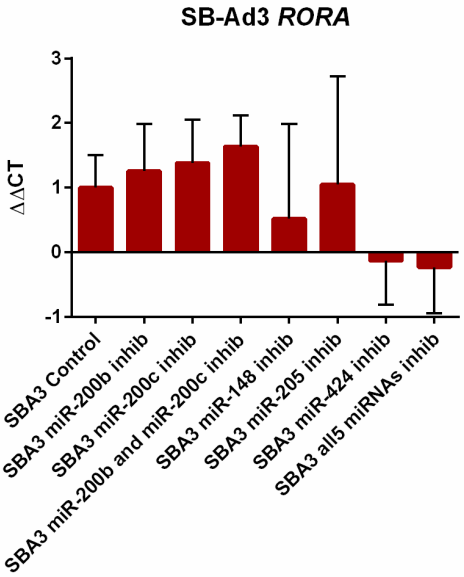
SBA3 miR-424 inhib
SBA3 all5 miRNAs inhib


RUNX1a H9
ΔΔCT
3
2
1
0
H9 Control
H9 miR-200b inhib
H9 miR-200c inhib
H9 miR-200b and miR-200c inhib
H9 miR-148 inhib
H9 miR-205 inhib
H9 miR-424 inhib
H9 all5 miRNAs inhib


SB-Ad3 RUNX1a
ΔΔCT
5
0
-5
-10
-15
SBA3 Control
SBA3 miR-200b inhib
SBA3 miR-200c inhib
SBA3 miR-200b and miR-200c inhib
SBA3 miR-148 inhib
SBA3 miR-205 inhib
SBA3 miR-424 inhib
SBA3 all5 miRNAs inhib


RORA H9
ΔΔCT
2.0
1.5
1.0
0.5
0.0
H9 Control
H9 miR-200b inhib
H9 miR-200c inhib
H9 miR-200b and miR-200c inhib
H9 miR-148 inhib
H9 miR-205 inhib
H9 miR-424 inhib
H9 all5 miRNAs inhib


SB-Ad3 RORA
ΔΔCT
3
2
1
0
-1
SBA3 Control
SBA3 miR-200b inhib
SBA3 miR-200c inhib
SBA3 miR-200b and miR-200c inhib
SBA3 miR-148 inhib
SBA3 miR-205 inhib
SBA3 miR-424 inhib
SBA3 all5 miRNAs inhib

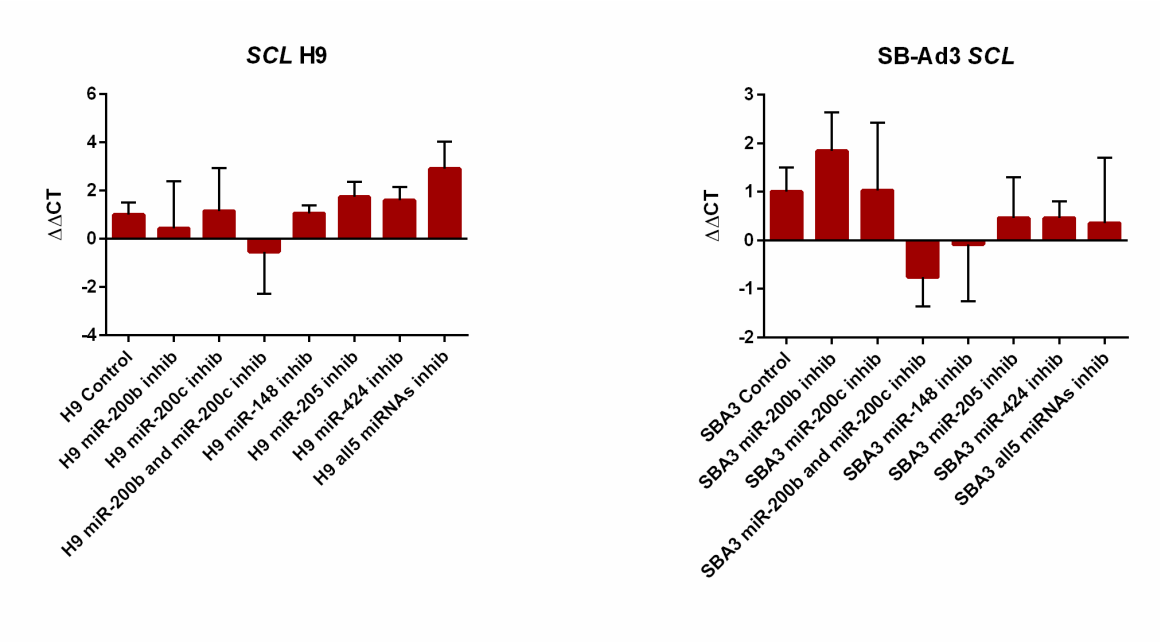


Figure 5.8: qPCR data showing gene expression in hiPSCs and hESCs at day 12, 48hr after inhibition. Data is shown as mean ΔΔCT values ±SEM, n=3.

The robust change in miRNA expression has resulted in very small changes in phenotype and gene expression. Large changes in gene expression are not expected after miRNA inhibition because miRNAs function as 'buffers' or 'fine tuners' of gene expression, rather than as master regulators [349, 350]. It is also important that there were many other mis-regulated miRNA with similar functions, (i.e. EMT suppressors, see **results section 4.2.1**). Stabilising the cells in the epithelial or mesenchymal state is one of the best studied and most important functions of miRNA, and there are dozens of miRNAs which are known to have a role in preventing EMT [351], so inhibiting as few as five may not have been enough to overcome the factors stabilising the cells in an adherent endothelial/epithelial phenotype.

Redundancy between different miRNAs controlling the same process is common enough that miRNA knockdown mice often have no phenotype [352, 353] and this is recognised as one of the principal challenges in the field of miRNA research. Epithelial cells usually benefit from maintaining a stable phenotype, EMT is a major factor in oncogenesis and metastasis, and EMT suppressing miRNAs may be one of the most important factors in maintaining this stability and preventing tumourigenesis. One of the ways that miRNAs are able to do this is though auto-regulatory feedback loops, for example the miR-200 group inhibits *ZEB1* and *ZEB2*, and these transcription factors inhibit miR-200 expression [354]. **Figure 5.9** shows the complexity of the genetic network which governs miR-200c expression alone, this network of factors means that the change in expression of only one factor may not be enough to affect the

overall phenotype.

Figure 5.9

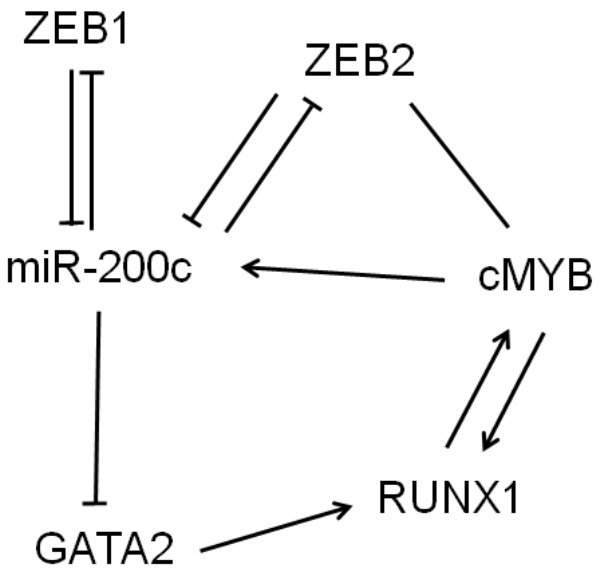


Figure 5.9: Schematic of relationships between genes regulating miR-200c

6. Effect of hypoxia on hematopoietic differentiation

Hypoxia plays a key role in many of the cellular processes involved in this project, including HSC maintenance, hematopoietic differentiation, EMT and the regulation of many miRNAs. Studying the role of hypoxia in *in vitro* hematopoietic differentiation may help to improve the function of HSCs produced from pluripotent stem cells.

There are two possible mechanisms by which hypoxia might improve the generation of multipotent hematopoietic progenitors. Firstly, hematopoiesis requires VEGF pathway activation and although VEGF is a cytokine which is present in the media throughout differentiation it is possible that it is more effective to activate this pathway endogenously. *HIF-1α* directly induces endogenous expression of several receptors and ligands in the VEGF pathway [355] in addition to multiple other hematopoeisis promoting pathways including Notch, EPO and CEBP signalling (reviewed by Liu et al 2012 [356]).
Secondly hypoxia is a well established promoter of EMT [356, 357], which my miRNA data suggests is the primary point-of-failure in *in vitro* hematopoietic differentiation (**results section 4**). It was hoped that *HIF-1α,* by acting as a transcription factor for EMT promoting factors would indirectly reduce the expression of EMT blocking miRNAs. This might be more effective than inhibiting individual miRNAs because hypoxia is known to produce a global effect in terms of gene expression so there is less potential for functional redundancy between miRNAs reducing the effect of inhibition. *HIF-1α* is known to have numerous direct miRNA targets including miR-200b and miR-424 [398].

***HIF-1α* mediates cells' response to hypoxic conditions:** Cells must be able to sense hypoxia and respond by making adjustments to their metabolism and gene expression. *HIF-1α* is the master transcription factor responsible for this process. It is constitutively expressed but is rapidly broken down if oxygen levels are normal [358, 359]. Under normoxic conditions oxygen-dependent proteins hydroxylate prolyl residues on the *HIF-1α* protein, allowing von Hippel Lindau factor to ubiquitinate it [359-361]. In hypoxic conditions *HIF-1α* is stabilised and forms a heterodimer with *HIF-1 β*, and the complex acts as a transcription factor regulating hundreds of genes [358]. *HIF-1α* stabilisation has a global effect on both gene and miRNA expression, affecting many cellular processes including glucose metabolism [362], the cell cycle [363], angiogenesis [364], morphogenesis [365, 366], inflammation [367], and hematopoiesis [367].

6.1 The role of *HIF-1α* in HSC biology

There are several compelling lines of evidence that *HIF-1α* is critical in the maintenance of HSC. The HSC niche is hypoxic, with HSC clustering in the most hypoxic areas of the bone marrow, as shown by pimonidazole staining [368, 369], *HIF-1α* expression [368], and direct measurement of oxygen tension in the bone marrow [370]. *HIF-1α* expression is important for the maintenance of the HSC population; HSC differentiation occurs along an increasing gradient of increasing oxygen tension [371] and stabilisation of *HIF-1α* increases HSC quiescence and survival [372].

The key effect of *HIF-1α* is on cellular metabolism: Hypoxia switches metabolism from mitochondrial-dependent oxidative phosphorylation to glycolysis, which is less efficient but requires less oxygen and produces fewer potentially mutagenic reactive oxygen species. HSC have a glycolytic metabolism which aids in maintaining a stable genome and is essential for keeping them in an undifferentiated and quiescent state [373-375]. Preventing mitochondrial respiration completely blocks HSC differentiation [376, 377].

HIF-1α can sometimes be stabilised even under normoxic conditions by cytokine signals such as SCF [378] and TPO [379] and this aids in maintaining the stem cell state when HSC leave the bone marrow niche [380]. This makes the picture more complex as it shows that *HIF-1α* is controlled by factors other than hypoxia, but it underscores the importance of *HIF-1α* in HSC biology. Danet et al. have shown that hypoxia also helps in maintaining HSC *ex vivo* [381, 382].

Most interesting from the point of view of those trying to produce HSCs from pluripotent stem cells is that hypoxia has also been shown to be important in hematopoietic development in the embryo: *HIF-1α* expression is essential for HSC formation in zebrafish [383] and murine [384] embryos. *HIF-1α* is also an essential factor in HSC mobilisation [355, 385] which makes it a potential target for improving the motility of P-HPCs.

6.2 Hypoxia promotes EHT

Hypoxia is important for the maintenance and production of HSC *in vivo*, but it may also be helpful in the specific problem of producing HSC *in vitro* from pluripotent cells because it is likely to encourage the process of EHT, which has been identified as the major block in P-HPC production (see **results section 4**). Hypoxia promotes EMT [386, 387], which is much like EHT in that it involves dramatic changes; for example cell-cell junctions are removed and motility is increased, the cell changes from a flat, polarised cell morphology to a rounder, non-polar one and proliferation and resistance to apoptosis are increased. miRNA data from

this project shows aberrant high expression of miRNA which are known EMT inhibitors (see **results section 4**) and hypoxia might potentially reduce the expression of these miRNAs.

EHT is also a similar process to angiogenesis. Angiogenesis is the process by which blood vessels expand their reach, primarily in response to signals emitted by hypoxic tissues. Endothelial cells become less adhesive and more proliferative and migrate towards the hypoxic tissue. Hematopoietic cells and endothelium have a common evolutionary [388] and developmental [74] origin and both angiogenesis and hematopoietic development are controlled by the same pathways and signalling molecules [389, 390]. For example the VEGF pathway, which is activated by *HIF-1α* is essential for both angiogenesis [391] and hematopoietic development [392]. The NF-KB pathway [393-395], EPO [396, 397] and GM-CSF [390] are also essential for both hematopoietic development and angiogenesis, so it's reasonable to hypothesise that a condition which promotes angiogenesis might also promote hematopoiesis.

Given the connection between hypoxia and EHT it is possible that hypoxia would improve the ability of hESC and hiPSC to produce functional hematopoietic progenitors. This might be more effective than inhibiting individual miRNAs because hypoxia is known to produce a global effect in terms of gene expression so there is less potential for functional redundancy between miRNAs reducing the effect of inhibition. *HIF-1α* is known to have numerous direct miRNA targets including miR-200b and miR-424 [398].

6.3 Measuring the effect of hypoxia on hematopoiesis

The effect of hypoxia on hematopoiesis was tested on both the hESC and hiPSC cell lines. $CoCl_2$ is commonly used by researchers to mimic the effect of hypoxia [399], and is more practical to use for many experiments, as different concentrations can be tested in parallel and a specialised hypoxic incubator is not needed. Cobalt mimics hypoxia by binding to *HIF-1α* and preventing its ubiquitination [400] but since hypoxia may affect the P-HPCs by *HIF-1α* independent mechanisms [401] and $CoCl_2$ can potentially affect other cellular pathways [402] differentiation was also done in a hypoxic incubator at 5% O_2 so as to test the effect of true hypoxia. The effect on hematopoietic differentiation was tested using the same techniques described in previous chapters: colony forming assays, flow cytometry and qPCR of gene and miRNA expression.

Preliminary tests were done to optimise the concentration of $CoCl_2$ and the time-points at which it should be added to the differentiation cultures. These tests found that while $CoCl_2$ was present in the culture throughout development from day 0 until they were analysed at day

12 strong measurable effects on numbers for hematopoietic progenitors were seen, but there was little effect at all when $CoCl_2$ was added or removed at other time-points during development. It was also found that the concentration which had the maximum effect on numbers of hematopoietic progenitors was 0.1ug/ml $CoCl_2$ (data not shown). Therefore the analysis in this chapter was done on cells which were exposed to either 5% O_2 or 0.1 µg/ml $CoCl_2$ throughout development.

The effect of hypoxia on *HIF-1α* expression was tested by qPCR (Figure 6.1). The 5% O_2 condition shows a slight increase in *HIF-1α,* a larger increase would not be expected as *HIF-1α* is constitutively expressed and is mostly regulated by oxygen levels in a post-transcriptional way, by degradation or stabilisation of the protein [358, 359].

Figure 6.1

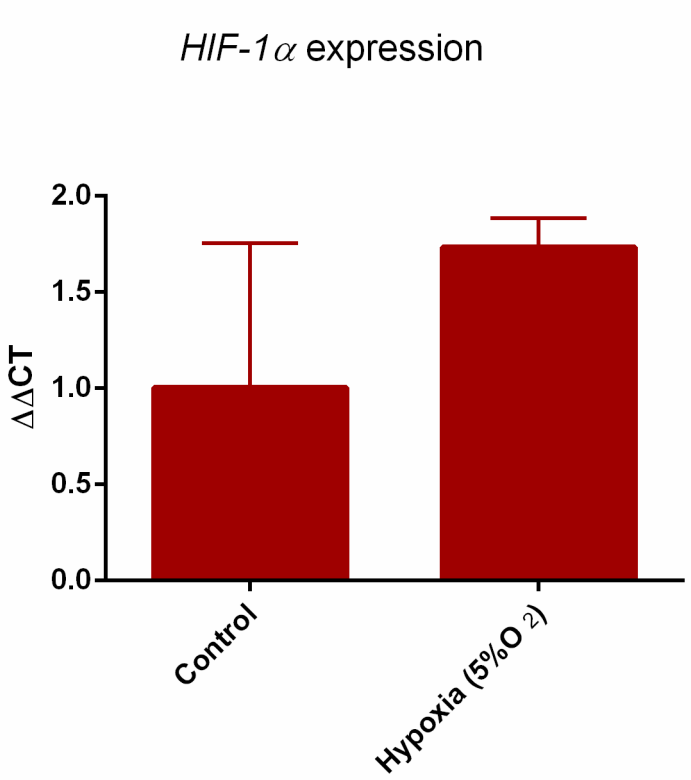


Figure 6.1: graph showing relative *HIF-1α* expression in induced pluripotent stem cells differentiated to day 12 in normoxia and hypoxia conditions. Expression calculated using the ΔΔCT method.

CFU assays show increases in hematopoietic progenitors after treatment with hypoxia or $CoCl_2$: Both cell lines show an overall increase in the number of hematopoietic colonies produced by both the hypoxic (5% O_2) and hypoxia mimic ($CoCl_2$) conditions (see **Figure 6.2**). The increase in the number of colonies is comparable for both lines, the apparent

differences shown in the chart are due to the figures being adjusted for passage number (see **results chapter 3.3**). When this data is broken down by colony type the results are more interesting, showing different effects for the two different conditions (**Figure 6.3**). Hypoxia produces a greater number of erythroid colonies, both BFU-E and CFU-E. Although the increase isn't statistically significant in all the experiments it appears to be consistent across both cell lines. This is completely unsurprising as hypoxia is one of the major drivers for erythropoiesis *in vivo*. Hypoxia also promotes a significant increase in CFU-M across both cell lines, which is a less intuitive result. However it is the $CoCl_2$ induced increase in the numbers of CFU-GM and especially CFU-GEMM which are the most interesting for this project because numbers of multi-potent progenitors can be considered stand-ins for HSCs (see **results section 3.2.3**). Both cell lines show a greater than two-fold average increase in CFU-GEMM numbers in cells treated with $CoCl_2$.

$CoCl_2$ and hypoxia have different effects: $CoCl_2$ appears to have very little effect on erythropoiesis, and a much greater effect on the numbers of CFU-GM and CFU-GEMMs, whereas the results from the experiments where the cells were treated with hypoxia show the opposite. It is possible that this is due to the differences in biological action described above, but it would be unlikely to have so different an effect. It is more likely that the concentration of $CoCl_2$ used for these experiments corresponds to a different concentration of oxygen than the 5% used in the incubator. Blood cells regulate their response to O_2 levels very precisely and are likely to respond differently to different levels, for example HSC are stable at the slightly hypoxic physiological levels of O_2 concentration typically found in the bone marrow but will respond urgently to unusually O_2 low levels by producing large numbers of erythrocytes.

Effect of hypoxia on cell surface marker expression: The results of the flow cytometry experiments using the Kennedy 2012 markers are not clear. From the CFU data you would expect to see more CD235a+ cells in the hypoxia treated cell population as this is an erythroid marker, but this does not appear to be the case for either cell line (see **Figure 6.4a**). Nor do either hypoxia or $CoCl_2$ treated populations have a higher proportion of hematopoietic cells overall. The $CoCl_2$ treated SB-Ad3 cells have a significantly higher proportion of definitive progenitors, and the H9 $CoCl_2$ treated cells have a significantly higher proportion of megakaryocyte erythroid progenitors and a significantly lower proportion of multi-lineage myeloid progenitors, but none of these results are replicated in the other cell line. The cell surface markers may not correspond to the colonies formed because the colonies have been

cultured in a different cytokine mix for a further 14 days, but overall the results of this experiment are inconclusive.

However, since there was a clear increase in the number of CFU-GEMM colonies in the $CoCl_2$ treated cells they were also analysed for the markers described by Notta et al. as being specific for LT-HSC [159]. There is a lot of variation in the numbers of this rare cell population but the H9 cells showed an increase of over 2-fold in $CoCl_2$ treated cells (see **Figure 6.4b**). The results for the SB-Ad3 cells are skewed by a major outlier, but aside from this they also show an increase of a similar amount. Although I have not demonstrated that the pluripotent-derived cells expressing this combination of markers are LT-HSCs capable of long term engraftment, this is an increase of a similar amount to the increase in CFU-GEMMs and supports the colony assay data which suggests that treating cells with $CoCl_2$ during hematopoietic differentiation can increase the number of progenitors with multi-potent hematopoietic potential.

miRNA and gene expression during hypoxic hematopoietic differentiation: miRNA expression of the key miRNAs which were chosen for inhibition in **results chapter 4** was analysed, in the hope that hypoxia might bring their expression more into line with the expression in cord blood and bone marrow hematopoietic progenitors. Unfortunately the effect on most of the miRNA was small, with only miR-148a being significantly affected, when exposed to hypoxic conditions (see **Figure 6.5**). The lack of overall change in miRNA expression suggests that the positive effect that $CoCl_2$ and hypoxia have on colony numbers is due more to the effect of stabilising progenitors against differentiation rather than enhanced EHT. Much like the miRNA expression, hypoxia had much less effect on the expression of hematopoietic genes than was expected, none of them were significantly changed (see **Figure 6.6**).

Although these experiments didn't produce the results that were expected in terms of changing gene or miRNA expression the considerable increases in CFU-GM and CFU-GEMM produced by $CoCl_2$ exposure are a promising result and this technically simple method of boosting the numbers of multi-potent progenitors may be helpful in future experiments.

Figure 6.2

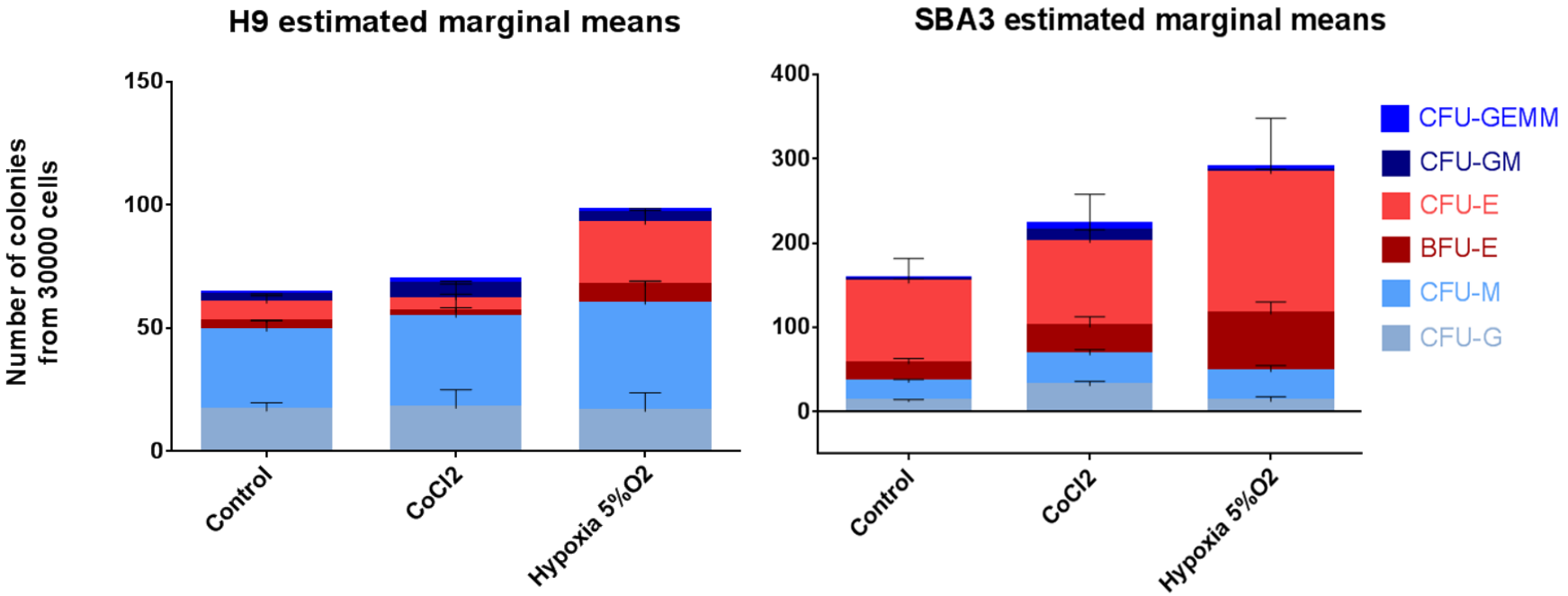


Figure 6.2: Bar charts show a comparison of total CFU numbers under different experimental conditions in two cell lines. Colony numbers are counted from a starting population of 30000 cells, the means are normalised for the effect of passage number using the estimated marginal means method ±SEM. The cells were differentiated to day 12 according to the protocol given in **methods section 2.1.2** and then cultured in methocult medium for 14 days before the colonies were scored. Three experimental conditions were tested: a control, 5% O_2 throughout differentiation and 0.1 μg/ml $CoCl_2$ throughout differentiation. n = 3.

Figure 6.3a

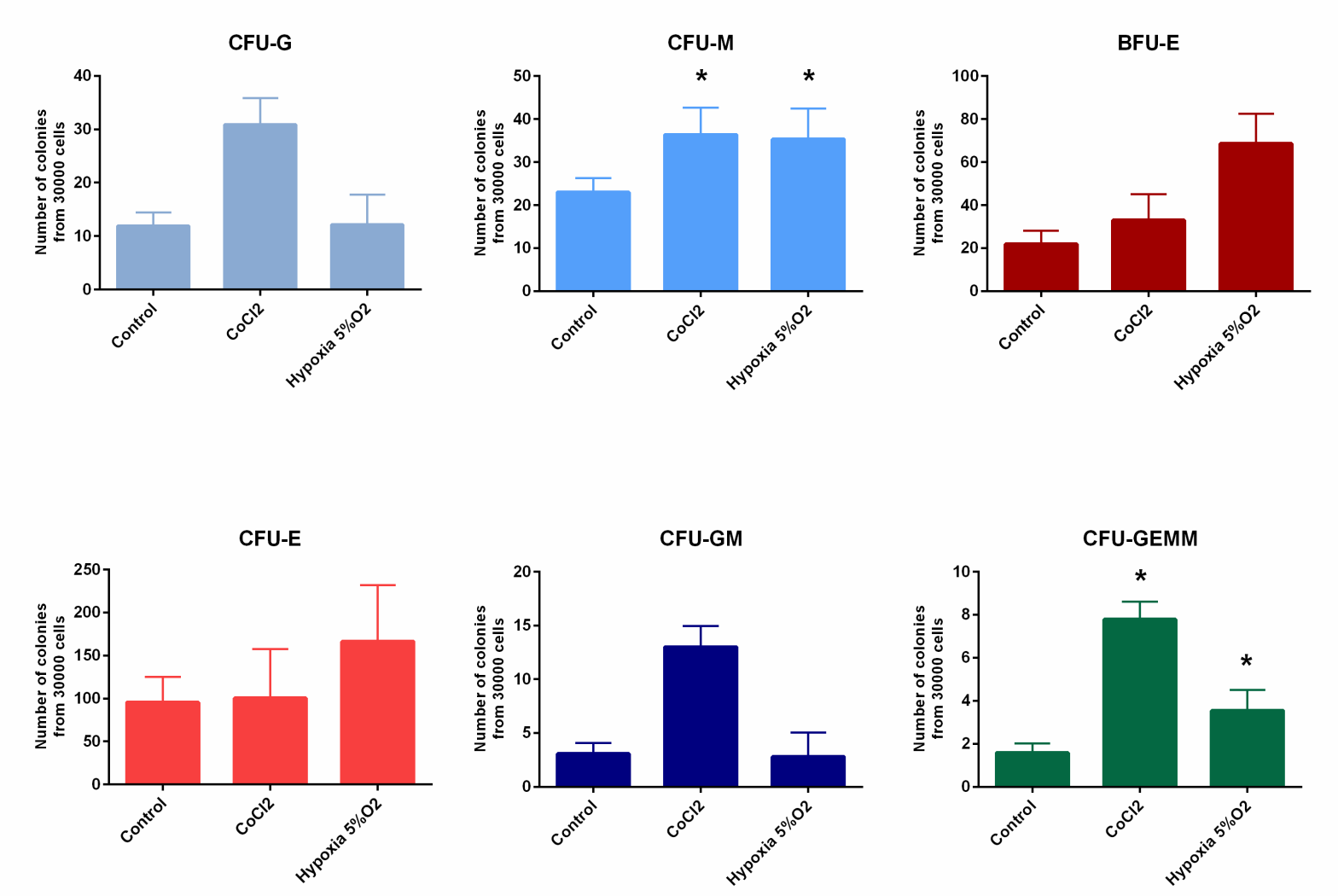

CFU-G
Number of colonies from 30000 cells
40
30
20
10
0
Control
CoCl2
Hypoxia 5%O2
CFU-M
Number of colonies from 30000 cells
50
40
30
20
10
0
*
*
Control
CoCl2
Hypoxia 5%O2
BFU-E
Number of colonies from 30000 cells
100
80
60
40
20
0
Control
CoCl2
Hypoxia 5%O2
CFU-E
Number of colonies from 30000 cells
250
200
150
100
50
0
Control
CoCl2
Hypoxia 5%O2
CFU-GM
Number of colonies from 30000 cells
20
15
10
5
0
Control
CoCl2
Hypoxia 5%O2
CFU-GEMM
Number of colonies from 30000 cells
10
8
6
4
2
0
*
*
Control
CoCl2
Hypoxia 5%O2

Figure 6.3b

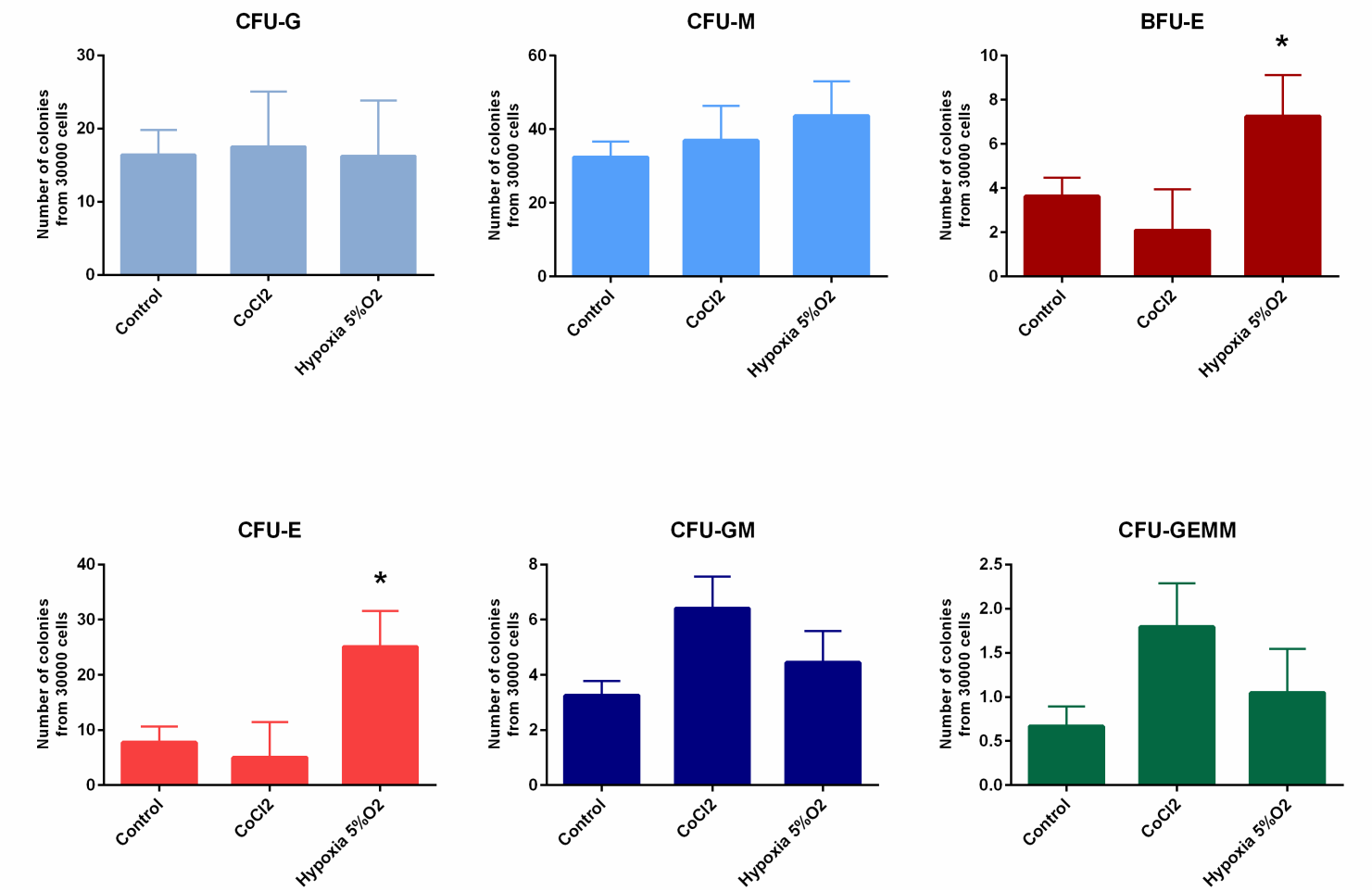


Figure 6.3: Bar charts show a comparison of individual colony types produced by the experiment shown in **figure 6.1**. Colony numbers are counted from a starting population of 30000 cells, estimated marginal means ±SEM, n = 3. Graphs show a control, 5% O_2 throughout differentiation and 0.1 µg/ml $CoCl_2$ throughout differentiation. **a:** SB-Ad3 cells, **b:** H9 cells.

Figure 6.4a

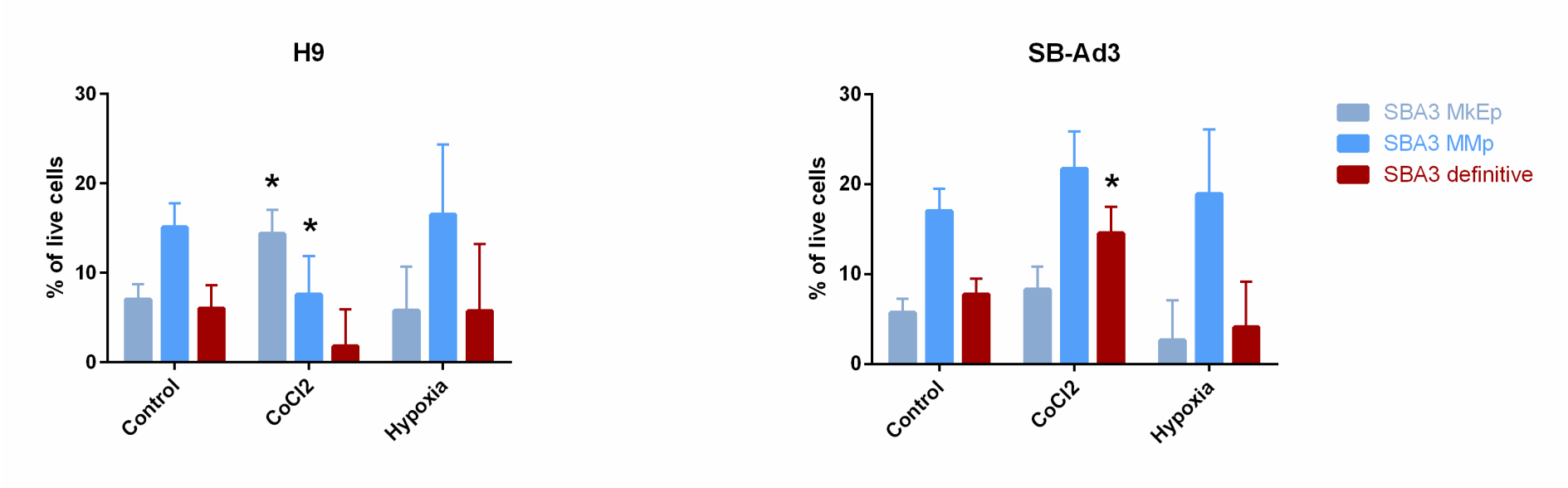


Figure 6.4a: Graphs showing percentage of live cells expressing Megakaryocyte erythroid progenitors (MkEP) = CD43+CD34+CD235+CD41a+CD45-; Multi-lineage myeloid progenitors (MMp) = CD43+CD34+CD235-CD41a-CD45-; Definitive hematopoietic progenitors = CD43+CD34+/-CD235-CD41a-CD45+. Cells were generated by differentiating two pluripotent cell lines until day 12 under three experimental conditions: a control, 5% O_2 throughout differentiation and 0.1 µg/ml $CoCl_2$ throughout differentiation. Data is shown as estimated marginal means normalised for the effect of passage number ±SEM, n=3.

Figure 6.4b

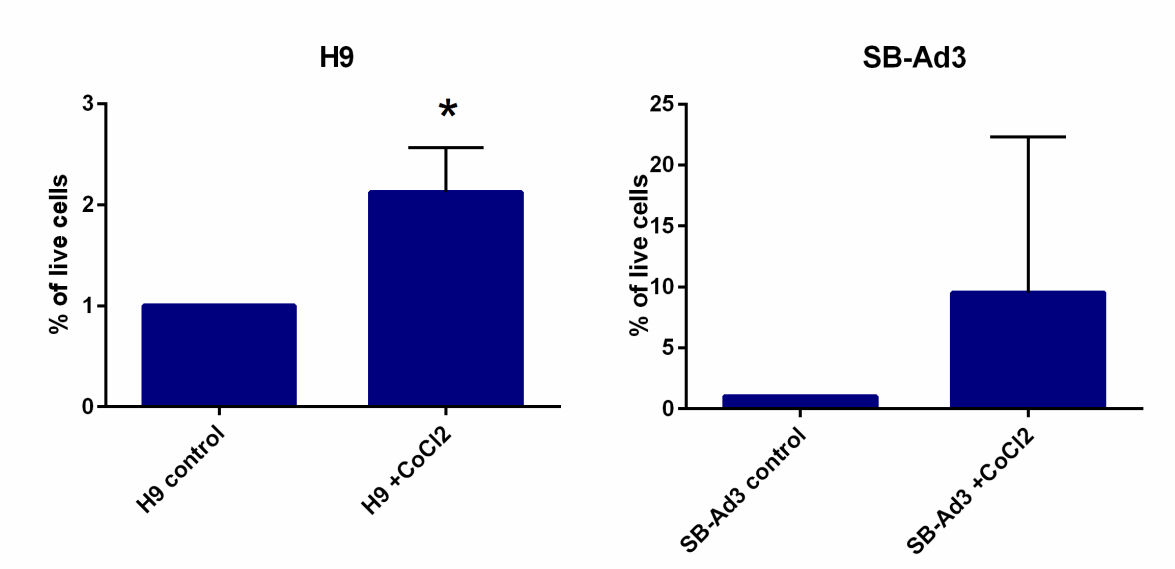


Figure 6.4b: Graphs showing fold-change in percentage of live cells expressing markers for LT-HSC (CD34+CD38-CD45RA-CD90+CD49f+). Cells were generated by differentiating two pluripotent cell lines (SB-Ad3 cells and H9 cells) until day 12 under either control conditions or treated with 0.1 µg/ml $CoCl_2$ throughout differentiation. Data is shown as mean fold-change ±SD, n=3.

Figure 6.5

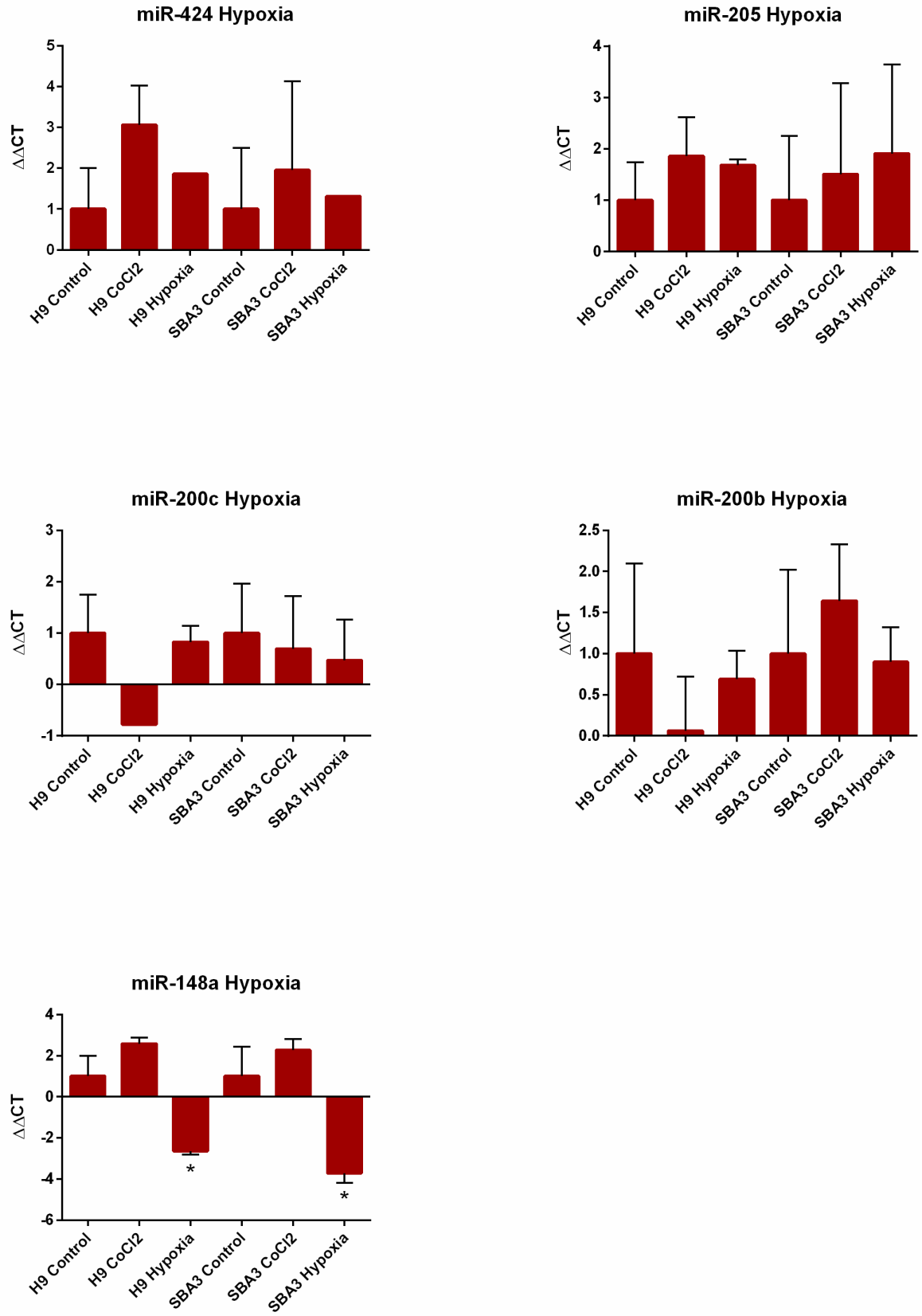
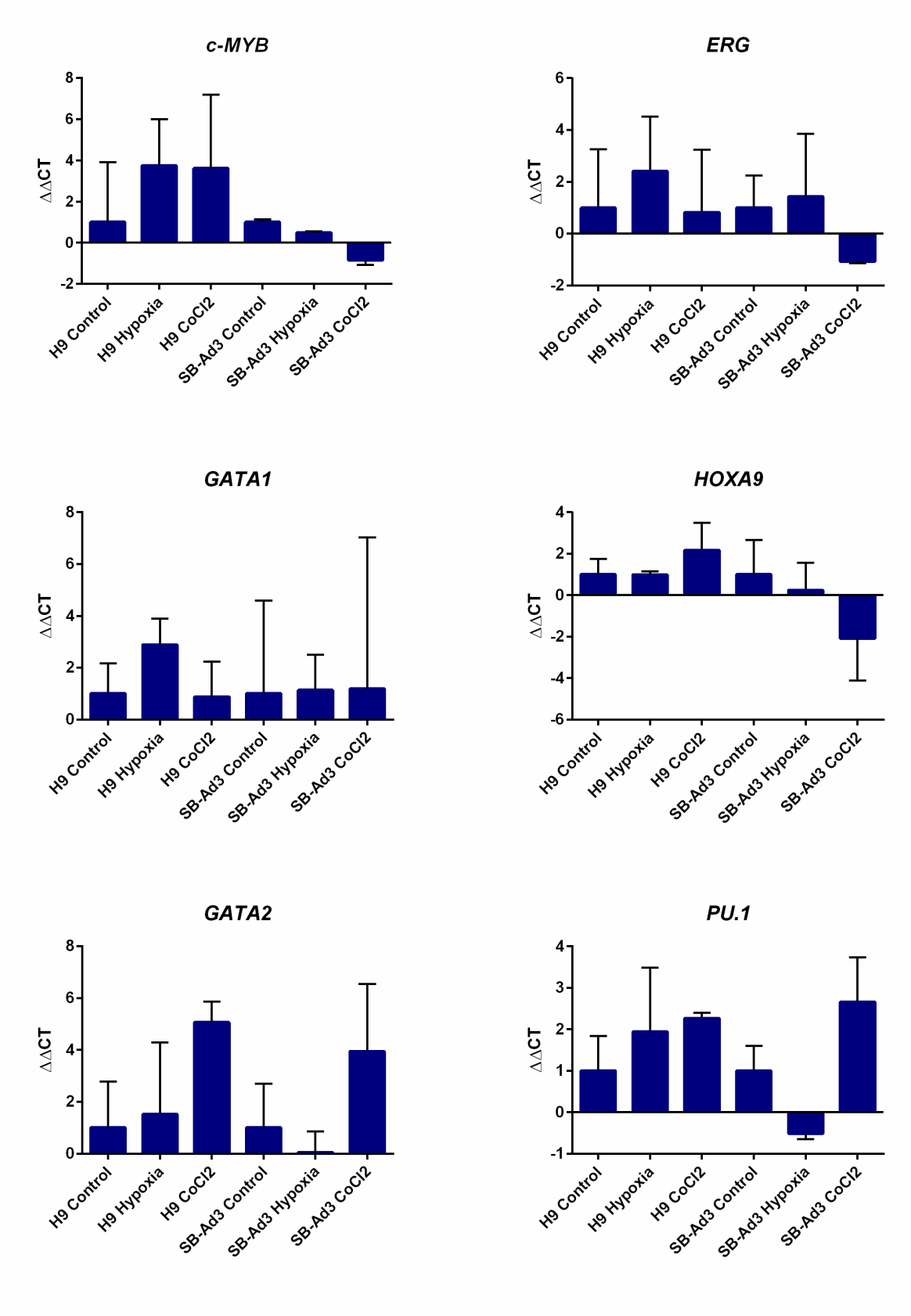


Figure 6.5: qPCR expression analysis of miRNA normalised to RNU48 and RNU44 in SB-Ad3 and H9 cells after 12 days of differentiation under the effects of 0.1 µg/ml $CoCl_2$ or 5% O_2 throughout differentiation normalised against control conditions. Data is shown as mean±SEM, n=3.

Figure 6.6


c-MYB
ERG
GATA1
HOXA9
GATA2
PU.1
ΔΔCT
H9 Control
H9 Hypoxia
H9 CoCl2
SB-Ad3 Control
SB-Ad3 Hypoxia
SB-Ad3 CoCl2

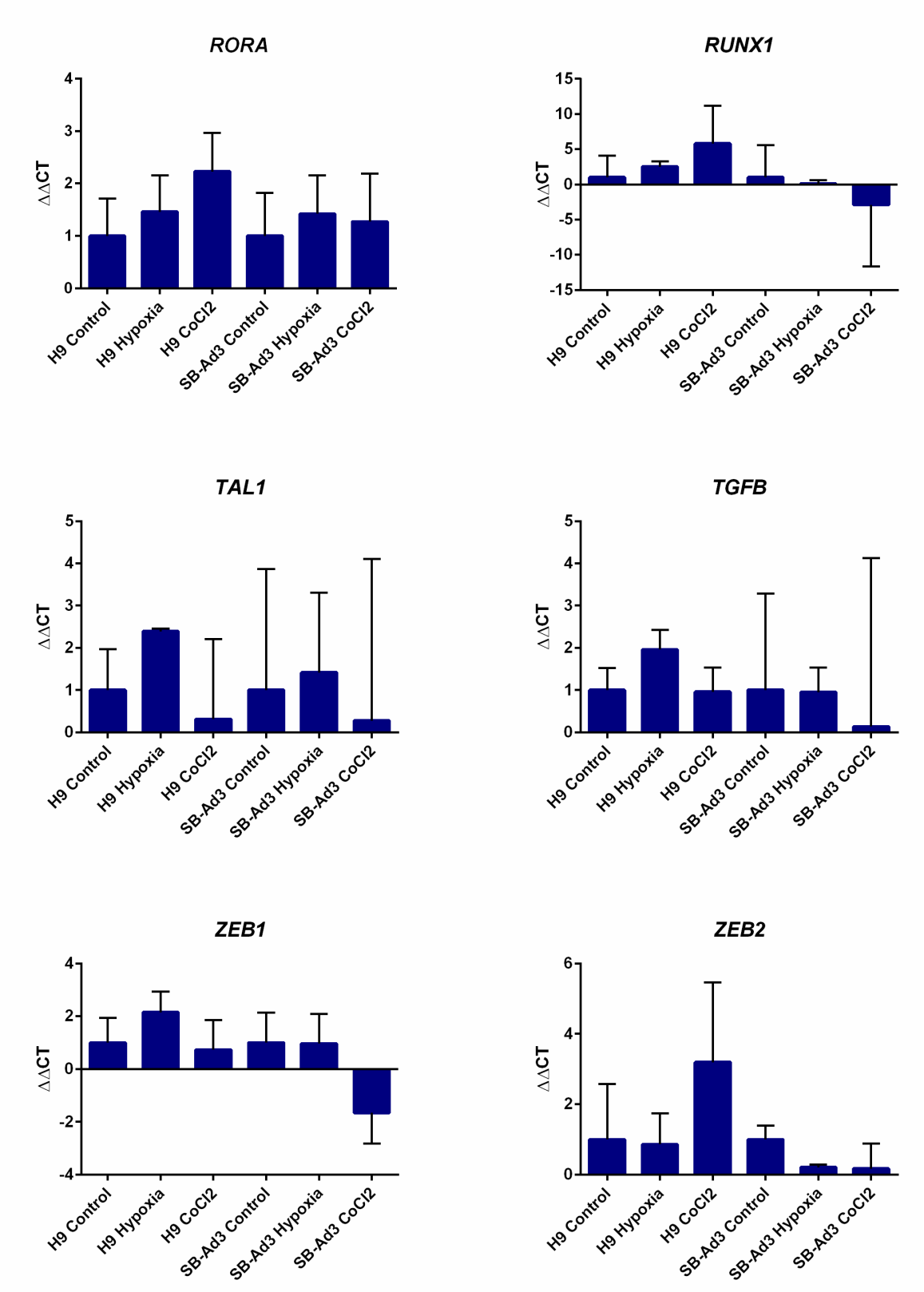


Figure 6.6: Gene expression analysis of miRNA normalised to GAPDH in SB-Ad3 and H9 cells after 12 days of differentiation under the effects of 0.1 µg/ml $CoCl_2$ or 5% O_2 throughout differentiation or control conditions. Data is shown as mean±SEM, n=3.

7. Discussion

7.1 Recent progress in the *in vitro* production of LT-HSCs:

Manufacturing HSC from pluripotent cells has been a long term goal of stem cell research. A great deal of progress has been made in this field in the last few years, allowing long term repopulating HSC to be produced from human cells for the first time. Although there is progress in elucidating the inductive factors which trigger HSC formation in the embryo, the most exciting results have been produced by direct reprogramming induced by the ectopic expression of various transcription factors (see below, **section 7.1.2**). In spite of this progress the methods used still require the cells to be co-cultured with animal cells either *in vitro* or *in vivo*, suggesting there are key factors produced by the hematopoietic niche which have yet to be discovered.

7.1.1 Inductive factors for HSC development

In vitro hematopoietic development closely follows the development of hematopoietic cells in the embryo, and mimicking the signals developing HSCs receive in the embryo should allow them to be produced *in vitro.* An incomplete understanding of hematopoietic ontogeny and the signalling pathways and transcription factors involved has hampered the efforts towards this but recent developments are building a more complete picture. PSC differentiation systems are an excellent model for analysing the factors necessary for differentiation as any factor can be easily added or removed. This kind of experiment has demonstrated the importance of stage-specific Activin-nodal signalling and Wnt3 signalling [8, 49], as well as other factors such as notch signalling [403], mechanical forces and nitric oxide signalling [404, 405] in the specification of definitive hematopoietic cells.

Other systems such as animal models of embryogenesis can also be helpful in elucidating these pathways. More information should eventually make it possible to promote the formation of HSC from pluripotent stem cells, and progress has been made on screening small molecules which can promote HSC formation [406]. For example evidence showing that hypoxia (as mimicked by $CoCl_2$) promotes HSC formation in the zebrafish embryo has been shown [383, 407] but it has now been demonstrated to promote hematopoiesis in human cells (see **results chapter 6**). Further progress towards learning all the inductive factors necessary for HSC formation will be aided by model systems with reporter genes marking the earliest hematopoietic cells [149, 408, 409], but there is still much work to be done, and it is clear that producing HSC from pluripotent stem cells will not be as easy as producing other hematopoietic cell types.

7.1.2 Direct reprogramming towards HSCs

Recent experiments using direct reprogramming to make HSCs have been more successful than the previous approach of using cytokines to direct differentiation. Direct conversion from fibroblasts to hematopoietic cells was first achieved by Szabo et al. in 2010 [410], by ectopically expressing *OCT4* and culturing the cells with hematopoietic cytokines. Direct conversion from fibroblasts using various combinations of transcription factors (see **Table 7.1)** has allowed multi-potent hematopoietic progenitors to be made [411, 412] but there has been more success in reprogramming cells from cell types which are from lineages to more closely related HSC such as committed blood progenitors [413] or endothelial cells [414]. Expressing *FOSB, GFI1, RUNX1*, and *SPI1* in human endothelial cells is the most successful attempt at manufacturing human HSC to date, achieving relatively high levels of multi-lineage engraftment over serial transplantations [414]. The authors suggest that using starting cells which are epigenetically and transcriptionally similar to HSC allows them to be more easily reprogrammed. Certainly chromatin remodelling plays an important role in the development for hematopoietic cells, from controlling the original hematopoietic specification of mesoderm by *HOXB4* expression [415] to down-regulating endothelial genes and up-regulating hematopoietic ones during EHT [416-418] and in maintaining HSC in the adult [152, 419-421], so reprogramming to HSC would have to not only make sure that the right transcription factors are expressed but also make sure that the enhancers of hematopoietic genes had the correct histone modifications.

Interestingly, although there are several papers showing direct conversion to HPSCs no consensus has been formed on which transcription factors are necessary for the conversion. Different transcription factors would be expected when using different starting cell types but there also appears to be some differences caused by overlapping functions or stochastic difference based on cell line. That being said there are some transcription factors which many of the papers have in common, which are known key regulatory factors of haematopoiesis and particularly EHT, such as *GATA2, LMO2, GFI1 and RUNX1* (see **results section 3.2.4**).

Table 7.1: Factors used for direct reprogramming

Starting cell type	Transcription factors	Other factors	Outcome	Reference
mESCs	*HoxB4*	Co-culture with OP9 cells and hematopoietic	Long term multi-lineage hematopoiesis.	[50]

		cytokines.		
Human fibroblasts	*OCT4*	Culture with hematopoietic cytokines.	CD45+ cells, low levels of mostly myeloid engraftment.	[410]
Murine fibroblasts	*Gata2, Gfi1b, cFos,* and *Etv6*	Co-culture with stromal cells.	Definitive hematopoiesis (based on gene and marker expression), mostly myeloid cells in CFU assays.	[412]
hESCs and hiPSCs	*ETV2, GATA2; TAL1, GATA2*	Culture with hematopoietic cytokines.	Definitive hematopoietic cells based on marker expression and CFU assays, no transplants were performed.	[422]
Murine fibroblasts	ERG, GATA2, LMO2, RUNX1c, and SCL	Culture with hematopoietic cytokines.	Low levels of short term engraftment.	[411]
Murine committed lymphoid and myeloid progenitors	*Hlf, Runx1t1, Pbx1, Lmo2, Zfp37,* and *Prdm5*	Immediate transplant after transfection.	Low levels of multi-lineage engraftment after serial transplantation.	[413]
HUVECs (human umbilical cord vascular endothelial cells)	*FOSB, GFI1, RUNX1,* and *SPI1*	Co-culture with E4ECs (immortalised human endothelial cells).	Definitive cells positive for LT-HSC markers, transplant into NSG mice at relatively high levels over serial transplants, mostly myeloid cells.	[414]

7.1.3 The hematopoietic niche in HSC formation

Co-culture with stromal cells such as OP9s has long been used to differentiate cells into hematopoietic cell types (see **introduction section 1.3.2**), and recent papers have emphasised the importance of the hematopoietic niche in HSC formation. The niche has been replicated in several ways, including by teratoma formation, in animal bone marrow and with co-cultures of niche derived cells.

Differentiation of pluripotent cells *in vivo:* HSC have been made from pluripotent cells which were injected directly into animals and allowed to form teratomas. The hESCs in the teratomas were directed towards the hematopoietic lineage by co-injection with OP9s [47, 48] or with hematopoietic cytokines [48] and the teratomas were shown to contain bone marrow-like tissues, around which the HSC were clustered. These experiments showed colonisation of the animals' bone marrow and spleen with human hematopoietic cells, which could engraft after secondary transplant, although in low numbers and with mostly myeloid lineage differentiation. These experiments show that HSC can be made from human pluripotent stem cells, but that there are key factors in the *in vivo* system which are necessary for successful differentiation into HSCs. A similar experiment found that pluripotent stem cells which were differentiated towards the hematopoietic lineage could successfully differentiate into HSC when transplanted into a mouse [46], but only when the cells were at day 3 of differentiation (which corresponds to the early hemogenic endothelium stage). Cells injected after that stage were unable to form HSC, suggesting that there are conditions *in vivo* which are permissive of HSC formation which are absent in their *in vitro* culture system. Riddell et al [413] transplanted the committed progenitors they had transformed immediately after transfecting them with their transcription factors, possibly allowing the hematopoietic niche to direct the reprogramming of the cells.

HSC using the vascular niche: The vascular niche is especially important in the differentiation and maintenance of HSC. Engraftable multi-potent progenitors have been made from hiPSCs using co-culture with endothelial cells expressing notch ligands *JAG1* and *DLL4* [423]. Sandler et al. [414] made LT-HSC by directly reprogramming endothelial cells but their method requires co-culture with immortalised endothelial cells.

Although differentiation *in vivo* or with co-culture of cells from the hematopoietic niche have been the most successful attempts at creating HSCs in the lab it is not a clinically viable strategy to use animals, animal cells or immortalised cell lines, both because it is risky and because the results are less reliable than in feeder free systems. These experiments are

promising however, because they will allow the factors which make up the HSC permissive niche to be discovered.

7.2 Is miRNA manipulation a viable technique for HSC induction?

Work in this project has been equivalent to direct reprogramming from HE cells or hematopoietic progenitors using miRNAs rather than transcription factors. Other papers have shown that the more similar the cell type to HSC the easier reprogramming is (see above, **section 7.1**) and the success of reprogramming endothelial cells or committed hematopoietic progenitors shows that our approach has promise. Having said that, these successful attempts used starting cells which were derived *in vivo* rather than from pluripotent stem cells so they would not have had the same epigenetic profile as endothelial cells and hematopoietic progenitors derived *in vitro* from pluripotent cells [424]. In that sense the experiments in this project are more like the experiments where *HOXB4* expression was used to make ESC derived hematopoietic progenitors into functional HSCs [50, 425].

Reprogramming cell fate with miRNA: It is possible to reprogram cell fate with ectopic miRNA expression; in combination with Yamanaka factors *SOX2*, *KLF4*, and *OCT4*, expression of the miR-302 cluster is known to enhance the efficiency of reprogramming to a pluripotent state [426], and the combination of mir-200c, mir-302 and mir-369 can induce pluripotency without the need for any transcription factors [21]. miRNA have also been used for direct reprogramming. This is usually done in combination with transcription factors, for example fibroblasts can be turned into neurons with the ectopic expression of miR-124, *MYT1L* and *BRN2* [427], or fibroblasts into monocytes using miR-125b and *SOX2* [428]. In these experiments the miRNA function as enhancers of reprogramming which is done by the transcription factors, but it is also possible to directly reprogram cells with miRNA alone, for example cardiomyocytes can be made from fibroblasts with a combination of miR-1, miR-133, miR-208, and miR-499 [429].

miRNAs are certainly important in the differentiation of hematopoietic cells (see **introduction section 1.4.4)**, but although the results of this project show that a number of miRNAs are mis-regulated, attempting to fix the problem by manipulating the expression of miRNAs may not be the best approach, because miRNA are modulators and have only a small effect on any particular gene's expression (see **results chapter 5**). miRNA-reprogramming without transcription factors typically occurs at very low efficiency. Although the experiments in this project should be helped by the fact that the cells are reprogrammed from hematopoietic progenitors, reducing the 'distance' in gene expression, they may also be hindered by low efficiency leading to only a tiny number of cells being transformed, which

would lead to a small effect size perhaps undetectable in such a 'noisy' experimental system with large amounts of variability. Although miRNA can control genes which affect epigenetic factors this is unlikely to be a short term effect, so will not help to overcome the epigenetic differences between *in vitro*-generated hematopoietic progenitors and LT-HSC.

The majority of experiments where transcription factors were used to reprogram cells to hematopoietic cells also used a more systematic approach, screening dozens of transcription factors by ectopically expressing all of them at once to create a large effect and then eliminating the ones that are unnecessary. If it was possible to eliminate the expression of all the candidates flagged by the microarray data a larger effect may have been seen. This was not practical using our techniques as large amounts of lipofectamine are toxic to the cell.

It may also be particularly difficult to change the expression of the genes targeted by the miRNAs identified as mis-regulated in this study because the miRNAs are part of a network of tumour suppressors, and the cell benefits by having multiple redundant regulators regulating the stability of potentially oncogenic genes. The technique that was used for miRNA inhibition was transient, and it may not have had a strong enough effect for long enough to overcome the barriers to EHT.

7.3 HSC emergence, tumour suppressors, and EHT

7.3.1 Tumour suppressors prevent the formation of HSC *in vitro*

The formation of HSCs in the embryo necessitates that at some point cells should lose contact with and leave their parent tissue and survive doing so with their self-renewal capabilities intact. This process is something that is undesirable under any other circumstances as it is equivalent to the metastasis of cancerous cells. It is therefore not surprising that HSC formation is so tightly controlled both spatially and temporally (see **introduction section 1.3.1**). It is also not surprising that it is so difficult to produce HSC *in vitro* in comparison to hematopoietic lineages without self-renewal capability such as primitive hematopoietic progenitors and lineage restricted definitive progenitors.

The data on microRNA expression produced in this project bears this out, miRNA with functions as tumour suppressors are the largest group which are highly expressed in the *in vitro* cultures in comparison to HSC derived from bone marrow or cord blood. Unfortunately this means that attempting to down-regulate these miRNAs engages multiple robust anticancer mechanisms, which appear either suppress EHT, preventing the cells from acquiring a motile phenotype and homing capabilities, or to allow EHT but remove the cells' self-renewal capacity, allowing them to form hematopoietic colonies *in vitro* but preventing

them from permanently repopulating bone marrow when transplanted. This is supported by the evidence that loss of tumour suppressors such as p53 dramatically improves the efficiency of direct reprogramming to HSCs [411] and the fact that no tumours have been reported in any experiment where P-HPCs have been transplanted into immune-deficient mice.

7.3.2 HSC form via a multi-step specification process

The question is how nascent HSC in the embryo can overcome the anti-cancer mechanisms. Evidence suggests a multi-step specification process, where HE capable of forming HSC (HSC-HE) is specified early in the developmental process, but which only form HSC when a variety of conditions are met. The HE is 'primed'- epigenetic and transcriptional factors are put in place which would allow self-renewing cells to undergo EHT, and then 'triggered' by a selection of factors present in only the dorsal aorta.

HSC-HE is specified early in embryogenesis: Haemogenic endothelium is a separate lineage from other types of endothelium [61], and it has been shown that HE cells are an intermediate stage to becoming hematopoietic cells rather than being bi-potent progenitors as was previously believed. However it also seems that there are multiple lineages of HE which give rise to different types of hematopoietic progenitors.

mESC which are differentiated towards the hematopoietic lineage can engraft in a recipient animal and form functional HSC, but only if they are transplanted at day 3 of differentiation, when they are still in the early hemogenic endothelium stage; if they are transplanted at a later stage they will not generate HSC [46], which suggests that the *in vitro* differentiation protocol is missing a factor which must be present at the very earliest stages of hematopoietic specification. Experiments in transgenic mice show that the HE which generates EMP and the HE which generates HSC are distinct lineages [430] and *in vitro* experiments on hESCs demonstrate that endothelial progenitors for the definitive and primitive hematopoietic lineages are determined as early as day 3 of differentiation by the presence or absence of Wnt and activin-nodal signalling [8, 49]. The idea that HSC-HE is separate from other HE lineages is supported by the direct reprogramming experiments by Elcheva et al [422] which show that *ETV2* and *GATA2* promote conversion to HE which generates definitive cells whereas *TAL1* and *GATA2* promote conversion to HE which generates definitive cells.

It is clear that the specification of HSC-HE occurs early in development, well before the emergence of HSC. Unfortunately there are no known markers which can differentiate HSC-HE from other lineages so although they differentiate early it is difficult to separate them in the embryo or *in vitro*, making the progression from mesoderm to HSC-HE difficult to study.

Signalling in the AGM triggers EHT of HSC: HSC-HE once formed is not destined to become HSCs, it can only do so when triggered by a combination of mechanical, chemical and biological signals found only in the AGM for a short time in embryogenesis. Signalling from the sub-aortic mesenchyme is required for HSC formation [431], as are the mechanical stresses caused by blood flow in the lumen of the aorta [405]. Signalling by SCF, BMP4, the Wnt pathway, retinoic acid and signalling from the sympathetic nervous system (see review by Rowe et al for list of all known factors [3]) are either necessary for HSC formation or promote it, although this should not be considered a complete list of necessary factors as there may be others which are not yet known. This multiple step process for the specification of HSC allows very precise control over when and where they can form, protecting the organism from the effects of allowing self-renewing cells to undergo EMT.

7.4 Future work

Evidence from this project suggests that the major cause of the failure of motility and engraftment shown by P-HPCs is due to over-expression of tumour suppressor factors. miRNA inhibition may not be the best solution for this problem because the tumour suppressor network is more robust than was initially apparent. Although the P-HPCs produced by treating pluripotent cells with cytokines share many common attributes with LT-HSC in terms of gene and marker expression, *in vitro* differentiation capacity etc. there are powerful mechanisms preventing the production of motile self-renewing cells in culture.

In order to overcome this problem some groups have turned to direct conversion, which has the advantage of being rapid and the possibility of using cells which are epigenetically similar to the desired cell type, or even of converting mononuclear blood cells, avoiding the necessity of inducing EHT altogether. However there are some potential problems with this approach. Most methods have used transcription factors introduced to the cells in integrating viruses, opening up the possibility of insertional oncogenesis [432]. Although there are methods to introduce genes into cells without integrating them into the genome it remains to be seen if they will be efficient and effective enough for reprogramming cells to HSC. There is also a problem that many of the reprogramming techniques use pluripotency factors, which are potentially oncogenic [433].

With or without ectopically expressed transcription factors, it is still necessary to use animals or animal cells to complete the transformation into HSC which means there are essential factors which are unknown and will have to be elucidated before these techniques can be used clinically. Systematically studying embryonic and *in vitro* hematopoietic differentiation and the ways in which they diverge transcriptionally and epigenetically should be the priority in

this field. This work is already being done in murine hematopoiesis from mESCs [434] but should be continued in human cells. Analysis of miRNA expression should be included in this work, but changing miRNA expression alone is not likely to have a sufficient effect, it would probably be more effective to analyse the transcription factors which control miRNA expression and how they, in turn, are regulated.

References

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