Plasticell

Differentiation of human ES cells into megakaryocytes and platelets in protocols derived by multiplexed CombiCult technology.

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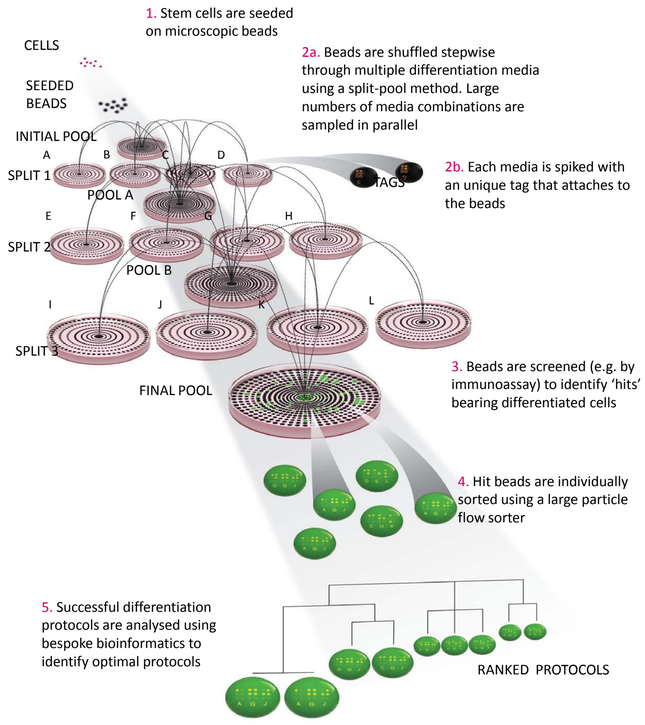
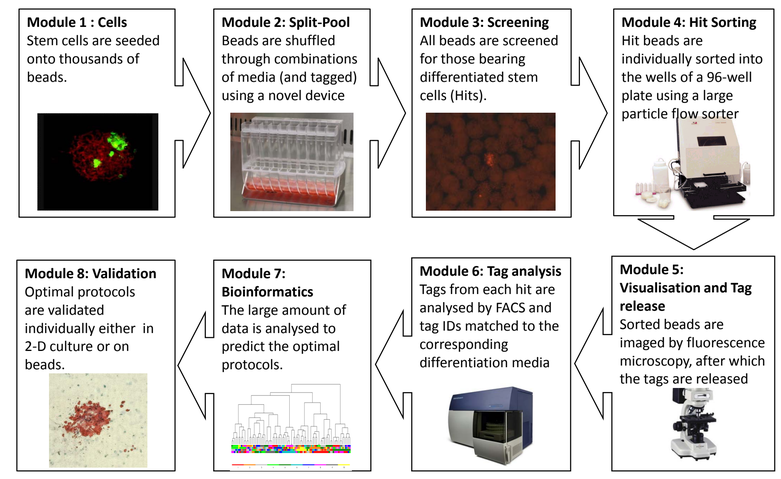
1. Introduction:

- **Combinatorial Cell Culture™ (CombiCult™) is a bead-based, combinatorial technology specifically developed for discovery of novel stem cell differentiation protocols.**
- **Stem cells on beads are exposed to multiple combinations of media, containing active agents such as growth factors or small molecules. The optimal combinations for effective differentiation can be deduced reliably, rapidly and cost effectively.**
- **A disposable split-pool device that enables efficient bead manipulation, and a custom developed bioinformatics software for rapid and reliable protocol selection have accelerated CombiCult™ workflow.**
- **CombiCult™ has been used successfully by many customers and collaborators, validating the technology and showing proof of its many applications:**
  - **Finding multiple new protocols**
  - **Obtaining higher quality/yield of cells**
  - **Reducing cost/time of cell manufacture**
  - **Eliminating undefined variables (e.g. serum), growth factors (e.g. using small molecules) or animal derived components**
  - **Elucidating the importance/timing of signalling pathways**

2. CombiCult™ Technology:



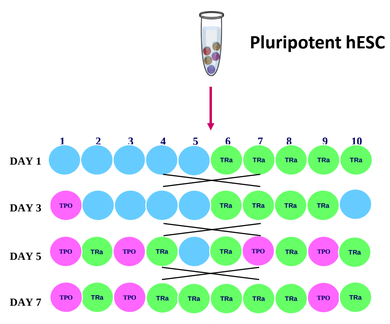
3. CombiCult™ Workflow:



4. Combicult™ Screen for protocols that direct differentiation of hES cells into megakaryocytes and platelets in the presence of TPO receptor agonist

- **Human embryonic stem (hES) cell derived hematopoietic progenitors represent a renewable source of material for use in screening and evaluation assays in the search for novel therapeutic and regenerative drugs. Here we describe the discovery of novel serum-free, feeder-free protocols that direct differentiation of hES cells to megakaryocytes and platelets.**
- **Additionally, these protocols feature the replacement of commonly used cytokines with small molecule bioactives.**
- **Optimal differentiation protocols were discovered using a high throughput CombiCult™ screen:**
  - **Human ES cells SHEF 1 were grown on microcarriers and shuffled randomly through 40 different culture conditions, with concomitant labelling of the beads using nanomaterial tags.**
  - **A small molecule TPO receptor agonist was included in about half of the tested media compositions.**
  - **Ten thousand distinct protocols were sampled in one experiment (Fig 1).**
  - **Following screening to identify beads bearing cells positive for the megakaryocyte specific marker cd41a (Fig 2) and analysis of the tags to deduce cell culture history, 85 unique protocols were identified (Fig 3 (A)).**
  - **Candidate protocols were ranked using a bespoke bioinformatics program Ariadne™ (Fig 3(B&C)).**
  - **Top ranking protocols were further validated in immunostaining, FACS analysis and MegaCult colony forming assays.**

Fig 1. Overview of Combicult™ screen
40 media compositions were tested in 10,000 combinations over a 4-step, split pool experiment – testing a total of 10,000 different protocols. Green depicts media containing TPO receptor agonist, pink - media containing TPO, blue - not containing either.



DAY 16- Detection of Megakaryocytes on microcarriers with cd41a antibody

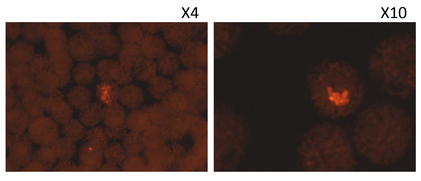


Fig 2. Examples of 'hit' beads picked for tag analysis
On Day 16 cells on microcarriers were stained with cd41a/AlexaFluor594 antibodies. Individual microcarriers bearing cd41a positive cells – 'hit beads' - were picked for tag analysis

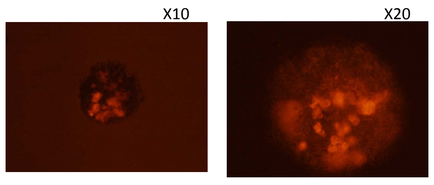
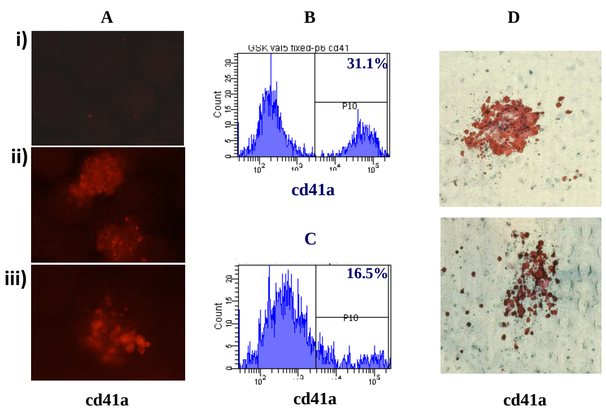


Fig 3. Protocol analysis by Ariadne™
The culture history of each hit bead was deduced from analysis of tags. The significance of each protocol was determined using Plasticell's Ariadne software.
(A) Diagram showing overlay of all positive protocols. The height of each rectangle corresponds to the number of hits generated by that culture medium and the opacity of the linkage lines is proportional to the number of hits generated by a particular combination of two media. (B) Dendrogram shows the hierarchical clustering of protocols. Colours on dendrogram refer to the media compositions used in any one split. (C) Similarity Matrix is a pair-wise comparison of each protocol with every other protocol

Fig 4. Validation of selected protocols
Differentiation into megakaryocytes was assessed by a variety of detection assays:

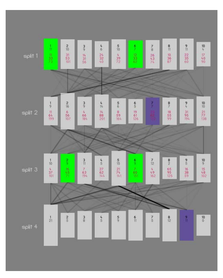
A. Immunostaining on beads .
i) AlexaFluor595 only (X10);
ii) anti-cd41a/AlexaFluor595 (X10);
iii) anti cd41a/AlexaFluor595 (X20).
B. Immunostaining and FACS analysis of cells shed off beads. Gates were defined by staining of the samples with corresponding isotype controls.
C. Immunostaining and FACS analysis of cells differentiated in MethoCult media.
D. Colony formation in collagen based MegaCult media. Cells were differentiated on beads until day 9 then seeded into collagen-based semi-solid media supplemented with the cytokines specified in the final step of the differentiation protocol. Megakaryocyte colonies were stained with cd41a/Alkaline Phosphatase kit (StemCell Technologies).



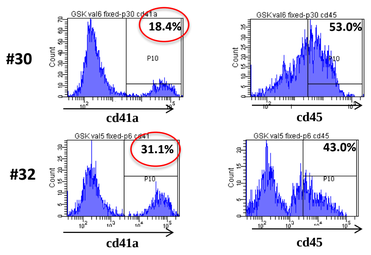
5. Characterisation of megakaryocytes and platelets derived from hES cells by CombiCult protocols

Fig 5. CombiCult screen uncovered highly efficient protocols for production of mature megakaryocytes from hES cells

Example: X/7/X/9
Protocol #30: 1/7/6/9
Protocol #32: 6/7/2/9



(i) High proportion of cd41a and cd45 positive cells (FACS analysis)



(ii) High number of colonies in MegaCult

Protocol	# of cd41+ Colonies in MegaCult*/per 10^5 cells
#30	173
#32	313

(iii) CombiCult derived protocols produce mature megakaryocytes positive for both cd41a and cd42b markers and generate platelets in MegaCult

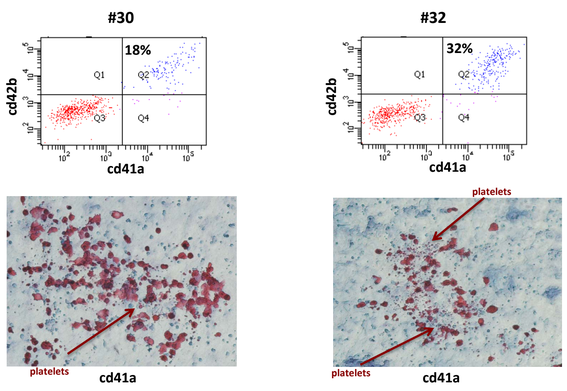


Fig 6. TPO agonists promote hES derived megakaryocyte differentiation in vitro
The efficiency of megakaryocyte differentiation was highly dependent on presence of TPO or TPO receptor agonist (A/B) in the final stage of differentiation. Replacement of TPO in final media with another TPO agonist compound (labelled B) led to increased yield of cd41a positive cells as shown by MegaCult assay (A) and FACS analysis (B).
✦ - marks original protocols.

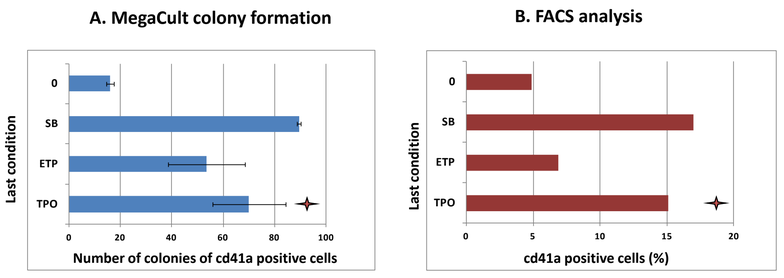
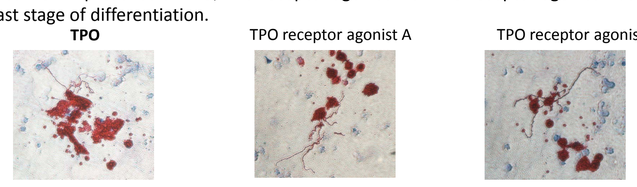


Fig 7. Megakaryocytes derived from hES cells in presence of small molecule TPO agonists produce platelets in vitro

A. cd41a staining of Megakaryocytes/platelets in MegaCult assay. Colonies of megakaryocytes/platelets were formed from progenitor cells differentiated following protocol #32 in presence of TPO, TPO receptor agonist A or TPO receptor agonist B at the last stage of differentiation.



B. FACS analysis of platelets produced by megakaryocytes derived from hES cells by protocol #32 in presence of TPO, TPO receptor agonist A or TPO receptor agonist B. After 13 days of differentiation on microcarriers megakaryocytes were placed on OP9 cells for platelet production. ES derived platelets were gated on size using human blood platelets.

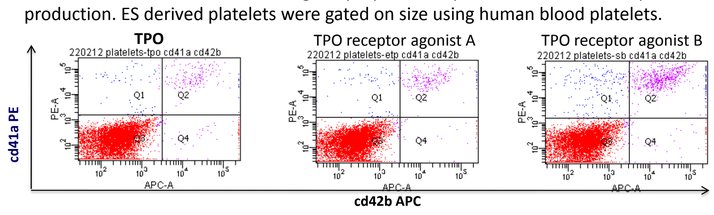
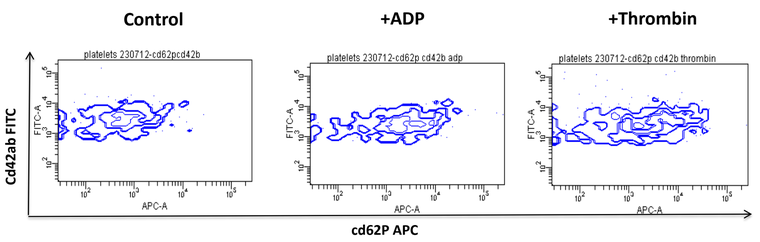


Fig 8. Megakaryocytes derived from hES using CombiCult protocols produce functional platelets
Platelets collected from Megakaryocytes/OP9 co-culture were incubated with 20uM ADP or 1 unit/ml thrombin. Expression of cd62P (P-selectin) (marker of activated platelets) was monitored by FACS analysis. Platelets were gated on size and cd42b expression.



Summary:

1. **Plasticell's screening technology, CombiCult™, was used to screen 10,000 combinations of 40 media compositions in order to discover optimal serum-free, feeder-free protocols for the differentiation of hES cells into megakaryocytes and platelets. Small molecule TPO receptor agonist was included in about a half of the tested media compositions. 85 candidate differentiation protocols were identified by the screen.**
2. **Candidate protocols were ranked using a bespoke bioinformatics program Ariadne™. Validation of the 17 top ranking protocols identified 8 novel protocols that generated megakaryocytes in more than two validation assays.**
3. **Two most efficient protocols were chosen for further characterisation. These protocols yielded high proportion of mature megakaryocytes that generated functional platelets when placed onto OP9 feeder cells or in semi-solid collagen based media.**
4. **The efficiency of megakaryocyte differentiation was highly dependent on the presence TPO or TPO receptor agonist in more than one (notably the final) stage of differentiation (Fig 6), making these protocols suitable for further development of an hES-based assay for screening and evaluation of novel TPO receptor agonists.**