EX VIVO BLOOD PRODUCTS MANUFACTURE

A discussion beginning July 2014 between Reg Harris and Rob Thomas¹ with updating notes in 2016

Reg Harris Rob Thomas is doing research towards the in vitro manufacture of various blood cell lineages, including RBCs [red blood cells, erythrocytes]. This leads to investigations relating to [ref: EPSRC Annual Report 2012/13] ‘different types of blood cells’. So, Rob, what cells, exactly, are being focused on?

Rob Thomas I have two main programmes. The first programme is a 5 year EPSRC Fellowship with a team of two researchers + PhDs. This is targeted at developing the underpinning technology to support in vitro blood lineage development (of any type) i.e. stirred tank control modifications etc. We are using a non-RBC lineage as an exemplar in response to clinical demand (favoured target is myeloid population to support cord blood transplant engraftment).

The second is a Wellcome Trust translational fund award to develop manufacturing for first-in-man clinical trials of RBCs. This is a large consortium led by Marc Turner of SNBTS [Scottish National Blood Transfusion Service]. The process is based on a ~month long differentiation from pluripotent cells (embryonic currently and for trial purposes, but intention to move to iP5 for commercial feasibility). This is a transition from my previous work with DARPA [Defence Advanced Research Projects Agency] which was based on CD34+ cells from umbilical cord blood, although the latter stage of the process is similar (the embryonic cells are differentiated to an approximately CD34+ equivalent state over the first 10 days).

RH I imagine that for RBCs the clear track from progenitors to final cell, via -blasts and reticulocytes, confers, in relative terms, a certain straightforwardness in process. Relative, that is, to manufacture of the likes of granulocytes.

RT Yes and No! The multi-step lineage transition is well established and the culture is suspension-based offering advantages in tracking and scalability. However the control required to reproducibly achieve it, and particularly achieve very late stage maturation and survival (i.e. membrane reduction post-enucleation) is not yet fully understood. Also, significant population heterogeneity increases complexity due to feedback between more and less mature cells.

RH So, then, how do you orchestrate, in synchrony, the three lines from the myeloblast? And how do you separate the monoblast line from the myeloblast?

RT I think that the points above have a common answer; most protocols rely on hitting cells with a high level of lineage-selective GFs [growth factors]. EPO [erythropoietin] is clearly key for RBCs. So you are out-competing other lineages through vast relative expansion of selected cell types.

RH As for lymphocytes, are there ways of facilitating the production of T-cells, B-cells and NK [natural killer] cells separately?

¹ Dr Rob Thomas, Centre for Biological Engineering [CBE], Wolfson School of Mechanical and Manufacturing Engineering, Loughborough University, Leicestershire, UK. Deputy Director EPSRC Centre for Innovative Manufacturing in Regenerative Medicine.
RT Yes, there are published protocols that select for various sub-pops – NK cells have received significant attention recently.

RH As for the case of RBCs, possibly platelet production, with a clear run from myeloid SCs, is relatively straightforward....?

RT We have a collaborator focussed on forward programming for platelet production (Dr Cedric Ghevaert, Cambridge University). I think the major issue is enabling their formation from Megs [megakaryocytes] without subsequent platelet activation.

In 2016 RT confirms that this issue is still extant and that platelets in their activated state would render therapy unworkable.

RH Perhaps it’d be appropriate at this point to insert, as a quick reference, a simple picture of the origins and differentiation of the blood cells we’ve been talking about [and a few more]. The following is my best effort using Word:
So, continuing.....possibly ‘manufactured’ blood will, by design, be less than '100% pure', and contain tiny numbers [way way less than normal blood] of all non-erythrocytic cells, and that the necessary numbers would be built up naturally on an as-needed basis in vivo.

RT There is probably no issue with some less mature red cells in the final product. HOWEVER, purification will cause an issue here. Realistically we are going to have to pass through a purification step out of the bioreactor. Currently the only suitable tech is leukofilters that will strip out nucleated cells (and this is very problematic as most ‘manufactured RBCs’ would be stripped out as well due to not being fully mature – even umbilical cord blood has a large proportion of red cells retained by leukofilters). A new separation technology may be needed to achieve this. Alternatively we could just irradiate, but then the benefit of any remaining nucleated cells is lost anyway.....Downstream processing remains an issue here and will largely determine what can be transplanted (even supposing a clinical argument could be made for a heterogeneous population, DSP to address other safety concerns will restrict the options).

RH Another question in my mind relates to whether, in the final clinical setting, where 'whole blood' is prescribed, this needs to contain all cell types, or whether in some instances key cellular contents are able to be injected separately e.g. forget basophils, just inject the heparin and histamine [and serotonin?] independently.

RT Not sure where you’re going with this. Producing whole blood is a non-starter I think due to the complexity/heterogeneity. Producing individual components that can be combined for clinical effect seems plausible, if increasingly expensive(!). Where the clinical effect of a blood cell population is based entirely on secreted factors it may be possible to administer these instead, but you would not have the potential for the longer term paracrine regulation of the factors that a cell transplant could provide.

RH How do you handle the likes of EPO and CSFs [colony stimulating factors]? i.e. adding them in to the pre-manufactured blood, getting the 'mix' and amounts right, process control, etc? Or are they left to handle themselves?

RT There are supplementation regimes throughout the culture process. In the very late stage maturation there are very few if any additives in most protocols beyond base media – cells will release their own cytokines, but most will be removed in downstream wash/formulation. There doesn’t seem to be any requirement to formulate into final product. A DoE [Design of Engineering] approach would be ideal, but this is difficult given shear number of variables and length of differentiation. In all honesty, most protocols have arisen through fairly ad hoc experimentation and are probably sub-optimal. Cytokine QC is a major issue – I know that pharmacopeia descriptions are being discussed for certain cytokines, but batch variation is a massive problem currently where more attention is needed.

RH Ditto relating to haemostasis e.g. designing the manufactured blood to expedite the use of vWF [van Willebrand factor], PAFs [platelet stimulating factors], calcium, clotting factors, etc? [I'm imagining that the body's 'natural' system may be too slow in a supercritical life-and-death clinical situation].

RT Can’t comment on this – not part of my current focus.

RH How, indeed if it's even relevant, do you manage the manufacturing process to account for different blood types?

RT Interesting question. There is obviously a large call for O negative Rh negative universal donor blood. This could be supplied from a single cell line of appropriate genotype. However, the price point is not likely to be viable for some time. High frequency transfusion patients that are sensitised to minor antigens are
more promising, but this requires a very specific starting cell line or some form of GM, and multiple different starting materials. This is part of the economic viability argument currently being developed.

*In 2016 higher-value markets for transfusion medicine are being contemplated.*

**RH** Would your manufactured blood, whether whole or RBCs with specified leukocytes, be offered to the clinic in plasma suspension? And would that plasma be 'standard' or be [able to be] offered with some specified balance of constituent proteins and other solutes?

**RT** Formulation is currently under discussion – it would likely be one of the currently available solutions (i.e. SAGM [saline-adenine-glucose-mannitol]) used for the preparation and storage of donated RBCs – this could potentially be modified to accommodate specific attributes of manufactured product. A problem with solutions for prepping adult donor RBCs is they may not be optimal for manufactured RBCs (or any other source) – this again highlights potential differences in manufactured and donated product, also of relevance to other aspects of downstream processing, where sharing technology with established blood processing may need further review.

**RH note:** Most Hb [haemoglobin]-based O₂ carriers are not stable at room temperature in liquid form. A majority of potential blood substitutes need refrigeration, or freeze-drying, meaning they have to be reconstituted with plasma. Native Hb, being tetrameric, dissociates into dimers in the body that are excreted through the kidneys with a half-life of about 30 minutes. A number of different approaches have been taken to stop this dissociation e.g. polymerisation with glutaraldehyde [pentane-1,5-dial] to make large polymers of Hb that have a ‘respectable’ half-life [18-24 hours in the body]. In Hemopure [made by OPK Biotech from bovine blood], for example, the polymerised Hb is then put into a physiological buffer, which contains salts and lactates to maintain heart function. At this stage the product still contains about 30% stabilised Hb tetramers - and while they may not dissociate in this form, they are still problematic because they can enter blood vessel walls and scavenge NO. NO naturally causes blood vessel walls to relax, so if too much NO is scavenged, relaxation is prevented, making it more difficult for blood to flow, resulting in an increase in BP [blood pressure]. A final clean-up stage removes most of the tetramer Hb, significantly reducing the BP effect. Once transfused into the body, polymerised Hb works in the same way as a RBC i.e. it binds to O₂ in the lungs, carries it to the appropriate site in the body and then releases it. What makes it different from RBCs is that the Hb is free in the plasma - rather than bound within the cell. The advantage of the Hb being in the plasma is that the O₂ it carries is closer to the sides of blood vessels. O₂ binding and release in the plasma is more efficient than in the red cell, so Hemopure releases O₂ more quickly than donor blood once it gets to the target tissue. At end of its life, Hemopure is broken down by the normal biological pathway for Hb.

**RH** Is dried/preserved blood easily and safely rehydrated to its original state?

**RT** A bit out of my domain – not as far as I’m aware.

**RH** I’m trying to picture what, exactly, blood manufacturing capacity might mean. I grapple a bit with producing blood at a greater rate [viz cells per unit time] than in the body’s natural processes. Presumably,
in response to a traumatic event, EPO is produced and acts according to 'need', up to some upper possible limit. Cell differentiation and proliferation occurs with exactly the requisite quanta of energy [always the minimum to do the job], again up to the limit of ATP [adenosine triphosphate] production via the usual intracellular pathways. And the production of CSFs would have its own constraints. This then leads me to ponder whether your mission includes making blood 'faster', therefore making greater volume in a given time or, alternatively, whether you are simply developing a resource [of blood-producing 'machines'] that generates the product at an acceptable [human-like?] rate to add to the accessible pool.

**RT** Realistically the latter. We are currently developing some interesting models of this. The numbers are, as you can imagine, challenging. To get to sensible production we need technology that can sustain cells at higher density with more efficient use of cytokines. Current culture limits in the order of $10^8$/ml still require 20L end volume to produce 1 unit, and this presumes pure production and zero downstream processing loss. As you say, the target is to expose the cells to their optimum level of cytokine for the desired effect, at a concentration of cells that means no cytokine is wasted through time-dependent degradation – this requires waste handling technology... this sort of development is a major thrust of my Fellowship programme.

**RH** Does the technology you’re developing make use of microfluidics? Endothelium-like enclosures? I suppose this is leading me up to asking what Loughborough’s envisioned final manufacturing technology will look like....?

**RH Note**......and this is linked with other points above.....one biotechnology firm [which will remain unidentified here] reports that it has demonstrated proof of concept for taking umbilical cord cells, expanding them and differentiating them [into RBCs] in useful volumes. The firm says the first step is to isolate haematopoietic stem cells from an umbilical cord. They are then cultured on ‘Nanex’ technology, which is a polymer nanofibre substrate that expands the stem cells much more rapidly than normal cell culture techniques. The firm reports that In 10 days there’s a 300 to 350-fold increase in cells and over a month hundreds of thousands, or a million-fold increase. Its second step is to recreate the differentiation process [using ‘special’ bioreactor technology] that occurs normally in the body, in vitro. Using a special cocktail of GFs that can be changed over the course of several weeks, most of these stem cells are driven to form RBCs that are functionally indistinguishable from the RBCs that can be produced natively. Finally, the cells are filtered and put in plasma or fluid before they can be transfused directly into the body. The end goal is to carry out the complete process onsite in remote areas such as in hospitals in war zones. As the firm would

In 2016 RT says that if it were possible to produce cells at the rate that the body can we would not need to do anything. But in a manufacturing situation there are inhibitory factors in play that have not been identified. He refers to a *Journal of Tissue Engineering and Regenerative Medicine* 2016 paper [Bayley, R; Ahmed, F; Glen, K; McCall, M; Stacey, A; Thomas, R] ‘The productivity limit of manufacturing blood cell therapy in scalable stirred bioreactors’ which, in its abstract, states that ‘.....the properties of erythroblasts are such that the conventional constraints on cell manufacturing efficiency, such as mass transfer and metabolic demand, should not prevent high intensity production; furthermore this could be achieved in industry standard equipment. However, identification and removal of an inhibitory mediator is required to enable these economies to be realised’. RT’s work is looking to address this challenge.
The ‘functionally indistinguishable’ claim is a difficult one to sustain. It is obviously limited to the range of assays that are conducted in vitro. A clinical trial with recovery to assess circulation time would be required to confirm this in addition to a wide range of in vitro functional and identity assays.

I believe we need to retain as much proven manufacturing technology as we can with built in control systems – basic stirred reactors for example, but engineer in additional control as required – the latter in terms of GF presentation etc is, again, a major aim of my Fellowship work. Having worked closely with a number of systems that attempt to ‘mimic’ in vivo environments with complex structure I feel that they often do not account for pragmatic upscale manufacturing requirements, and likely monitoring and control requirements. There is also a tendency to start from the premise that a lot of complexity is necessary, without attempting to define the components of complexity required and engineer into the simplest, and therefore most cost effective/reproducible, scalable environment.

In 2016... a Biotechnology Journal 2016 paper [Worrallo, MJ; Moore, RL; Glen, KE; Thomas, RJ] ‘Immobilised hematopoietic growth factors onto magnetic particles offer a scalable strategy for cell therapy manufacturing in suspension cultures’ confirms in its abstract that ‘Hematopoietic therapies require high cell dosages and precise phenotype control for clinical success; scalable manufacturing processes therefore need to be economic and controllable, in particular with respect to culture medium and growth factor [GF] strategy’. The aim of this work was ‘to demonstrate the biological function, and integration within scalable systems, of a highly controllable immobilised growth factor (iGF) approach’. The authors state that ‘This immobilisation approach has the potential to reduce the manufacturing costs of scaled cell therapy products by reducing GF quantities and offers important process control opportunities through separation of GF treatments from the bulk media’. 

~///~