Shaping erythropoiesis under in vitro flow

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Chapter 6

General Discussion

Cultured red blood cells (cRBC) are promising for cell therapy applications. This involves transfusion of matched blood to allo-immunised polytransfusees, but also novel applications made possible by technological advancements with RBCs for targeted drug delivery or as antigen vehicles [1]. Taking the limitations of the current donor dependent system into account, the interest in developing a large scale culture system for RBC is very high. As culture upscaling will undoubtedly require some form of stirred bioreactors, this brings up novel questions about mechanical properties of erythroid cells and their physiological response to mechanical stress.

With this thesis I tried to further bridge the gap between mechanical and biological investigations regarding red blood cells (RBCs) and their precursors. A plethora of information is being generated about RBC in fluid physics and molecular biology realms, but often the information remains within the discipline and unequipped for clinical applications. Additionally, with the discovery of mechanosensitive ion channels, the direct translation of mechanical stimulation to physiological regulations are beginning to be unraveled. And with the advancement of bioreactor technologies, robust cell therapy is within reach. In short, understanding the flow mechanics and the implications of mechanical stress on RBC and their precursors are more relevant than ever. In this thesis a range of topics all with potential applications in a large-scale manufacturing process of RBCs or diagnostics of red blood cell defects were covered. Here the expansion, differentiation, filtering, and fill/finish steps of the potential large scale process is discussed. At the end, the implications of a mechanically stimulated culture setup on signalling cascades are explored and the potential path of cRBC to clinic is briefly reviewed.

Expansion of Erythroblasts in a Bioreactor

Considering the amount of RBCs needed for a transfusion unit (about 2×10^{12}), it is clear that employing a bioreactor is necessary (also discussed in detail in the Introduction). In order to achieve such high numbers the proliferative expansion phase must be utilized for best yield. Current *in vitro* static culture of erythroblasts and the *in vivo* expansion in the bone marrow both can be considered as low mechanical stress environments. The physiology and proliferation characteristics of erythroblasts under repeated long-term mechanical stress need to be elucidated before efficient bioreactor systems can be implemented.

In chapter 4, we investigated the effects of mechanical stress on expansion stage erythroblasts. We showed that the mechanical stress in a shaker flask induced similar Ca^{2+}-dependent signalling cascades as direct activation of PIEZO1. We tested the hypothesis by intracellularly chelating Ca^{2+}, and using the direct channel agonist Yoda1. For full confirmation, complete PIEZO1 knockout or direct channel inhibitors need to be utilized. There is currently no known direct PIEZO1-specific inhibitor available. GsMTx-4,

which is sometimes used as an inhibitor, is a molecule that incorporates itself in the lipid membrane dissipating the distribution of tension forces [2]. We did not find the inhibition effects of GsMTx-4 to be stable or consistent for our mechanically stimulated experiments. Currently, research within the group uses Crispr-Cas9 efforts for stable knockout of the PIEZO1 in erythroblasts. Having a knockout would provide evidence whether PIEZO1 is the only, or the major, transducer of mechanical stress on erythroblasts. All in all, understanding specific PIEZO1 kinetics is essential knowledge but knockout or channel inhibition techniques are likely not scalable in an economically viable way. Therefore our efforts focused on mitigating the short and long-term effects of mechanical stress, rather than working towards eliminating mechanosensing altogether.

Ideally a single closed system of culture vessels would be used from start to finish, to minimize the risk of contaminations, low recovery rates or the need for open handlings. We mimicked those conditions, utilizing shaker flasks both for expansion and differentiation stages of the culture in Chapter 4 and 5. To simplify, and keep the shear stress and gas exchange rates comparable throughout, we used similar culture volumes and revolutions per minute (RPM) for both of these experiments, but it is important to note that these parameters have room for optimization. For example, volume could be increased as the cell numbers increase, and the RPM could be increased day by day which will be required in larger culture vessels.

Our lab previously showed that CD14+ monocytic cells help increase the yield of erythroblasts by affecting CD34+CD36- HSPC and CD34+CD36+MEP [3]. One thing to note here is that most CD14+ cells are adherent cells. Non-stationary handling of these cells can result in loss of this population [4,5]. The CD14+ monocytic cells are, however, only required during the first few days after seeding PBMCs. Presently we keep the first stage of the culture in a stationary set up. From the process development point of view, this is not a big issue as the volumes and cell concentrations in the first stage are manageable with a stationary set up (e.g. CellSTACKS by Corning or Nunc Cell Factory by Thermo Scientific). In this first stage minimal handling is required (only one media replenishment on Day2). On Day7, the culture will be transferred into the stirred bioreactor for the expansion phase.

In bioprocesses mass transfer rate (kLa, used for gas to liquid transfer) is used to estimate mixing efficiency. For most cases kLa is used to calculate oxygen supply efficiency in the liquid. For erythroblast cultures, the oxygen consumption rate can be reported as relatively low in comparison to industrial cell lines. O_2 uptake rates of CHO and erythroblasts were measured as $8 \times 10^{-5} \, \mu g/cell$ day and $1.2 \times 10^{-8} \, \mu g/cell$ day respectively [6,7]. However it's important to mention that compared to Baylet et al., we observed a higher O_2 consumption rate for proerythroblasts for our dexamethasone (Dex) synchronized culture (data not shown). The rate decreased with maturation as the mitochondrial mass

reduced and metabolism switched more towards glycolysis (data not shown). In addition to estimating O_2 transfer, kLa can be used as the common denominator for scaling up and estimating mixing necessary for appropriate agitation [8–10]. We approximated the kLa for the setup we utilized for different RPMs and for different volumes (Figure 1). For the experiments from Chapter 4 and 5, kLa was approximated to be around 0.05 s^{-1}. Such calculations can estimate how much faster the orbital shaking needs to be when the culture volume is increased within the same vessel [11]. kLa based approximate scalability is applicable to more complicated bioreactor setups like STRs as well, whether it is calculated or computationally simulated [12,13].
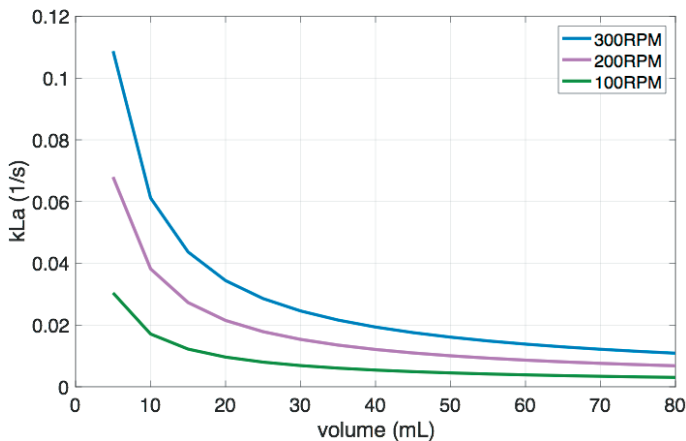
Figure 1. kLa (s^{-1}) for oxygen approximated for 125mL Erlenmeyer flask (Corning) at different RPMs and volumes. Such calculations can be used to determine the suitable RPM for a higher volume (calculated from Klockner 2013[58], via self-written code on MATLAB).

The oxygen consumption is most likely not the limiting factor upon orbital shaking as long as the surface for gas exchange and RPM are sufficient. The cell density of the culture during the expansion phase, however, will be limiting proliferation rates for which the mixing and perfusion capacity of the bioreactor will need to be tested. When erythroblast concentration is above ~2 million/mL in the current static expansion system, spontaneous differentiation can occur [14]. During our preliminary trials with stirred tank reactors (STRs) and optimized perfusion schemes, cell concentrations of ~10 million/mL could be maintained without spontaneous differentiation (data not shown), indicating that the limiting feature is exhaustion of factors or buildup of inhibitory agents in the media. Bioreactors are well suited to control the supply of nutrients or stimuli to the culture thus preventing depletion. Especially in concentrated cultures, the feeding strategy needs to be carefully designed to fit the culture's needs [15]. In general there are three feeding strategies: batch, fed-batch, and continuous [16–18]. In batch systems all nutrients are provided at the beginning of the culture and the seeding density needs to be kept low to provide space for proliferation. Fed-batch systems supply nutrients to the culture as needed, commonly top-up of media is used

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to bring down the cell concentration. Both strategies are limiting as toxins or inhibitors will build up, prohibiting the culture from reaching sufficiently high concentrations. The most promising strategy would be continuous culture. Perfusion systems provide constant supply of fresh media while cell free supernatant is being removed. Continuous feeding can also reduce costs by supplying the specific nutrients that are depleted instead of complete media. For a perfusion setup, a filtering system needs to be established for the removal of supernatant so that the total culture volume can be maintained.

Various filtering methods are available for cell retention in perfusion systems, but it is important to note that most filters were optimized for production cell lines such as CHO cells or recently for lymphocytes for cell therapy. Due to their small size and high deformability, sized-based filters are likely to cause low recovery for cRBCs. In larger scale STRs, hollow fiber filters are used with a diaphragm pump which alternates back and forth (XCell ATF, Repligen). Moreover acoustic retentate filters are increasingly used, as they are non-clogging, provide low shear and can be operated with cultures of a wide range of volumes (e.g., BioSep, Applikon)[19]. These systems are mainly designed with permeate collection in mind, for example antibody production from cell-lines, therefore using these systems for the filtration/purification of the cRBC final product would need further optimization.

**Differentiation of Erythroblasts in a Bioreactor**

Within the scope of this thesis, orbital shaker flasks were utilized as a model bioreactor system, also during differentiation. This scaled down model allowed us to perform various optimization experiments, and investigate the long term implications of non-stationary setup on differentiating cells. For the orbital shaker experiments from Chapter 4 and 5, RPM was chosen to create a high shear, turbulent environment, similar to what can be expected near the propeller region of a STR. Considering the high cell concentrations and the size of the tank necessary for this process, it is expected that high impeller speeds will be used to reach full agitation. We aimed to simulate the high shear regions of a potential STR, to observe the greatest effect. Our studies on employing STRs are ongoing in conjunction with scaled down experiments from shaker flasks, by utilizing these findings in the optimization of our bioreactor.

After the desired cell concentration is achieved with the expansion phase, differentiation can be induced by replacement of media supplemented with Omniplasma, an increased concentration of EPO (5U/ml), and holotransferrin. In particular removal of SCF and Dex is needed to initiate good differentiation [14,20]. In a manufacturing setting, this wash step can be done on functionally closed, automated cell processors (e.g., LOVO, Fresenius Kabi). And then the cells can be placed back into the bioreactor, resuspended in differentiation

media. Alternatively, after inoculation a high perfusion rate could be implemented to remove the media with SCF and Dex, and replace with fresh differentiation media. We show in Chapter 5 that orbitally shaken conditions resulted in accelerated differentiation, so we expect also in a bioreactor setup the mechanical stimulation will yield earlier enucleation, saving on processing time and cost.

Deformability and Final Product Filtration

A mechanically important distinction between erythroblasts and erythrocytes is deformability. Erythroblasts, with their large nuclei, have a relatively low deformability, whereas erythrocytes have very high deformability and can squeeze through vessels and slits in the spleen, much smaller than their own size. We visualized this deformability distinction with an ektacytometry microscope, also called ARCA (Figure 2) [21]. We also quantified the deformability of erythroblasts, mature erythrocytes and glutaraldehyde rigidified erythrocytes on LORRCA (RR Mechatronics) (Figure 3). In Chapter 2 we described RBC fixation with glutaraldehyde, we determined that fixation occurs as a binary process but within a narrow range of glutaraldehyde concentrations pseudo semi-deformable cells could be formed. The term pseudo is used as this low concentration of glutaraldehyde fixation was not permanent. Although those RBCs could be maintained at low deformability, they were not viable in the long term and could be lysed upon washing with distilled water. Terminally fixed RBCs (glutaraldehyde concentrations 0.01% and above) do not lyse upon contact with water. So these semi-fixed RBCs with very low concentrations of glutaraldehyde (0.001% and below) could be used for fluidic studies in the place of erythroblasts. For example, cell-cell collisions of erythroblasts and RBCs can be approximated via stiffened RBCs to study margination and displacement [22]. Although not a direct replica, both exhibit low level deformability and osmotic fragility.
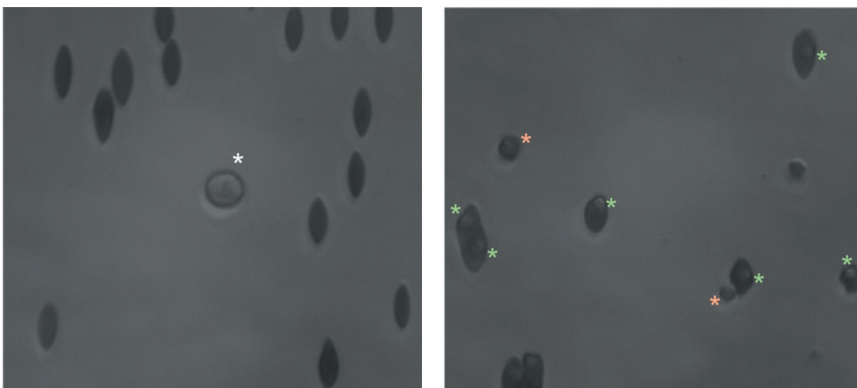
Figure 2. On the left: non-deformable behaviour of erythroblast (marked with white star) amongst mature RBCs. On the right: enucleating erythroblasts with condensed nuclei (marked with green stars) and non-deformable pyrenocytes (marked with orange stars).
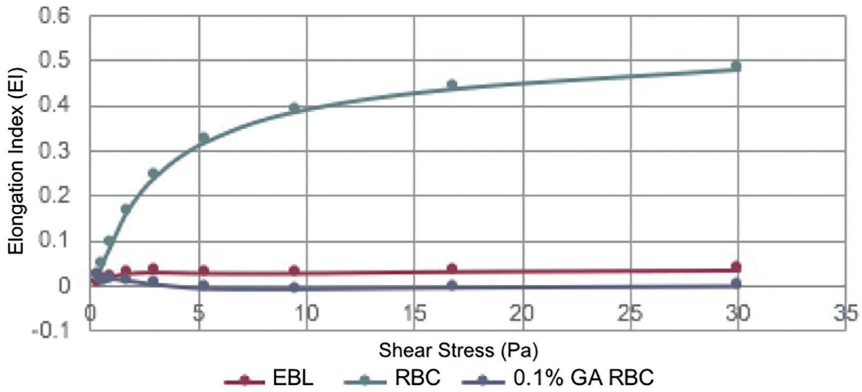
Figure 3. Deformability of erythroblasts (EBL) compared to mature RBCs and glutaraldehyde (GA) treated fixed RBCs.

At the end of the differentiation phase of the culture, the enucleation frequency can exceed 90% of the erythroblasts. Yet, the expelled pyrenocyte, the remaining of nucleated cells, and aggregates must be filtered prior to administration of the blood product to the patient to avoid potential complications like nonhemolytic febrile transfusion reactions (NHFTRs) [23]. Considering the size of the operation needed to produce a transfusion unit, it is very important to start developing robust purification and filtering strategies for the final product.

Leukodepletion filters are routinely used for donor blood transfusions to remove white blood cells. But these filters are designed specifically for donor material, and underperform for cRBCs with ~30% recovery [24]. A potential reason for this can be the disintegration of pyrenocytes at the filtering process, and/or overloading of the system as these filters are designed to filter whole blood with very low percentages of nucleated/low-deformability cells. Ruptured pyrenocytes expose DNA, which result in DNA aggregates of varying sizes, and potentially block the filter [25]. Additionally cRBCs, similar to reticulocytes, can have lower deformability and higher sensitivity to mechanical stress, meaning cells could lyse or simply get stuck to a larger extent in the filter compared to mature erythrocytes [24]. An alternative to deformability based filters could provide great benefits like reducing clogging of the filter and the associated cell loss.

We can expect disintegration of pyrenocytes in a bioreactor as well as shaker flasks. Notably, in orbital shakers we observed some clumps adhered as a ring around the perimeter of the free surface as this is where the centrifugal force is the highest [26]. Disintegrated nuclear material is sticky in nature and it entangles cells in DNA into clumps, similar to what was mentioned by Timmins et al. [25]. A potential improvement could be to incorporate DNAse in the process. For different applications, like rAAV vector production, DNAse digestion has been studied in detail and implemented [27,28]. Our initial "clean up" trials removed the

clumps in the shaker flasks with Pulmozyme (DNAse based drug). Providing transfusion material free of any aggregates or clumps is an utmost patient safety concern, but also preparing the culture for better, non-clogging final filtration is a concern for bioprocessing efficiency.

Currently large processing volumes, up to thousands of liters, are often used for protein or antibody manufacturing. So their downstream processing is designed to retain the supernatant, and remove the cells. This of course is the opposite for any advanced therapy medicinal products (ATMPs). Microfluidic separation has been proposed to mimic upstream and downstream units, as well as filtering systems for bioprocesses [29,30]. By employing laminar flow via microfluidics, high levels of precision for cell retention/separation can be achieved. In Chapter 3, we showed that in a sudden contraction-expansion channel, non-deformable cells could be trapped in the vortices. Multi-orifice channels that are explored for enrichment of circulating tumor cells [31,32], could be employed for separation of nucleated cells and pyrenocytes. Margination of stiffened RBCs, whether due to chemical treatment, similar to Chapter 2, or an infection like malaria, have been investigated in microfluidic channels as well as in vivo [33–36]. The mixture of pyrenocytes and enucleated cRBC can be approximated as a mixture of platelets and RBCs. Both platelets and pyrenocytes exhibit non-deformable behavior and pyrenocytes are roughly twice the size of platelets. Microfluidic studies of platelets and RBC can be indicative of fluid dynamics of pyrenocytes and cRBC. For example, i) Závodszky et al. investigated RBC-platelet collisions [37] ii) Gifford et al., developed a microfluidic concentrator, as a continuous high throughput method for separating platelets from whole blood [38]. Combining the data from RBC-platelet separation studies with multiorifice microchannels, a continuous separation could be designed as a non-invasive filtration method for the final culture.

Effects of Long Term Signalling Due to Mechanical Stress

Excessive mechanical stress can manifest in physical damage to the cell, in the worst case a rupture of the membrane. We wanted to test this limit, by adhering erythroblasts on the bottom of a microfluidic chip, and then flushing the chip with high flow rates. As the shear stress is highest at the walls of the channel, this setup gave us the opportunity to observe erythroblasts under high shear. Shear stress up to ~8.5 Pa was tested without physical disintegration of the cell (Figure 4). Such a high shear stress is unlikely to occur in a bioreactor set up, with the possible exception of the immediate region of the impeller [39,40].
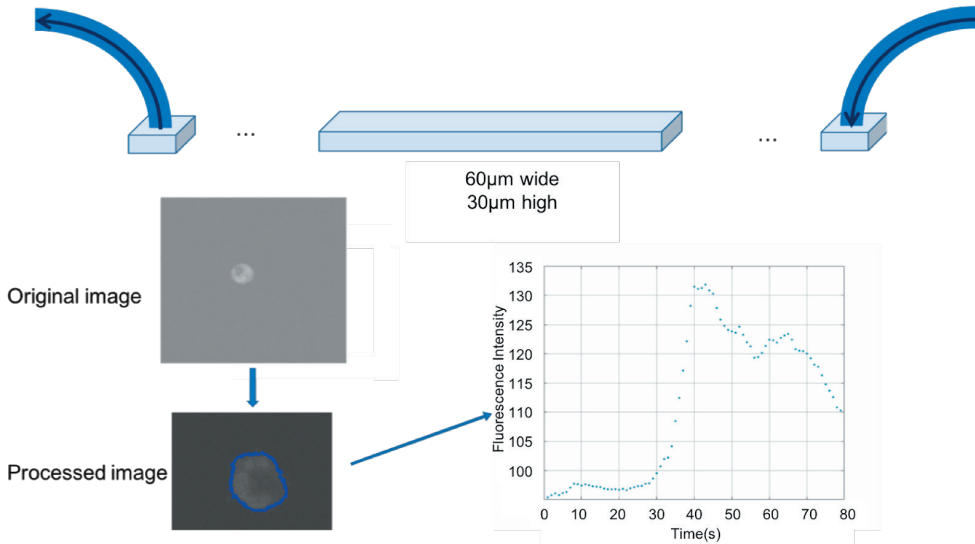
Figure 4. Example of the Ca^{2+} measurements conducted on erythroblasts in a microfluidic channel. Polydimethylsiloxan (PDMS) microfluidic channels were fabricated through standard soft lithography techniques [59]. The channel was then coated with tissue adhesive Cell-Tak, and loaded with a diluted solution (0.1x10^6 cells/mL) of erythroblasts that were pre-stained with Ca^{2+} marker Fluo4 (1μM). After the cells were allowed to settle and adhere at the bottom pressure was applied by fluid flow. A self-written Matlab code was used to trace the cell and process the signal. ~25% increase in fluorescence could be detected indicating a Ca^{2+} influx into this cell.

A more relevant outcome of mechanical stress in bioreactors are the cellular responses downstream of mechanosensitive channels. In this thesis we discussed a number of Ca^{2+} dependent signalling cascades that were activated by mechanical stress, and compared with cells that were stimulated by Yoda1, which is a direct agonist of PIEZO1. We observed a clear relation between mechanical stimulation and dephosphorylation of NFATc2, as well as phosphorylation of ERK and STAT5, all of which could be blocked by intracellular chelation of Ca^{2+} demonstrating a clear connection between Ca^{2+} influx due to mechanical stress and these signalling cascades. The pathways that remained continuously phosphorylated, could make candidates to be used as biomarkers for mechanical stress.

We observed in Chapter 4 that when cultured in an orbital shaker, proliferation was reduced and CD49d/CD235a maturation was increased. Because maturation of erythroblasts ends their proliferative stage, the decrease in total cell yield could result from the accelerated maturation. This idea is strengthened by our findings in Chapter 5. RNA profiles and membrane protein expression levels clearly suggested that differentiation is accelerated in shaker flasks. We hypothesize this physiological regulation could be due to one or more signalling pathways being activated downstream of PIEZO1.

NFATc2 can inhibit erythropoiesis by decreasing KLF expression[41]. In Chapter 4, We observed that Yoda1-induced NFATc2 activation could be blocked by the use of a

Calcineurin inhibitor, FK506. Potentially, such inhibitors could offset the effects of mechanical stress in a bioreactor set up. Similar inhibition of dephosphorylation of NFATc2 was also achieved by intracellular chelation of Ca^{2+}, but this would be a less favourable option for the manipulation of this signalling cascade because: i) Ca^{2+}, as a universal secondary messenger, is likely to affect the homeostasis in multitude of unintended ways, ii) the cell could rapidly replenish its Ca^{2+} with influx from the extracellular space, as the difference between intra and extracellular Ca^{2+} concentrations are very large (~100nM intracellular vs. ~1mM extracellular) [42,43].

In addition, phosphorylation of ERK is known to block terminal differentiation [44,45]. We observed different kinetics for ERK phosphorylation: with Yoda1 stimulation, phosphorylation dissipated after 10 minutes, whereas with the orbital shaker the phosphorylation remained for at least 60 minutes (Chapter 4). We did not observe an inhibition of erythroid differentiation when erythroblasts were differentiated in the orbital shaker. In contrast, we observed faster enucleation and maturation as confirmed by surface markers as well as RNA-seq data (Chapter 5). Based on these findings, we hypothesized that the kinetics of ERK phosphorylation is slower in the orbital shaker compared to Yoda1 stimulation.

Phosphorylation of STAT5 induces proliferation, differentiation, and restrains apoptosis by regulating transcription of genes like Bcl-xL and SOCS proteins that are essential for erythropoiesis [46,47]. STAT5 activation, as well as Bcl-xL overexpression can maintain erythropoiesis even in the absence of EPO receptor [48,49]. In Chapter 4 we observed an increased maturation of erythroblasts during the expansion phase after 4 days of culture in a shaker flask, and in Chapter 5 we observed overall accelerated differentiation. Although mechanical stimulation increased STAT5a/b phosphorylation both during expansion and differentiation (Chapter 4 and 5), we did not observe an increase in the known target genes of STAT5 upon differentiation in the shaker flask. The kinetics of STAT5 activation, and the long term effects on transcription regulation by STAT5 remain to be clarified.

Intracellular Ca^{2+} is tightly regulated by pumps, transporters and Ca^{2+} storing organelles like mitochondria and ER [50]. In mature RBCs such organelles are not present, yet the Ca^{2+} homeostasis is essential, e.g., for RBC volume regulation. Ca^{2+} signalling can modify other ion regulators such as the Gardos channel, regulate the cytoskeleton remodeling, or may control transcription factors and thereby the transcriptome of erythroblasts [51]. Additionally, Ca^{2+} influx can happen through other pathways, namely through glutamate gated N-methyl-D-aspartate receptor (NMDAR) which supports the survival of proerythroblasts [52,53].

The integration of signal transduction pathways that are regulated by Ca^{2+} influx will be different from the individual activation of these pathways as indicated above. Thus,

although activation of, e.g., ERK by itself may inhibit erythroid differentiation, the combination of ERK with other Ca^{2+}-induced pathways may be entirely different. Here we opened the box of mechanical stress-induced signalling pathways and provided a first analysis of the adaptations within erythroblasts on a transcriptional level. The importance of these adaptive responses during erythropoiesis will need to be further investigated to assess the necessity to interfere/fortify those responses to generate cRBC in stirred bioreactor settings.

Regulations and Economics of Scaling Up

Compared to other cell therapies currently being developed, RBC therapies could have a number of advantages regarding regulatory affairs. To begin with, transfusion of donor derived RBCs is routinely performed for about a century. The knowledge on compatibility of transfusion products (e.g., blood group antigens) is enormous, and is primarily applicable to transfusion of cRBCs. Transfusions can be considered low risk, as cRBCs can be expected to be naturally cleared from the body in a maximum of 120 days. Additionally, mature RBCs lack genetic material negating any risk of oncogenic transformation of these cells.

In most developed countries, donor based systems can meet the transfusion need. However, there are multiple shortcomings of a system that is entirely donor dependent, as discussed in the Introduction. Although bioreactor based culture systems can be costly, developing and optimizing these technologies will have extensive impact within the cell therapy field. In this thesis we primarily focused on erythroblasts, but findings regarding the signalling cascades (Chapter 4) and maturation trends (Chapter 5) could be relevant to culture other cell types, like T, B or NK that are derived from multipotent HSCs. It is difficult to bring down the cost of our culture system lower than the donor-derived blood transfusion products for most cases. However in some cases, such as alloimmunized patients that are in need of regular transfusions, it could be one of the few options available. Importantly, RBCs are great tools for drug delivery methods including antigen presentation or enzyme replacement. They can function as vehicles which require large scale culture of RBC albeit that therapeutic loaded erythrocytes may not require a complete transfusion unit.

Currently, genetically engineered cRBC that contain the enzyme PAL are being explored in Phase Ib Trials (RTX-134). These RBC are expected to degrade toxic phenylalanine in the circulation of patients with phenylketonuria (NCT04110496) [54]. For this clinical trial, CD34+ cells were isolated from an O, Rh negative donor and, after lentiviral genetic editing, cultured in bioreactors to produce cRBC. The company behind these systems, Rubius Therapeutics, reported the intention to move up to 1000L bioreactors with their contract manufacturing organization. This therapy would be an alternative to enzyme replacement therapy, where the costs can be comparable or even higher to the projected

costs of large scale cultures of RBCs [55,24]. The same company also has two other clinical trials for which they are currently recruiting patients: (i) RTX-240 in which cRBC are engineered to express 4-1BBL and IL-15, with the goal of inducing an innate immune response for patients with solid tumors and/or AML (NCT04372706) [56], and (ii) RTX-321 in which cRBC express 4-1BBL, IL-12, and HPV-16 for patients with HPV16+ cancers (NCT04672980) [57]. Advancement of bioprocessing technologies can be expected to bring down the cost of in vitro culture rapidly. Not only larger, but more efficient bioreactors are developed and adapted for clinical use. Future availability of up-and-coming technologies, such as genetically modified/loaded RBCs for targeted drug delivery, establish that interest in erythroid cell therapies is growing. The results presented in this thesis aid the development of such products and therapies, by advancing the knowledge of physiological responses to mechanical stress and the biomechanics of RBCs and erythroblasts.

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