Transfusable Blood: A Thesis Looking at New, Necessary, Potential Sources

Bachelor thesis for the study life sciences & technology at the University of Groningen

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Contents

Introducing the problem

Ever since Karl Landsteiner discovered how the ABO blood type system functioned within human blood back in 1909, blood transfusions have been a staple of modern medicine (1). Ranging in use from emergency applications such as during hemorrhagic shock, to assisting during surgical procedures, to treatments of diseases involving chronic anemia (2)**.** Each year over 110 million donations of blood are collected across the globe, making up the source of the transfused blood (3)(4)**.** But even with these impressive amounts being collected by donation centers, supply cannot meet demand at every turn as donations are far from evenly spread between countries and populations (3)**.** To make things worse the future stability of the blood supply is under stress from various sources, and should these threats go unanswered, lifesaving procedures might be forced to grind to a halt. With the mean age of the world's population rising (4)(5)(6) there will be fewer people able to donate blood. Whilst also increasing age group that disproportionally receives the most transfusions annually (7). Even as some countries have reduced the age restrictions on donation viability this is only a temporary solution (7). Another far less controllable or predictable threat to blood safety will come from emerging infectious diseases that can slip through conventional screening tests. Leading to an increase in the risk of transfusion transmitted infections over the course of the current century (8)(9). The first incidences of which are already seen having an impact as the bacteria *Trypanosoma cruzi* (10) and *Babesia Microti* (11), both having affected the safety of donated blood in the past years. Whilst even more recently the Zika virus outbreak in the Americas is still leaving its mark. As it has been found in donated blood in the United States (12) Puerto Rico (13) and Brazil (14)(15). Combining these prospects and reports it becomes clear there will be a vested interest in exploring alternate sources for transfusable blood in order to support a growing worldwide demand with slimming traditional sources. Giving rise to the following question: What are possible future proof options, and our current understanding of them, that will allow for a steady supply of safe transfusable blood allowing for transfusions to remain a corner stone of modern medicine?

Exploring Current Options

When looking for options that can support or substitute the current donation-based system there seem to be two ways to go about acquiring the needed blood, either synthetic or biological. Over the last decade these two possibilities have been explored, with varying success. The first of these goes under the name of Haemoglobin-Based Oxygen Carriers (HBOCs). Their general premise is based on the *in vitro* generation of molecules mimicking natural haemoglobin function (17)**.** Using these might have been the way to go was it not for a study done in 2008 by Natanson et al (18) pointing out risks in HBOC use and causing interest to shift away from the synthetic path. Unwarranted perhaps as a review was able to flip the results of Natanson entirely by excluding a questionable compound form the study (19). Sadly when this criticism was shown the damage had already been done setting HBOCs back years (20). The second option with potential to support global blood supply would be through, a biological approach, attempting to culture red blood cells (RBC) (21)(22). Using human cells cultured *ex vivo* to mimic natural haematopoiesis and by doing so function as biological blood factories (23). Since knowledge on cultured RBC is significantly more in-depth compared to HBOCs, cultured RBC will be the focus of this thesis. Now that the initial proposition has been made towards a solution, we should also consider whether overhauling our current system is truly more desirable than improving what we already have.

To Improve Screening and Safety or to Culture

Culturing RBC *ex vivo* might seem like a lot of work when comparing it to the simpler idea of improving upon current screening techniques and quality safeguards, making one perhaps wonder if this is really the way to go. However, looking deeper into what cultured RBC can offer, their potential is greater than simply offering extra supply to the current system.

Firstly, cultured RBC could allow for generation of specific blood-types in accordance with the ABO system and its subtypes. For example, allowing for rare phenotypes such as the universal donor O^- to become more readily available. A blood-type that has a notoriously low representation amongst the world population with approximately ~7% of people expressing this type (24)**.** Building on this principle with enough research we can reach a point of haematopoiesis understanding that could even allow for patient-specific blood culturing. Which would mean that people could receive transfusion with what would be in essence their own blood, given that transfusions are planned in advance, as is the case with chronic anemia patients (27). Combining these two possibilities could severely reduce the occurrence of transfusion based reactions, such as cases of haemolytic transfusion reactions (25). During haemolytic transfusion reactions either the transfused blood or recipients blood is destroyed at an accelerated pace leading to various internal complications (26). Furthermore, with the ability to tailor blood for transfusions on a patient by patient basis, could reduce the strain on those that undergo frequent transfusions, such as patients suffering from chronic anemia. Patients suffering chronic anemia face the risk of various possible complications, one of those being the risk for alloimmunization (27)(28) which might be lessened or prevented if transfusions were preformed using one's own blood. Eventually, if technology allows for it, the ability of patient specific blood being used in planned surgeries could reduce the strain on the body after procedures, improving recovery time.

As described in the introduction there is a growing risk of emerging infectious diseases getting into blood meant for transfusion and past screening leading to transfusion transmitted infections. Since laboratories allow for germ free working conditions, they would also allow for cultured RBC to be produced far away from bacteria and viruses, giving a safety guarantee to cultured blood which

donated blood does not have. Even after blood has been screened and cleared for infectious agents it can still hold one more risk: allergens that are carried in the blood of the donor. These allergens can persist during storage and by doing so can eventually be introduced into a patient system. As it is possible for these proteins to not be deactivated during storage they can lead to allergic transfusion reactions in the recipient, with similar consequences as haemolytic transfusion reactions (29)**.** As culture conditions can be closely monitored and maintained, the risk of allergens getting through to patients would become minimal.

Furthermore problems such as natural disasters, political instability, and disease outbreaks might cause swings in donor availability and participation (3). Through setting up conditions for culturing RBCs with long-term planning in mind these swings can be mitigated, leading to a more reliable supply, as well as the potential to scale supply based on projected needs.

Finally, during the time RBCs spend in storage, erythrocytes undergo both morphological changes, losing their biconcave shape, as well as biochemical changes, (e.g. lowered intracellular haemoglobin-count) (30). Leading people to believe that some transfusion complications can, in part, be attributed to RBC age, however the ideal or correct age for transfusion is still being debated. As some studies suggest that younger RBCs, or shorter storage periods, are preferred meaning the cells have undergone minimal changes (31)(32)(33). Whilst others say that during storage missed infectious agents can be deactivated, leading to the idea that longer storage periods are more ideal (34)(32)(35). Regardless of what conclusion this discussion will end on, through proper planning and culturing RBC can be used at the exact right time in their lifespan to optimize their functionality after transfusion.

Surmised culturing RBCs may not only strengthen our supply of transfusable blood but also comes with various side benefits, see figure 1 for a visualized recap, that cannot be achieved by simply improving on screening and safety protocols. This brings us to the next stage of answering our central question, what kind of cells can serve as the best starting point in culturing RBCs?

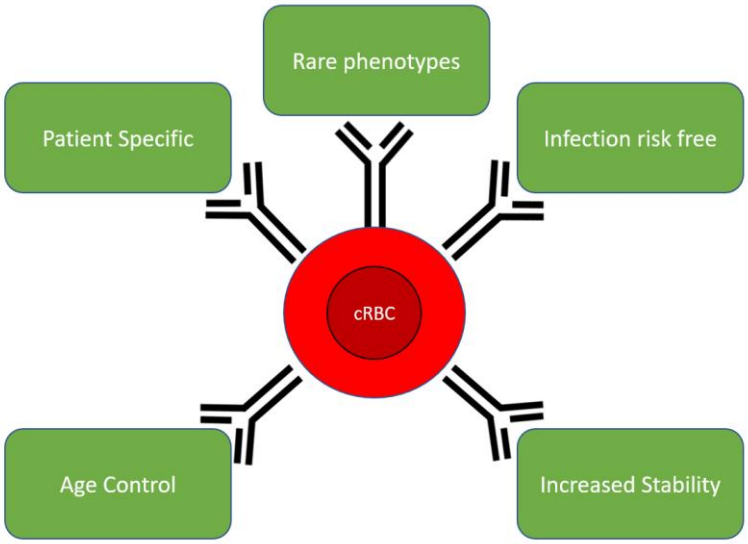

Figure 1 additional benefits that cultured RBC can offer over improved screening and safeguards aside from alleviating our current donation-based blood supply. cRBC are cultured red blood cells.

Haematopoietic stem cells, cord blood cells and induced pluripotent stem cells, finding the optimal choice

To culture RBC *ex vivo* we first need to examine what cell type is best suited to start with. Ideally these cells would meet the following properties: 1. Cells would be easy to obtain from donors whilst causing minimal discomfort and requiring minimal recovery time. 2. Cells would retain suitable culturing quality for extended periods of time. 3. Cells must possess the required genetic makeup to allow for proper differentiation down RBC lineages.

The first option that could meet some of these criteria could come from the widely used bone marrow transplants which involve haematopoietic stem cells (HSC) from a donor being transplanted into a patient. The goal of this procedure is for the transplanted HSC to perform normal haematopoiesis in the patient as first described by Lorenz *et al.,* in 1951 (36). As HSC stand at the top of haematopoiesis pyramid it is in their natural "behavior" to produce, amongst other cell types, RBC (37). With this trait in mind, it would mean that manipulating them into producing RBC *ex vivo* might be easier given the right conditions, which includes the need to reproduce the osteogenic niche in laboratory settings. The major downside that may come with HSC comes when looking at the method by which they are collected. As the cells have to be obtained straight out of the bone marrow of a donor, a procedure not without considerable risk, unlike regular blood donations, which are relatively safe by comparison (38)**.** Also, recovery time before someone can resume everyday life is vastly different between an HSC donation and a blood donation. Whereas after a regular blood donation donors are often time able to resume everyday life the same or following day (2)**,** recommended recovery period for bone marrow donations can range from one to seven full days of minimal exertion (37). Combining these two incumbencies to donors it can be expected overall participation will be lower when compared to current blood donations. Another limitation when working with HSC is that should the ability to generate patient specific niches is impaired as well. As patients are rarely considered healthy enough to undergo the HSC collection procedure (38), limiting the applicational potential of HSC gained from cultured RBC even further.

An alternative that has been of the some interest in studies is the idea of using cord blood cells, cells obtained from the umbilical cord of a baby post birth (39). A large upside of this source would be the ease at which the cells of interest can be collected, as there is minimal to no risk when collecting these cells from the placenta and umbilical cord (40). But the convenience of collection also displays its limitations as a starting point for culturing RBC. A person is only born once meaning there is also only one chance in a person's life for collecting cord blood, making it hard to build database that would cover the entire population and allow for patient-specific culturing. The limitation of building such a database could be alleviated over time as storage prices continue to go down and the ability to store cell collections for larger parts of the populace (41). But ethically cord blood is not as simple as HSC, since HSC donations occur through mature informed consenting donors this is not possible in cases of infants with regards to using their cord cells to culture blood for others (42). A final limitation when using cord blood cells to produce blood products is that RBC generated out of cord blood lineages do not display the same haemoglobin phenotype as RBC obtained through normal haematopoiesis (43). As adult RBC haemoglobin mainly consists of beta-globin whilst the RBCs obtained by Anstee *et al*., had a high expression pattern, upwards of 64%, of gamma-globin, also known as fetal globin (44). Anstee shows it is possible to generate regular adult RBC using cord blood right now, however the production of unwanted gamma-globin rich RBC shows that additional knowledge on haematopoiesis from cord blood is required for it to be an efficient option.

A final option that could fill the role for cultured RBC production *ex vivo* is through making use of induced pluripotent stem cells (iPSC) which are then turned into HSC to allow for haematopoiesis. Being the youngest of the three options as the possibility of creating iPSC from terminally differentiated cells was first proven in 2006 by Takahasi and Yamanaka. Through using four transcription factors (Oct3/4; Sox2; Klf4 and c-Myc) most terminally differentiated cells can be transformed into iPSC (45)(46). As the cells for generating iPSC can be virtually any cell type, a conscious choice can be made to take less invasive options such as fibroblasts, when compared to going into bone marrow to take HSC. Seeing as a donation of fibroblasts is both less invasive and therefore less dangerous. The kind of recovery after donating fibroblasts is vastly different and does allow for normal day-to-day activity (47), it can be reasoned that donor numbers will be significantly higher than for bone marrow donors. Seeing as a fibroblast sample puts less strain on a patient than HSC samples from bone marrow it will also be more likely that patients will be able to give up their own cells to use in RBC generation. Thus, not having to rely on a matched donor. The downsides to using iPSC for generating cultured RBC is that additional steps must be included in the culturing protocols. As now there must be accounted for two additional steps, namely: 1) going from terminally differentiated cells to iPSC, and 2) iPSC being transformed into HSC. This method also must contend with risks of teratoma formation (48). To this proposed problem are however two counter arguments to be made: First, in the case of cultured RBC transfusions, idealistically, cells containing a nucleus would not be transfused, therefore any tumorigenic risks are attenuated. Second, with recent advancements in iPSC understanding, targets have been uncovered that can allow for reduction in teratoma formation. As overexpression of p27 (48) and lysine-specific demethylase 1 inhibition (49) have been attributed to reduce teratoma generation. Alternatively at the end stage of *ex vivo* haematopoiesis targeting of anti-apoptotic factors such as surviving or Bcl10, through e.g. quercetin, can remove remaining nucleated cells from a batch (50). Aside from the risk of teratoma formation iPSCs and their derived differentiated cells seem to cause little to no immune responses in hosts should some still slip through (51)(52).

Comparing these three choices, the following can be surmised: HSC donations have many obstacles in terms of obtaining enough cells for culturing combined with a high level of inconvenience to donors making them an undesirable choice. Whilst the use of cord blood cells is too inaccessible in terms of ethical considerations, and to current generations until storage becomes more readily available and gamma-globin generation can be controlled better. Even though using iPSC would require additional steps and carry the risk of teratoma formation it would seem the most viable option to explore for now. With a cell type in mind we can now look more in-depth as to how (iPSC) cells can be manipulated into producing RBCs *ex vivo*.

Recreating haematopoiesis *ex vivo*, a matter of signals, time and markers

In order to manipulate iPSC into producing cultured RBC normal human haematopoiesis must be translated into laboratory conditions. Normal haematopoiesis relies on a cocktail of signaling molecules as well as direct contact signals generated by the surrounding tissues during different steps in order to drive cell specialization (53). Allow us to first look at the route of differentiation, amplification and specialization during normal haematopoiesis. At the top of the haematopoietic pyramid stand the HSC and it is with these cells that haematopoiesis begins (54). HSC are located within bone marrow, specifically the osteogenic niche, containing various signaling molecules, low oxygen saturation, and specialized stromal cells (55). HSC behavior within the osteogenic niche is dominated by a quiescent state (56), involving a low cell cycle speed and when active a great emphasis on self-renewal (57). From HSC haematopoiesis continues with differentiation into haematopoietic progenitor cells, losing some self-renewal capabilities and with their main purpose becoming amplification (58). Normally HSC differentiation into haematopoietic progenitor cells is regulated within the osteogenic niche (59)(60). Following the haematopoietic pyramid further down we can see that, in order to stay on the path towards RBC, haematopoietic progenitor cells must differentiate into erythroid progenitor cells. Continuing further erythroid progenitor cells then transform into reticulocytes, large cells that still retain their nucleus. Their purpose the synthesis of essential components for RBC function such as: haemoglobin for oxygen transport; pyruvate kinase and glucose-6-phosphate dehydrogenase for ATP maintenance and 2,3-DPG generation prevention (61). After the synthesis of these critical components reticulocytes undergo enucleation, the nucleus is expelled and the remaining cell starts to bleb into the familiar biconcave discoid RBC shape (62).

With this image of normal haematopoiesis in mind it is time to look at what signals cause this process to take place. As without defined factors HSC produce a wide array of possible cell lineages (63) which undesirable when the goal is to only produce RBC. As various studies have explored what signals are required for each step this part will draw parallels between those studies to extrapolate an as concise as possible signaling cocktail. To obtain the top of the pyramid, HSC, two routes have been described in studies differing in starting point but both attaining functional HSC namely: 1) Starting with iPSC obtained through the method described by Yamanaka et al 2007 (46) this method turns iPSC into HSC through introducing of select transcription factors (ERG; HOXA 5;9;10; LCOR; RUNX1 and SPN) (64). 2) Through using the transcriptional factors Fosb; Gfil; RUNX1 and Spill mature fibroblasts are directly transformed into HSC (65). After transforming into HSC these factors were not required to maintain this state. Many combinations of factors and signals have been studied to induce the various steps of haematopoiesis hinting at the possibility that not each factor is as essential as others. To obtain a more concise list various studies on haematopoiesis will be cross-referenced. As mentioned earlier in this section after obtaining HSC the next step is to trigger differentiated into haematopoietic progenitor cells. The most used factors for this step are: SCF; EPO; TPO; Il-3; Flt-3 (73-87). Following the generation of haematopoietic progenitor cells differentiation should be guided towards erythroid progenitor cells the most commonly used signals for this step are: SCF; EPO; Il-3 and hydrocortisone (73-87). Triggering reticulocyte differentiation from erythroid progenitor cells two signals seem to suffice namely: SCF; EPO (73-87). During all the observed studies cells were kept at 37°C under 5% oxygen. Lastly the haematopoietic process ends with enucleation of the reticulocytes, this has been a point of interest in various studies where successfulness has variety considerably (73-87). Resulting in RBC that still had nuclei or simply not shifting out the reticulocyte stage. Because of this future emphasis should be placed on enucleation signaling. Cross-referencing enucleation focused studies reveals the following factors of interest: Vaso Endothelial Growth Factor (VEGF); SCF; EPO; Il-3; IGF-II; follicle acid and vitamin B_{12} (88)(89) To note here is there is an argument to exclude SCF during enucleation as it has been show to slow down enucleation (66). These three studies demonstrate that there is more

research to be done before enucleation can be reliably achieved. A visual representation of differentiation signaling can be found in figure 2.

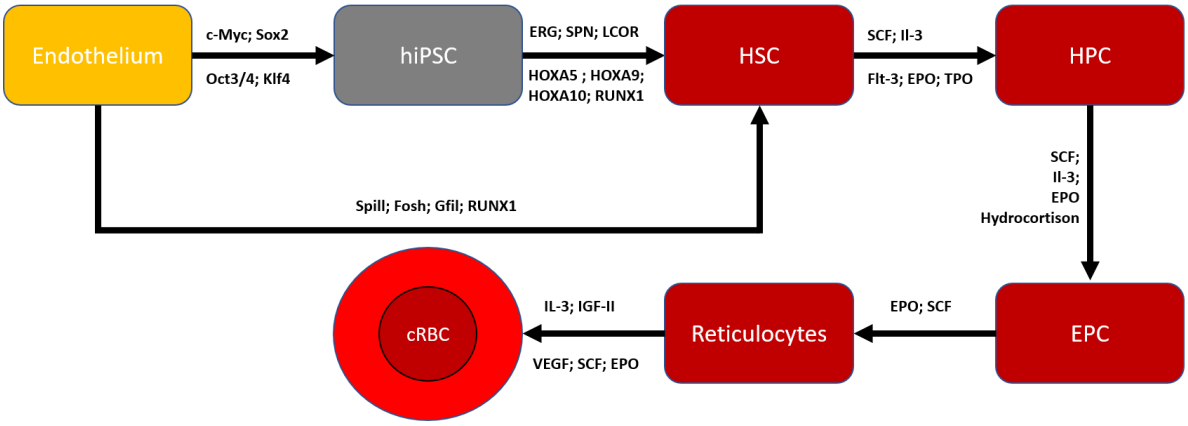

Figure 2 Surmised depiction of haematopoiesis as explored in laboratory conditions. hiPSC are human induced pluripotent stem cells, HSC are haematopoietic stem cells, HPC are haematopoietic progenitor cells, EPC are erythroid progenitor cells. Based on cross-referencing sources (46) (64-66) (73-89)

Aside from signaling cocktail composition, one other major factor that can influence culturing is timing, when to start the next step of differentiation. A better understanding of these timings may allow for better flow during culturing. Comparing the studies used for determining factors differentiation factors the following can be observed:

1) Overall the time it takes to differentiate RBC from either cord blood or iPSC is significantly different. In studies working with cord blood cultured RBC generation appears to range from 18 to 40 days (73-87). Whilst studies working with iPSC report timeframes ranging from 42 to 60 days (73-87). This supports arguments that if advancements can be made with regards to collection and storage for cord blood cells it would prove to be a better choice than using iPSC for culturing. One point of note is that these timeframes also accounts for iPSC induction.

2) Individual steps of haematopoiesis seem to vary between studies. These could be explained through differences in signaling composition, concentrations or perhaps genetic variation. With the initial step of HSC to haematopoietic progenitor cells ranges of 4 to 8 days (73-87) with one outlier of 14 days (85). HSC to erythroid progenitor cells appears to range from 5 to 10 days (73-87) erythroid progenitor cells to reticulocytes differentiation ranges from 4 to 8 days (73-87). Finally, reticulocytes to cultured RBC maturation ranges from 4 to 7 days (73-87).

However, it is important to note here that these studies emphasized techniques to generate cultured RBC and were not focused on cell count. Therefore, when projecting these findings onto culturing for medical use additional time should be accounted for with regards to amplification. With a better understanding of the timeframes each step seems to operate at one more factor should be considered, genetic variation between cultures.

Even if conditions were identical for differentiation there is still an influence exerted by genetic background of cells (67). To nullify this variable and attain certainty for when steps should be initiated screening of the samples should be performed. Using unique surface marker patterns; reverse transcription polymerase chain reaction (RT-PCR) or functional analysis test the various stages of haematopoiesis can be distinguished. Depending on what stage is being examined different methods can be used:

- iPSC identification: either through human embryonic stem cell specific surface antigens (e.g. SSEA-3; NANOG) (68) or through RT-PCR for embryonic stem cell-marker genes (e.g. OCT3/4; SOX2) (68). This step does not apply if culturing was to be done with cord blood.
- HSC identification: surface markers CD33; CD34; CD38; HLA-DR antigen (37).
- Haematopoietic progenitor cells identification: through surface markers CD33; CD34; CD38; CD71; HLA-DR antigen; (37) and CD33, CD13 (69).
- Erythroid progenitor cells identification: through surface markers lin^{-}SCA-1; c-kit; CD34; CD16/32 (70)
- Reticulocyte identification: primarily through cell surface makers CD47 (28) Glycophorin (71), Band 3, RhAG, GPC; (72).
- RBC can be identified through functional analysis, this in order to judge whether reticulocytes have indeed transitioned over into RBC. Also, to verify whether the RBC possess the necessary cellular components to function. For this mean cell volume, biconcave shape, mean cell haemoglobin, mean corpuscular haemoglobin concentration and lack of nucleus must be assessed (73-87).

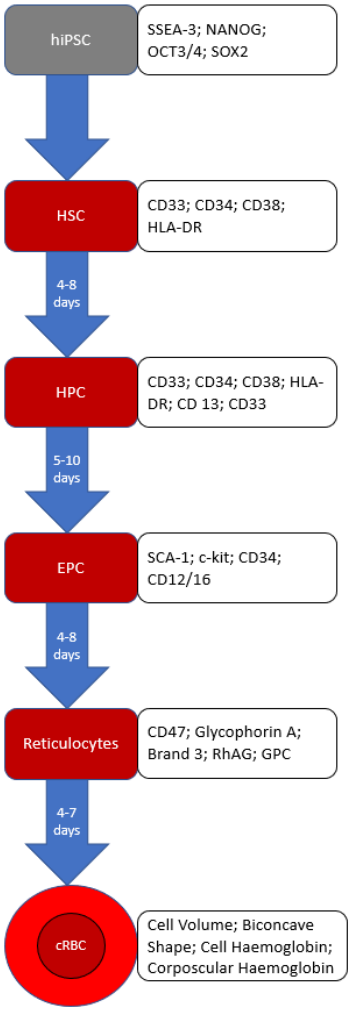

Figure 3 Estimated timeframe and markers of note for the individual steps of haematopoiesis. hiPSC are human induced pluripotent stem cells, HSC are haematopoietic stem cells, HPC are haematopoietic progenitor cells, EPC are erythroid progenitor cells.

It is clear various signaling molecule cocktails can result in the generation of RBC *ex vivo*. But with signaling alone a successful protocol cannot be generated, for this a keen understanding of timing and stage conformation are also desired. Despite some uncertainties a basic protocol using this information can be made and expanded upon.

With the caveat of genetic variation, it is interesting to look at possible manipulations that can be explored with future research to influence culturing. Such as altering differentiation, and amplification in order to cut the time it takes for RBCs to form. While actions such as immortalizing lineages could lead less need for donations in the long run.

Targeting cells to affect differentiation, amplification and immortalization

With the foundations for the method of culturing discussed a final point of interest is looking at possible future advancements. Thinking logically there are two ways in which the yield form culturing can be affected: 1) Increasing amplification speed and 2) reducing apoptosis rate. Both these processes may be manipulated through genetic or humoral signaling routes. Manipulating cells on a genetic level will lead to lasting changes, which in cases of medical procedures might lead to increased safety concerns for example with regards to tumorgenicity. Whilst humoral signaling would only persist whilst factors are present in the medium but might carry additional costs to maintain during growth. However, since RBC lose their nucleus and RNA during enucleation there would be little risk of altered genetic information being introduced into patients during transfusions. Meaning both the genetic and humoral solutions can be explored for a viable approach. Since changes are rarely without drawbacks it is worth looking in depth at the possibilities present for both humoral and genetic manipulation when wanting to affect cultured RBC yield.

Now the question becomes: At which stage can genetic and humoral manipulations exert their optimal effect? HSC are capable of self-renewal and have long lifespans whilst not functioning as the main stage of cell count amplification. It is for these reasons HSC should not be the primary focus when looking at increasing cell count. Reticulocytes are almost terminally differentiation and are spending a lot of time and energy on preparing for enucleation. So, it would seem unlikely meaningful gains can be made by increasing their lifespan, and since division has stopped attempting to induce amplification would simply do nothing. RBC possess no nucleus making changes amplification is simply impossible. And even if manipulations were possible within RBC with regards to lifespan this would be dangerous as the natural balance in count RBC that is maintained through generation and destruction within a person would be disrupted when transfused with long lasting RBC. Which would lead to patients developing highly viscous blood and in turn risking the clotting blood and blockage of blood vessels. Lastly are the haematopoietic progenitor cells and erythroid progenitor cells both are cells of the progenitor type meaning by name alone these cells should be of some interest when it comes to amplifying cell count. At first glance both might seem like equally viable options for manipulating lifespan and amplification, but there is a reason why affecting haematopoietic progenitor cells can be the more desirable target. Since not only do changes present in haematopoietic progenitor cells carry over into erythroid progenitor cells, by amplifying the amount of haematopoietic progenitor cells there would automatically be more cells that can then again be amplified as erythroid progenitor cells, allowing changes to “double-dip” as it were.

With haematopoietic and erythroid progenitors in mind it is time to look at what kinds of manipulations could lead to either increased amplification or to extended lifespan.

Looking at naturally occurring diseases we can find one possible biological trick that lead to increased RBC production namely *Polycythaemia vera*. Through a mutation in the tyrosine kinase JAK2 (V617F) a constitutive activation of the kinase is triggered leading to hypersensitivity to EPO, this in turn induces increased erythropoiesis (89). It has also been demonstrated that patients suffering from *Polycythaemia vera* their can have their cells undergo iPSC induction, supporting the idea that these cells are stable *ex vivo* (90). With modern techniques it would be relatively simple to induce such a select mutation into living healthy cells, for example through CRISPR-Cas, which would allow for increased division rates towards RBC if the model is viable *ex vivo* long-term.

It has been well established that during their progenitor stage cells have two options regarding their fate after their next cell division either: 1) continue propagating and by doing so remain at the progenitor stage. 2) Commit to differentiation and work towards terminal cell types (91). This

knowledge opens avenues utilizing signaling molecules to retain cells in their progenitor state and delay the onset of lineage commitment until a desired number of cells have been generated. Some of these signals have already been explored such as proinsulin (92) or combined manipulation through c-Kit and the erythropoietin receptor (93)**.** The downside of this is that culturing would still be reliant upon natural division rates. Meaning that amplification is perhaps not as quick as in other methods described here, but perhaps can be used in combination with methods influencing division speed for more meaningful gains in cell count.

Another method by which changes introduced into haematopoietic or erythroid progenitor cells affect the amount of RBC can be influenced is through immortalization. This would lead to each haematopoietic or erythroid progenitor cell being able to undergo more divisions before entering apoptosis. One method researched to cause immortalization within haematopoietic, or erythroid progenitor cells is through controlled use of the human papilloma virus (HPV), specifically through using HPV16 E6/E7. Through introduction of E6 into cells tumor suppressors TP53 and TP73 are inactivated as they get targeted by the 26S proteasome (94). Whilst E7 plays a part in the inhibition of antiviral and antiproliferative cell functions of INF-α (94). Combining these two functions studies have been able to generate haematopoietic / erythroid progenitor cells immortalized cell lines (43)(94)(23)**.** A downside to this method is concerns that may arise with the usage of the HPV16 as the virus' normal behavior causes uterocervical cancer and exerts a variety of oncogenic effects (95). Since this method would work with the introduction of new genetic information into human cells it might lead unpredicted behavior. A counter argument to this would be that when enucleation occurs happens all genetic information is expelled from the RBC therefore preventing any remnants of HPV16 being introduced into patients. To be certain though more research into this is required. A different possibility to immortalization in haematopoietic and erythroid progenitor cells could be through transfection of cells with *c-Myc* and *BCL-XL* genes, which have shown to increase lifespans of cells by about 1 month (96). According to Hirose the increased lifespan has been attributed to BCL-XL co-expressed with c-Myc as they mediate an anti-apoptotic effect.

One more way in which alteration to HSC could lead to an increase in RBC count is through manipulating their natural quiescence state in order to trigger division more frequently. Various studies have proposed different approaches to altering HSC quiescence. One possible way of reducing quiescence could be through preventing Tie2/Ang1 interactions on HSC surface (97). Other studies have put a focus on Wnt-signaling as a potential target. Reducing Wnt-signaling in HSC leads to increased cell cycling and a decline of regenerative function and reducing p21Cip1 expression (98). Other Wnt-family members might also be of interest as these genes are associated with transcriptional modulation, self-renewal and cell prolific effects. Wnt/β-catenin specifically having been incriminated in maintaining self-renewal (99)**.** These studies demonstrate that novel targets in HSC manipulation are out there and further research will allow for other avenues for affecting haematopoiesis to be uncovered.

These presented pathways showcase that optimizations can be made, on various fronts, to either speed up or scale up the process of culturing RBC.

Discussion

In the coming years the world's blood supplies will be challenged, as emerging infectious disease, and the aging world population will put additional stress on an already strained system. Leading to risks of safely transfusable blood shortages, which can halt important medical procedures and cost lives in the most extreme of cases. Meaning new methods of supporting the traditional donation based must be explored. These alternatives would not only prepare us for risks in our blood supply but can also serve other more niche functions that can widely benefit people, such as stocking up more niche blood groups or allowing patients that undergoing frequent transfusions to suffer less. In this thesis various obstacles and options were presented for the culturing of red blood cells *ex vivo*. Looking into additional applications for cultured RBC, weighing cell sources based on current risks and limitations, cross-referencing defined factors, timeframes and markers necessary to culture RBC, and exploring potential future targets for improvement.

With our current understanding of haematopoiesis the most likely candidate for culturing blood cells would be iPSCs. Even if we consider the risks that come with iPSC such as teratomas. With the possibility to shift to using of cord blood in the future as storage technology advances. Though choosing what cells to use is far from enough to make the RBCs we need, and a single concise protocol to do this has yet to be made as studies have various suggestions. But perhaps the most limiting factor for making such a protocol successful right now is time, the time it takes for cells to grow, divide and differentiate. With that key limitation in mind we explored possible targets that could speed up each of these tree steps in a protocol, however these are mere suggestions and would have to be studied more in depth before drawing conclusions. The purpose of this thesis has been to contribute meaningfully to finding a solution to preserve blood transfusions as a cornerstone of modern medicine. I think that that goal has been achieved, though not giving a perfect solution to the problem the information presented here might help move research closer to that.

Acknowledgement

This thesis could not have been completed without help from outside. Therefore, I would like to extend my gratitude to prof. dr. Rob P. Coppes for his incredible amount of patience while I worked on this piece and his concise feedback during meetings. Furthermore, I want to thank my brother Wouter T. Wink for his help with the flow, word choice and grammatical details. Finally, I wish to thank prof. dr. Ody C.M. Sibon for agreeing to be the second examiner for this piece after having worked with her during the Medical Cell biology research course.

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