UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Principi e Impianti di Ingegneria Chimica "I. Sorgato"

SCUOLA DI DOTTORATO DI RICERCA IN BIOLOGIA E MEDICINA DELLA RIGENERAZIONE

INDIRIZZO INGEGNERIA DEI TESSUTI E DEI TRAPIANTI

CICLO XXIII

MULTISCALE TECHNOLOGIES FOR HUMAN STEM CELL CULTURE IN SUSPENSION: APPLICATIONS FOR HEMATOPOIETIC AND CARDIAC DIFFERENTIATION

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Table of contents

Table of contents i

Sommario v

Introduzione ix

Summary xiii

Foreword xvii

Introduction xix

Chapter 1

Stem cells: clinical relevance and limitations 1

1.1 Introduction 1

1.2 Stem cells 2

1.2.1 Embryonic stem cells 2

1.2.1.1 Embryonic stem cell research in Italy *5*

1.2.2 Adult stem cells 5

1.2.3 Induced pluripotent stem cells 6

1.3 The clinical potentialities of hematopoietic stem cells 7

1.3.1 Recent perspectives for the obtainment of HSCs 8

1.3.2 Umbilical cord blood: a promising source of HSCs for transplantation 9

1.3.3 HSCs in vitro expansion 10

1.4 Tissue engineering of cardiac tissue 10

1.4.1 In situ cardiac tissue engineering 12

1.4.2 In vitro engineering of cardiac tissue constructs 12

1.4.3 Scaffold-free cardiac tissue engineering 13

1.4.4 In situ cellular cardiomioplasty 13

1.5 From discovery to clinical use 14

1.6 Aim of the thesis 15

1.7 References 16

Chapter 2

Design of a stirred bioreactor for human stem cell expansion 27

2.1 In vitro stem cell culture methods 27
2.1.1 Static cell culture 27
2.1.2 Dynamic cell culture: the bioreactors 28
2.2 In vitro hematopoietic stem cells expansion 30
2.3 The six-well stirred bioreactor 31
2.3.1 The bioreactor design 31
2.3.1.1 The stirring system 33
2.3.1.2 The gas inlet system 35
2.3.2 Study of oxygen solubility 35
2.4 Rational approach 36
2.5 Experimental plan and set up 36
2.5.1 Oxygen probe calibration 37
2.5.2 Oxygen solubility measurements 37
2.5.3 Oxygen transport 37
2.6 Results and discussion 38
2.6.1 Oxygen solubility 38
2.6.2 Transient of oxygen concentration 39
2.7 Preliminary biological experiments 40
2.8 Conclusions 40
2.9 References 42

Chapter 3

Application of the bioreactor for HSC expansion in hypoxic conditions 45

3.1 The stem cell niche and oxygen concentration 45
3.2 The role of oxygen in the maintenance of HSCs 46
3.3 Rational approach 48
3.4 Experimental plan 49
3.4.1 Umbilical Cord Blood derived CD34+ culture 49
3.4.1.1 Cell selection 49
3.4.1.2 In vitro cell culture 50
3.4.1.3 Cell characterization 50
3.4.2 Experiments at 20% oxygen partial pressure 51
3.4.2.1 Results and discussion 52
3.4.3 Experiments at 5% oxygen partial pressure 57
3.4.3.1 Results and discussion 57

3.5 Conclusions 59
3.6 References 61

Chapter 4

Biomaterials for human stem cell culture 65

4.1 Introduction 65
4.2 Hydrogels 66
4.2.1 Photopolymerization 67
4.3 Biomateral for single cell dispersion culture 67
4.3.1 Hyaluronic acid based hydrogel preparation 68
4.3.2 Analysis of hydrogel stability 69
4.3.2.1 Results and discussion 69
4.3.3 In vitro analysis of cell viability (cells cultured in monolayer) 72
4.3.3.1 Results and discussion 72
4.3.4 In vitro analysis of cell viability (cells cultured in 3D) 73
4.3.4.1 Results and discussion 73
4.4 Biomaterial for confined cell clusters culture 75
4.4.1 Polyacrylamide hydrogel preparation 75
4.4.1.1 Glass slide functionalization 75
4.4.1.2 Microstructured hydrogel photopolymerization 77
4.4.1.3 Hydrogel sterilization 78
4.4.2 Characterization of the polyacrilamide hydrogel 78
4.4.3 Optimization of the microstructured polyacrilamide hydrogel 79
4.4.3.1 Array design 79
4.4.3.2 Modulation of microwells depth 80
4.4.4 In vitro analysis of cell viability (cells seeded onto the microwells 82
4.4.4.1 Results and discussion 82
4.5 Conclusions 83
4.6 References 85

Chapter 5

Application of microstructured hydrogel for hES differentiation pattern control 87

5.1 Motivations and aim of the research 87
5.2 Cardiogenesis and embryonic cardiac progenitors 89
5.3 Cardiac differentiation of human embryonic stem cells in vitro 90

5.3.1 Stage protocol for hES-EBs cardiac differentiation 92
5.3.2 EB size control 95
5.3.3 Extracellular matrix interactions control 96
5.3.4 Cell-cell interactions control 96
5.3.5 Soluble factors control 97
5.4 Experimental plan 97
5.4.1 Results and discussion 98
5.4.1.1 Human embryonic stem cells in vitro culture 98
5.4.1.2 Generation of HES2-EBs 102
5.4.1.3 Microwells cell seeding 103
5.4.1.4 Cell viability assay 104
5.4.1.5 EBs size distribution 105
5.4.1.6 QPCR analysis 108
5.4.1.7 Microarray analysis 110
5.5 Future development: extension to iPS cells 112
5.6 Conclusions 113
5.7 References 115

Chapter 6
Conclusions 121

6.1 References 125

APPENDIX A "Norme in materia di procreazione medicalmente assistita" 127

APPENDIX B Parere del Comitato Nazionale per la Bioetica su ricerche utilizzanti embrioni umani e cellule staminali 137

APPENDIX C "Financing for research on embryonic stem cells: The situation in Italy and its origins" 143

APPENDIX D Extraction of RNA from hES – derived embryoid bodies 149

APPENDIX E Microscale in vitro model for screening pathological conditions on human embryonic stem cells-derived cardiomyocytes 153

Sommario

L'impiego di cellule staminali per scopi terapeutici è stato proposto per la rigenerazione o la sostituzione di tessuti danneggiati o malfunzionanti; tuttavia, i potenziali benefici presentano limitazioni legate principalmente alla scarsa disponibilità di cellule staminali e ad aspetti di sicurezza clinica connessi alla qualità delle cellule. L'ottimizzazione dei processi di espansione e differenziamento delle cellule staminali umane è una sfida sia ingegneristica che biologica.

Scopo del progetto di ricerca è lo sviluppo di tecnologie per la coltura di cellule staminali umane. In particolare, sono state sviluppate nuove metodologie che mettono insieme sistemi convenzionali di coltura con biomateriali e microtecnologie per migliorare l'efficienza e la qualità dei prodotti finali dei espansione e differenziamento delle cellule staminali. Le metodologie sono state progettate per due applicazioni: la prima consiste in un bioreattore a sei pozzetti con un volume di 10ml/pozzetto, per colture in sospensione di cellule staminali ematopoietiche derivate dal cordone ombelicale; la seconda è un hydrogel microstrutturato per il controllo del differenziamento cardiaco di corpi embriodi derivati da cellule staminali embrionali umane.

Un obiettivo generale è sfruttare i piccoli volumi richiesti dalle tecnologie proposte per la valutazione simultanea di un'ampia gamma di condizioni di coltura, soluzione che presenterebbe costi proibitivi con volumi di centinaia di millilitri.

Descrizione del progetto

Applicazione per il differenziamento ematopoietico

Nonostante gli sforzi compiuti nella ricerca sulle cellule staminali, le terapie cellulari non sono ancora applicate ampiamente in clinica, perché forti limitazioni riducono i loro potenziali benefici. In quest'ottica devono essere risolte alcune criticità, quali per esempio la necessità di avere un numero sufficiente di cellule che presentino il fenotipo appropriato e svolgano le funzioni biologiche desiderate.

Una prospettiva particolarmente promettente per quanto riguarda la terapia cellulare è il trapianto di cellule staminali ematopoietiche: questa soluzione presenta infatti ampia applicazione nel trattamento di tumori maligni e malattie ereditarie del sangue, così come

nella sostituzione di cellule danneggiate in seguito a trattamento chemioterapico. Le cellule CD34+ derivate da sangue di cordone ombelicale costituiscono una risorsa di cellule staminali ematopoietiche utilizzabili per trapianti. Tuttavia, sia per l'esiguo numero di cellule che possono essere ottenute da una singola unità di cordone, sia per la difficoltà di controllare il fenotipo cellulare risultante a seguito di protocolli di espansione *in vitro,* l'attuale impiego terapeutico di cellule staminali ematopoietiche isolate da sangue di cordone ombelicale è rivolto soltanto a pazienti pediatrici.

Per affrontare questa sfida, è stato realizzato un bioreattore con un sistema di agitazione per espandere cellule CD34+ derivate da sangue di cordone ombelicale e studiare gli effetti dell'ipossia nell'espressione di marcatori di staminalità. E' noto infatti che l'ossigeno favorisce il rinnovamento cellulare durante l'ematopoiesi.

Il sistema di mescolamento è stato realizzato in modo da poter essere applicato su di una piastra convenzionale a sei pozzetti, sia per uniformità con i sistemi di coltura statici convenzionali che per la conservazione dei protocolli di coltura. I volumi del bioreattore (10 ml/pozzetto) sono adatti per l'espansione delle cellule e per analisi multiparametriche mediante tecniche di citofluorimetria.

Il sistema di mescolamento favorisce l'omogeneità all'interno del volume di coltura, permettendo così di poter controllare la concentrazione di ossigeno disciolto nell'immediato microambiente cellulare. Non sarebbe possibile effettuare un controllo di questo tipo in condizioni di coltura statiche, nelle quali si riscontrerebbero gradienti di concentrazione nel medium.

Il bioreattore è stato anzitutto testato sulle cellule in atmosfera al 21% di ossigeno per verificarne la biocompatibilità e poter escludere eventuali effetti negativi sulle cellule dovuti all'agitazione; i risultati sono stati messi a confronto con quelli ottenuti in condizioni statiche. Verificati tali aspetti, il sistema è stato utilizzato al 5% di ossigeno per studiare gli effetti dell'espressione di c-kit nelle cellule CD34+, utilizzando le cellule coltivate in atmosfera al 21% come controllo.

Mentre è noto l' importante ruolo del c-kit nell'ematopoiesi, le dinamiche di interazione tra c-kit, l'ipossia, e lo Stem Cell Factor nelle cellule staminali ematopoietiche non sono ancora stati definiti.

Analisi di citofluorimetria evidenziano che cellule CD34+ coltivate in condizioni di ipossia rimangono vitali nel corso dell'intera durata degli esperimenti e presentano una maggiore espressione del c-kit rispetto alle cellule coltivate nelle condizioni di coltura convenzionali.

In questo studio, sfruttando il vantaggi offerti dall'impiego di tecnologie avanzate che hanno consentito di ottenere un ambiente di coltura altamente definito da un punto di vista chimico – fisico, siamo stati in grado di esplorare la relazione tra l'espressione di c-kit e le condizioni di ossigeno. Questi risultati aprono nuove prospettive nello studio dei meccanismi di interazione tra l'ossigeno e le vie biologiche dipendenti da c-kit a livello molecolare.

Applicazione per il differenziamento cardiaco

Il trapianto di cellule sta emergendo come possibilità promettente anche per la sostituzione di tessuto cardiaco danneggiato o malfunzionante in un cuore malato. Tuttavia, l'ottenimento di un numero adeguato di cardiomiociti richiesti in clinica è ostacolato dalla limitata disponibilità di questo tipo di cellule e dalla loro scarsa capacità proliferativa.

Cellule staminali embrionali umane, in grado di rinnovarsi indefinitamente mantenendo inalterata la loro capacità di differenziare in tutti i tipi cellulari dei tre foglietti germinativi, compresi i cardiomiociti, rappresentano una delle fonti più promettenti per lo sviluppo di nuovi approcci alla terapia cellulare. L'impiego di cellule staminali embrionali umane è limitato dal lento progresso nello sviluppo di protocolli per l'espansione e il differenziamento *in vitro*.

In questo contesto, abbiamo progettato un sistema di coltura che ci consentisse di esplorare gli effetti del microambiente cellulare sul differenziamento delle cellule staminali embrionali umane. *In vivo*, le cellule risiedono in nicchie specifiche proprie dei tessuti dei quali fanno parte e interagiscono con numerosi altri fattori, inclusi la matrice extracellulare, cellule vicine, e fattori solubili, mentre cellule isolate in sistemi di coltura convenzionali sono esposte ad un ambiente molto diverso rispetto a quello *in vivo*.

In particolare, abbiamo sviluppato un array di micropozzetti su hydrogel come piattaforma sperimentale. E' stato scelto l'hydrogel in poliacrilammide per la sua biocompatibilità, l'alta permeabilità, la capacità di assorbire grandi volumi d'acqua - requisiti fondamentali per ottenere un ambiente adatto a colture cellulari - per la superficie non aderente - adatta alla coltura in sospensione di corpi embriodi derivati da cellule staminali embrionali umane - e perché la geometria (di questo tipo di substrato) è facilmente modulabile.

Nel nostro sistema, è possibile ipotizzare che la diffusione di fattori solubili nell'hydrogel tridimensionale porti a un accumulo dei fattori stessi in tempi rilevanti per i processi di proliferazione e differenziamento cellulare. Nelle condizioni di coltura descritte, l'aggregazione di corpi embriodi è impedita e singoli corpi embrioidi possono essere isolati e raccolti con una micropipetta per ulteriori analisi, senza interferire con la coltura. Nello

studio, è stato analizzato il profilo di differenziamento dei corpi embrioidi confinati nei microwells di diversa profondità (450 μm e 1 mm) a confronto con quello delle cellule coltivate su piastre non aderenti convenzionali.

L'analisi dell'espressione genica dell'intero genoma mediante microarray dimostra che il controllo della disponibilità locale di fattori endogeni nel microambiente cellulare induce differenze significative nel differenziamento delle cellule staminali embrionali umane. IN particolare, il confinamento dei corpi embrioidi nei micropozzetti promuove l'espressione di geni coinvolti nei processi di specificazione della compartimentazione dell'embrione, mentre sistemi di coltura convenzionali promuovono l'espressione di geni coinvolti nello sviluppo del cuore. Ulteriori esperimenti sarano necessari per comprendere in modo approfondito i meccanismi alla base dei risultati ottenuti e per studiare la distribuzione spaziale di marcatori dei tre foglietti germinativi.

La tecnologia dei micropozzetti può essere utilizzata per ottenere un alto numero di corpi embrioidi separati e per valutare gli effetti del diverso microambiente sul differenziamento delle cellule. Questa tecnologia è inoltre adatta per modulare la disponibilità di fattori morfogenici e chiarire i meccanismi del differenziamento delle cellule staminali. I piccoli volumi richiesti per ottenere centinaia di corpi embrioidi rendono questo approccio uno strumento da prendere in considerazione quando si vogliano sviluppare protocolli di differenziamento *in vitro*.

Introduzione

L'uso di cellule staminali per il trattamento di malattie degenerative, congenite o dello sviluppo, per le quali non sono disponibili cure adeguate rappresenta uno tra gli scenari più promettenti per la medicina rigenerativa. Nonostante gli sforzi compiuti dalla ricerca in questa direzione, la terapia cellulare non è diffusamente applicata, poiché pesanti limitazioni ne ostacolano i potenziali benefici. Per future applicazioni in ambito clinico, sarà necessario anzitutto superare le difficoltà legate ad alcuni aspetti generali, quali ad esempio la necessità di disporre di un numero sufficiente di cellule che mantengano un fenotipo appropriato ed espletino le specifiche funzioni biologiche richieste. Se i pazienti potranno trarre beneficio o meno dI queste potenziali strategie terapeutiche, dipenderà in ultima analisi dallo sviluppo di tecnologie per ottenere cellule nelle quantità e qualità richieste, in una maniera affidabile ed economicamente sostenibile.

La mia ricerca si concentra sullo sviluppo di tecnologie per la coltura e il differenziamento di cellule staminali umane in condizioni di coltura definite e controllate.
La natura dello studio qui presentato è fortemente multidisciplinare: la stretta collaborazione con gli ingegneri del laboratorio, che hanno contribuito alla progettazione e alla realizzazione delle tecnologie utilizzate, ha permesso di applicare sistemi innovativi alla coltura di cellule rilevanti per la ricerca, quali le cellule ematopoietiche e le staminali embrionali umane. Obiettivi specifici della tesi sono la realizzazione di innovativi sistemi di coltura *ad hoc* per 1) l'espansione di staminali ematopoietiche in condizioni di ipossia e 2) il controllo del differenziamento cardiaco di corpi embrioidi derivati da cellule staminali embrionali umane.

Lo schema della ricerca è riportato nella Figura 1.
I passi principali per l'espansione delle cellule ematopoietiche CD34+ in condizioni di ipossia sono: la selezione, la coltura e la caratterizzazione di cellule CD34+ derivate da sangue di cordone ombelicale; la progettazione e lo sviluppo di un bioreattore con sistema di agitazione per la coltura di cellule staminali; l'applicazione delle tecnologie alle cellule; lo studio degli effetti dell'ipossia e infine, l'analisi dei risultati in termini di vitalità cellulare, grado di proliferazione ed espressione di marcatori specifici di differenziamento.
Le fasi principali del controllo del differenziamento cardiaco di corpi embriodi derivati da cellule staminali embrionali umane sono: l'espansione e la caratterizzazione di cellule

staminali embrionali umane; lo sviluppo di un hydrogel microstrutturato per la coltura di corpi embrioidi; l'applicazione dei nuovi strumenti tecnologici alle colture cellulari; lo studio degli effetti del controllo del microambiente cellulare sul differenziamento dei corpi embriodi e infine l'analisi del differenziamento cellulare.

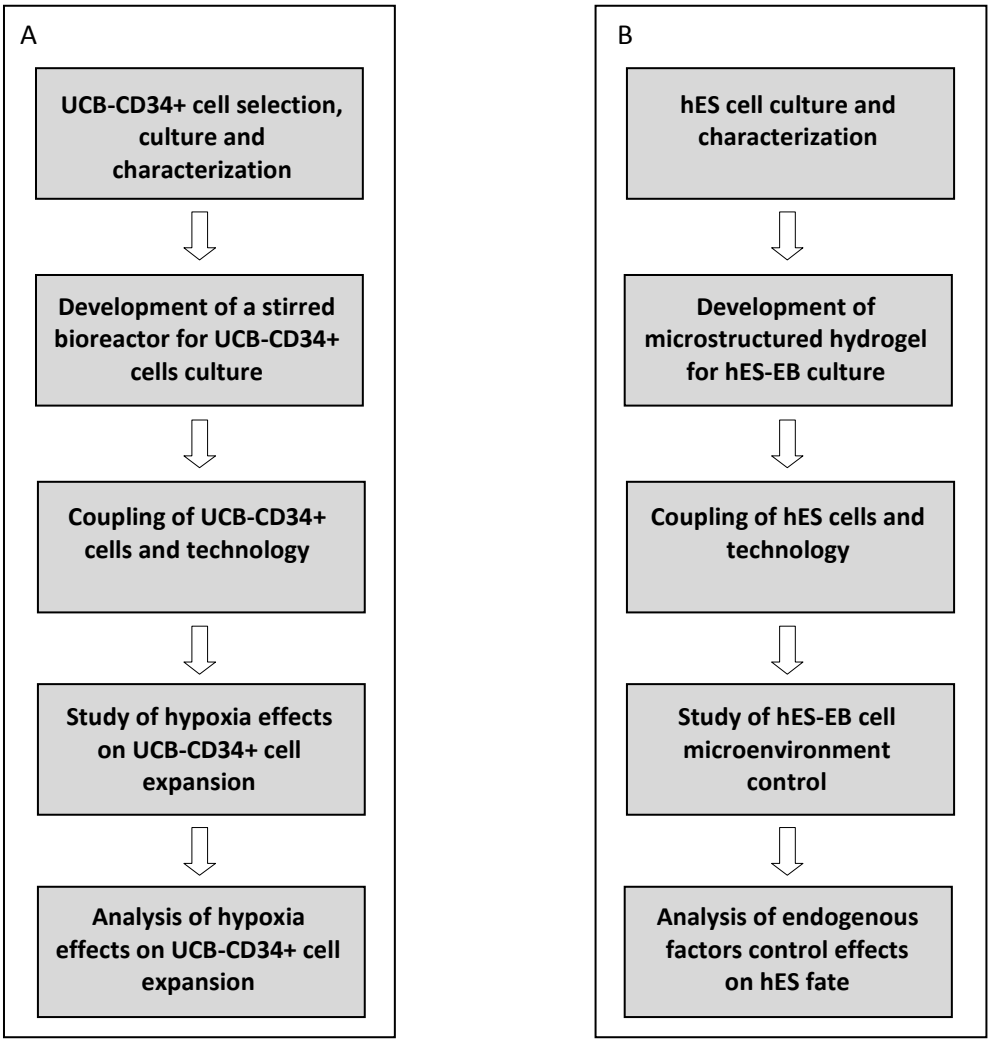

Figura 1 Rappresentazione schematica delle fasi principali del lavoro di ricerca svolto nel corso del Dottorato sullo sviluppo di tecnologie per: lo studio degli effetti dell'ipossia sull'espansione di cellule staminali ematopoietiche derivate da cordone ombelicale (A) il controllo del differenziamento cardiaco di corpi embriodi derivati da cellule staminali embrionali umane (B).

Schema della tesi

La tesi è così organizzata:

Nel capitolo 1 vengono illustrate le grandi potenzialità delle cellule staminali e le limitazioni al loro utilizzo clinico. Vengono presentate inoltre le possibilità introdotte dall'applicazione

di tecnologia alla biologia cellulare, con particolare attenzione agli ambiti cardiaco ed ematopoietico. Le appendici A e B riportano rispettivamente la legge italiana 40/2004 sulle "Norme in materia di procreazione medicalmente assistita" e il "Parere del Comitato Nazionale per la Bioetica su ricerche utilizzanti embrioni umani e cellule staminali". Il report sul Terzo Congresso Nazionale del Gruppo dei Ricercatori Italiani sulle cellule staminali embrionali umane "Financing for research on embryonic stem cells: The situation in Italy and its origins" è allegato come appendice C.

Nel capitolo 2 viene descritto un bioreattore adatto all'espansione di cellule per scopi clinici, dispositivo adatto alla coltura di cellule staminali in sospensione in condizioni definite e controllate.

Il capitolo 3 presenta i risultati di esperimenti biologici in cui cellule staminali ematopoietiche derivate da cordone ombelicale sono state coltivate nel bioreattore sviluppato e descritto nel capitolo 2 per lo studio degli effetti dell'ipossia sull'espansione di CD34+.

Il capitolo 4 descrive l'impiego di biomateriali per la coltura di cellule staminali disperse in singola cellula o in cluster fisicamente separati tra loro e per il successivo controllo del differenziamento.

Il capitolo 5 illustra il risultato della coltura di cellule staminali embrionali umane in un hydrogel opportunamente microstrutturato e il successivo controllo del loro differenziamento. Nell'appendice D è allegato il protocollo ottimizzato per l'estrazione di RNA da corpi embrioidi derivati da embrionali umane. Nell'appendice E è allegato l'articolo *"Microscale in vitro model for screening pathological conditions on human embryonic stem cells-derived cardiomyocytes"*.

Il capitolo 6 traccia le conclusioni del progetto di tesi, discutendo al contempo le prospettive future e le notevoli possibilità aperte dall'applicazione di strumenti ingegneristici alla biologia cellulare.

Summary

Stem cell based therapies have been proposed as promising solutions for the maintenance, regeneration or replacement of diseased tissues, but their potential benefits are limited mainly by scarse cell availability and clinical concern related to cell quality. Optimization of stem cell expansion and differentiation processes contains both an ingeneering and biological issues.

Aim of the presented research is to develop technologies for human stem cell culture. In particular new methodologies, that couple conventional cell expansion systems with biomaterials and microtechnology, are developed in order to improve the efficiency and the quality of stem cell products. The developed methodologies, a bioeractor with a working volume of 10ml/well and a microstructured hydrogel, have been designed to perform cultures of human cord blood-derived hematopoietic stem cells in suspension and for controlling cardiac differentiation of human embryonic stem cell-derived embryoid bodies, respectively.

A general aim is to yield the small volumes involved in the proposed technology to span simultaneously a wide range of cell culture conditions which would have prohibitive costs with volumes of hundreds of milliliters.

Project description

Application for hematopoietic differentiation

Despite the efforts in stem cell research, cell therapy is still not widely applied clinically, because severe limitations reduce their potential benefits. Some issues need to be solved in this sight, such as the need of having a sufficient number of cells that maintain the appropriate phenoype and the desired biological functions.

A particularly promising cell-based theraphy is **hematopoietic stem cell (HSC) transplantation**, because it presents wide applicability, for example in the treatment of blood cancers, hereditary blood disorders, or in the replacement of cells damaged by chemotherapy. Umbilical cord blood (UCB)-derived CD34+ cells are a source of hematopoietic stem cells (HSCs) potentially very useful for transplantation. However, the current therapeutic use of HSCs from UCB is limited to pediatric patients because of the low

cell count from single unit and the difficulty to control the resulting cell phenotype when cells are expanded *in vitro*.

In this scenario, we retain of great importance to improve the expansion rate of cell cultured *in vitro* and to develop a highly defined cell culture microenvironment to investigate critical parameters concerning differentiation.

To address this challenge, a stirred bioreactor for the expansion of UCB - derived CD34+ cells has been developed in order to investigate the effects of hypoxia on the expression of stem cell markers, as oxygen is recognized to favor stem cell self-renewal in hematopoiesis.

The stirring system has been designed on top of a standard six-well plate to favor continuity with conventional static conditions and transfer of culture protocols. The bioreactor volume (10 ml/well), is suitable for cell expansion and multi-parametric flow cytometry analyses.

The stirred system improves homogeneity within the culture volume thus allowing to control the effective O_2 concentration in the cell immediate microenvironment. This control is not possible in static cultures which are subjected to concentration gradients.

First, the stirred bioreactor is tested at 21% O_2 for biocompatibility and other possible negative effects on the cells compared to static conditions. Once verified these main aspects, the system is used to study c-kit expression of CD34+ cells at 5% O_2, using 21% O_2 cultures as a control. The important roles of c-kit in hematopoiesis have been thoroughly reviewed but the dynamics of connection of c-kit, hypoxia and the Stem Cell Factor (SCF) in HSCs, have not been elucidated yet.

Flow cytometric analysis show that CD34+ cells cultured in hypoxia conditions are viable for the entire duration of the experiments and maintained a higher expression of c-kit compared to those cultured in standard conditions.

In this work, taking advantage of advanced technologies which provided a highly-defined cell culture environment in terms of physical and chemical regulatory signals, we were able to explore the dependence of c-kit expression on oxygen conditions.

These results open new perspectives in the study of the mechanisms of interaction between oxygen- and c-kit-dependent pathway at a molecular level.

Application for cardiac differentiation

Cell transplantation is emerging as a promising possibility also **to replace scarred or nonfunctional myocardium in a diseased heart**. However, generating the amount of donor cardiomyocytes required for clinical applications has been hindered by the limited availability and proliferative capacity of these cells. Embryonic stem (ES) cells, which self-

renew indefinitely while retaining their capacity to differentiate into cell lineages of all three primary germ layers including cardiomyocytes, are one of the most promising source for the development of novel approaches in cell therapy. The use of human embryonic stem (hES) cells has been limited by poor progress in the development of robust protocols for cell expansion and differentiation.

In this scenario, we developed a culture system for investigating the effects of the microenvironment on hES cell differentiation. Traditional culture strategies do not help in elucidating these aspects. In particular we focus on the comprehension of the effects of soluble factors secreted in the cell microenvironment.

In vivo cells reside in the specific microenvironment of the tissue they belong to and interact with several factors, including the extracellular matrix, neighboring cells and various soluble factors, whereas isolated cells cultured in standard systems are exposed to a bulk environment which is very different from the environment *in vivo*.

In particular, we developed a microwell array on hydrogel as the experimental platform. The polyacrylamide hydrogel has been chosen because of its biocompatibility, its high permeability, its capacity of absorbing large volumes of aqueous solution thus creating a 3D microenvironment suitable for cell cultures, its non fouling surface which is suitable for hES-derived embryoid bodies (hEBs) culture, and because its geometry is easy to modulate.

In our systems, the diffusion of soluble factors through the 3D hydrogel is likely to lead to an accumulation of over times which are relevant for cell proliferation and differentiation. In the described cell culture conditions, aggregations of hEBs is avoided and single hEBs can be collected for further analyses using a micropipette, without disrupting the culture. In the present study, the differentiation profile of hEBs confined in hydrogel microwells of different depths (450 μm and 1 mm) compared to those cultured onto standard ultra low adhesive petri dishes is examined.

The analysis of the expression profile of the whole genome by microarray analysis demonstrate that the locally controlled availability of endogenous factors in the microenvironment elicit significant differences in hES differentiation. In particular, confinement of EBs in the microwells promotes the expression of genes involved in the pattern specification process, whereas conventional cultures promote the expression of genes of heart development. Further experiments are required to deeply understand the mechanisms underneath these results and to study the spatial distribution of markers of the three germ layers.

The microwell technology may be used to obtain many separated size-controlled hEBs and to evaluate the effects of different microenvironments on differentiation. This technology is also suitable to modulate the presentation of morphogenic cues and elucidate the mechanisms of stem cell differentiation. The small amounts of medium required for the obtainment of hundreds of hEBs makes this approach a tool to consider when developing *in vitro* differentiation protocols.

Foreword

During my PhD research, performed at the "Chemical Engineering Department "I. Sorgato" at the University of Padua and at the "Venetian Institute of Molecular Medicine" in Padua, I got the opportunity to work in a multidisciplinary lab where I could couple engineering tools to my educational biological background, thus having the possibility to explore new and exciting frontiers.

I am grateful to Ministero Italiano dell'Università e della Ricerca, Università degli Studi di Padova, Fondazione "Città della Speranza" and Fondo Sociale Europeo for the financial support to the research.

Due to the high interdisciplinarity of this topic, the work in this thesis has benefit from external collaborations and I would like to thank the following research groups:

- Prof. Paolo De Coppi, Department of Pediatric Surgery, Institute of Child Health, London, UK
- Prof. Luisa Barzon, Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Italy
- Dr. Barbara Di Camillo, Department of Information Engineering, University of Padua, Italy

During my PhD program the following publications have been produced:

1. C. Luni, M. Zagallo, L. Albania, M. Piccoli, M. Pozzobon, P. De Coppi, N. Elvassore *"Design of a stirred multi-well bioreactor for the study of c-kit expression in CD34+ cells from umbilical cord blood under accurate hypoxic culture conditions"* submitted to Biotechnology Progess

2. E. Serena, E. Cimetta, S. Zatti, T. Zaglia, M. Zagallo, G. Keller, N. Elvassore *"Microscale in vitro model for screening pathological conditions on human embryonic stem cells-derived cardiomyocytes"* to be submitted

3. M. Zagallo, E. Serena, G. Masi, L. Barzon, N. Elvassore *"Effects of the modulated availability of endogenous factors on hES-derived embryoid bodies differentiation"* to be submitted

4. M. Zagallo, C. Luni, E. Serena, E. Cimetta, S. Zatti, G. Giobbe, N. Elvassore *"Controlled cardiac differentiation of human embryonic stem cells – derived embryoid bodies in scalable bioreactors"* Cardiovascular Research, vol. 87, suppl. 1, pp. S110 – S110.

5. E. Serena, E. Cimetta and M. Zagallo "*Financing for research on embryonic stem cells: the situation in Italy and its origins*" Report on the 3rd Italian National Congress of the Group of Italian Researchers on Embryonic Stem Cells (IES Group) Rome, 1st July 2008. Notizie di Politeia. Rivista di Etica e Scelte Pubbliche" Vol. 91/Anno XXIV, 2008, pp. 110-113.

Part of the work in this thesis has been presented to several international conferences, including: Frontiers in Cardiovascular Biology (ESC), International Society for Stem Cell Research (ISSCR), First Internatonal Symposium on Human Embryonic Stem Cell Research, 3rd Italian National Congress of the Group of Italian Researchers on Embryonic Stem Cells.

Introduction

One of the most exciting frontiers in regenerative medicine is the potential use of stem cells for treating a host of congenital, developmental, or degenerative diseases for which no therapies are currently available. Despite the efforts in stem cell research, cell therapy is still not widely applied clinically, because severe limitations reduce its potential benefits. Some general issues need to be solved for future clinical applications, such as the need to have a sufficient number of cells that maintain the appropriate phenotype and perform the required specific biological functions. Whether or not these potentially curative strategies ever reach patients will ultimately depend on technologies for obtaining cells in the proper quantity and quality, in a robust and cost-effective manner.

My research focuses on the development of technologies for culturing and direct differentiation of human stem cells under defined and controlled cell culture conditions.
This work is characterized by a highly multidisciplinary nature: the tight collaboration with engineers of the laboratory, who contributed to the design and development of the technologies used in the present research, allowed the application of innovative devices to the culture of relevant cells, such as hematopoietic and human embryonic stem (hES) cells.
Specific aims of this thesis are the development of innovative *ad hoc* cell culture systems for 1) hematopoietic stem cell expansion in hypoxic conditions 2) and for controlling cardiac differentiation of hES cell-derived embryoid bodies.

The progression of the research is illustrated in Figure 1.
The main steps for the expansion of hematopoietic CD34+ cells in hypoxic conditions are: the selection, the culture and the characterization of UCB-derived CD34+ cells; the design and development of a stirred bioreactor for stem cell culture; the coupling of technology to the cells; the study of hypoxia effects and finally, the analysis of the outputs in terms of cell viability, proliferation rate and expression of specific markers of differentiation.
The main steps for the control of cardiac differentiation of hES cell–derived embryoid bodies are: the expansion and characterization of hES cells; the development of a microstructured hydrogel for hES-derived embryoid body culture; the coupling of technology to the cells; the study of microenvironment control effects on embryoid body differentiation and finally the analysis of cell differentiation.

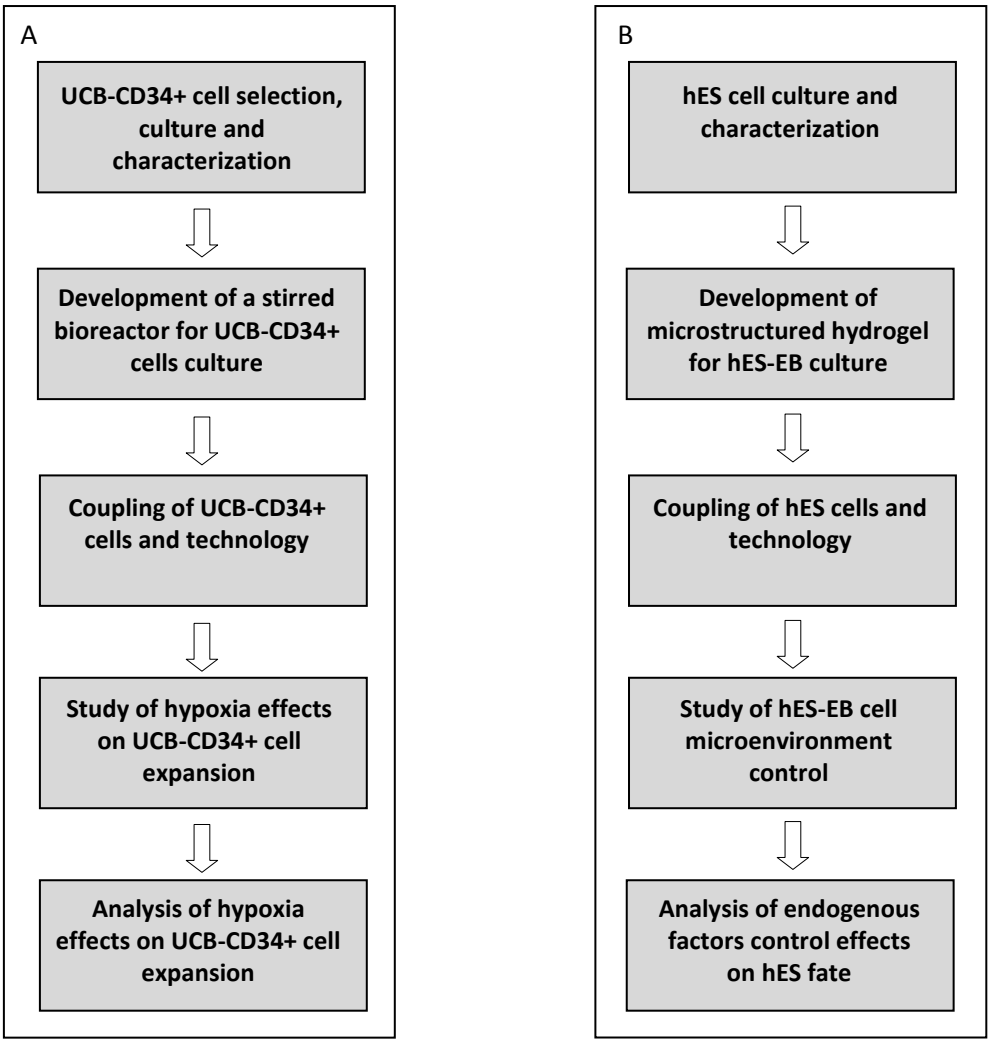

Figure 1 Schematic representation of the main phases of the PhD research for the expansion of cord blood-derived human stem cells in hypoxic conditions in a suspension stirred bioreactor (A) and for the control of cardiac differentiation of human embryonic stem cells (B).

Thesis outline

The thesis is organized as follows:

Chapter 1 reviews the great potential of stem cells and the limitations to their clinical use. It introduces the possibilities and advantages opened by coupling technologies to cell biology with regards to hematopoietic and cardiac applications. Appendix A and B report respectively the Italian 40/2004 law on "Norme in materia di procreazione medicalmente assistita" and the "Parere del Comitato Nazionale per la Bioetica su ricerche utilizzanti embrioni umani e cellule staminali". The report on 3rd Italian National Congress of the

Group of Italian Researchers on Embryonic Stem Cells ““Financing for research on embryonic stem cells: The situation in Italy and its origins” is attached in Appendix C.

In Chapter 2 a clinical-scale bioreactor is described. The apparatus is suitable for stem cell culture in suspension under defined and controlled cell culture conditions.

In Chapter 3 presents the results from biological experiments, where human umbilical cord blood (UCB) hematopoietic stem cells have been cultured on the developed bioreactor (described in Chapter 2) for the study of hypoxia effects on CD34+ expansion.

Chapter 4 overviews the use of biomaterials for the culture of single cell dispersion or physically separated human stem cell clusters and for the further control of cell differentiation.

Chapter 5 illustrates the results of hES cell culture within a proper microstructure hydrogel and the further control of hES cell differentiation. In Appendix D the optimized protocol for RNA extraction of hES cell-derived embryoid bodies is attached, Appendix E reports the manuscript *“Microscale in vitro model for screening pathological conditions on human embryonic stem cells-derived cardiomyocytes”*.

Chapter 6 draws the conclusions of the thesis project and discusses the future perspective and the wide possibilities opened by the application of engineering tools to cell biology.

Chapter 1
Stem cells: clinical relevance and limitations

This chapter introduces the potentialities and the limitations of human stem cells for clinical purposes with special attention to hematopoietic and cardiac applications.
After a brief introduction on regenerative medicine research field (§ 1.1), the different types of human stem cells that may be used for cell therapies approaches are presented. Special attention is addressed in these paragraphs to human embryonic stem cells since they constitute the basis of my research for cardiac application. The state of the art regarding induced pluripotent stem cells is also reported in details since these cells represent one of the most promising cell sources for regenerative medicine purposes and a future perspectives of this thesis (§ 1.2). The state of the art of hematopoietic (§ 1.3) and cardiac tissue engineering (§ 1.4) is then presented since it constitutes the context of my studies. The road map from discovery to clinics (§ 1.5) is reported in order to introduce and define the context of my research. The scientific motivation and the aim of this thesis together with the technological perspective that guide the studies are summarized in § 1.6. The cited literature is reported in § 1.7.

1.1 Introduction

One of the most exciting frontiers in regenerative medicine is the potential use of stem cells for treating a host of congenital, developmental, or degenerative diseases for which no therapies are available.
Many of the diseases that place the greatest burden on society are diseases of cellular deficiency. Heart failure, diabetes, stroke, hematological disorders, neurodegenerative

disorders, most cases of blindness and deafness all result from the absence of one or more critical populations of cells that the body is unable to replace [1].

The use of living cells as therapeutic agents for the maintenance, regeneration, or replacement of malfunctioning tissues has been proposed in last decades [2].

Stem cells are functionally undifferentiated cells that retain the ability to differentiate into one or more mature cell types under appropriate conditions, and to self-renew, representing a potentially inexhaustible cell source [3]. Stem cells play a critical and essential role in the human body not only by providing the starting material for organs and tissues but also for their continual maintenance, growth, and renewal throughout ontogeny. As the embryo and the fetus develop, stem cells are seeded into the various tissues and organs where they remain throughout life [4].

The goal of cell replacement is to develop therapies where stem cells are first induced to differentiate into specified cells of choice, then transplanted into patients to replace damaged or dysfunctional tissues. It is hoped that the replacement and integration of lost cells will be able to restore functions and behaviors compromised by the disease condition.

1.2 Stem cells

The international scientific community recognizes great potentiality to the applications of stem cells for cell therapy.

The main properties defining stem cells are:

- unlimited or prolonged self-renewal;
- ability to differentiate generating highly specialized cell types (plasticity) [5] [6]

Stem cells can be classified as embryonic or adult, depending on the developmental stage from where they were obtained. Embryonic and adult stem cells show different properties and developmental potentialities. The fact that ES cells can only be derived from early-stage embryos precludes the establishment of autologous cell lines for patients, and immune rejection hinders the use of nonautologous ES cell lines. New possibilities are available to overcome such limitations, since the obtainment of human induced Pluripotent Stem Cells (iPS), which are stem cell-like cells derived from reprogrammed somatic cells [7].

Usually, between stem cells and their terminally differentiated progeny there are intermediate populations of committed progenitors with a limited capacity for proliferation and a more restricted differentiation potential.

1.2.1 Embryonic stem cells

Human embryonic stem (hES) cells, which self-renew indefinitely while retaining their capacity to differentiate into cell lineages of of all three primary germ layers [8] constitute one of the most promising cell sources in regenerative medicine field and have been chosen in this study for cardiac tissue engineering application.

hES cells are derived from the inner cell mass of blastocyst-stage embryos [9] and can be propagated extensively in culture. The first derivation of mouse Embryonic Stem (mES) cells is dated 1981 [8], while the first human ES cell line was obtained years later, in 1998 by Thomson and colleagues. These human cell lines were derived from the ICM of the blastocysts of human embryos (Figure 1.1), generated by *in vitro* fertilization (IVF) for clinical purposes, and donated by individuals after informed consent and after institutional review board approval. The cells isolated from the blastocyst were cultured on mouse feeder layer for the establishment of an embryonic stem cell culture and for subsequent cells expansion (Figure 1.1). The undifferentiated hES cells and their clonal derivatives were shown to retain a normal diploid karyotype and display high level of telomerase activity during long-term propagation in culture. Pluripotencey of the hES cells was confirmed by injection into immunocompromised mice to produce teratomas containing differentiated derivatives of all three germ layers [10]. Pluripotency was also demonstrated *in vitro*.

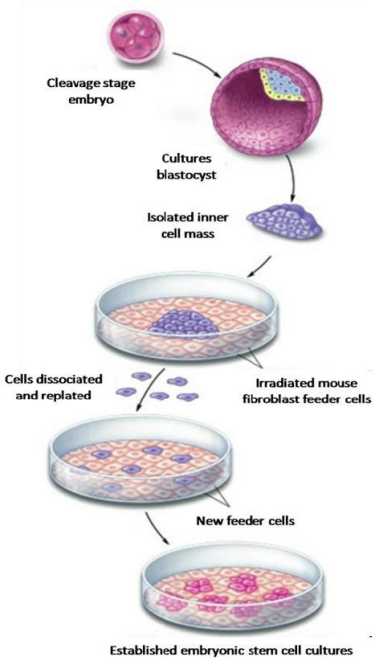

Figure 1.1 Techniques for generating embryonic stem cell cultures (modified from http://stemcells.nih.gov/info/scireport/appendixC.as)

When removed from the MEF feeder layer and cultivated in suspension, the hES cells tend to spontaneously form three-dimensional multicellular aggregates of differentiated and undifferentiated cells termed embryoid bodies, which contain cell derivatives of endoderm, mesoderm, and ectoderm origin (Figure 1.2) [11].

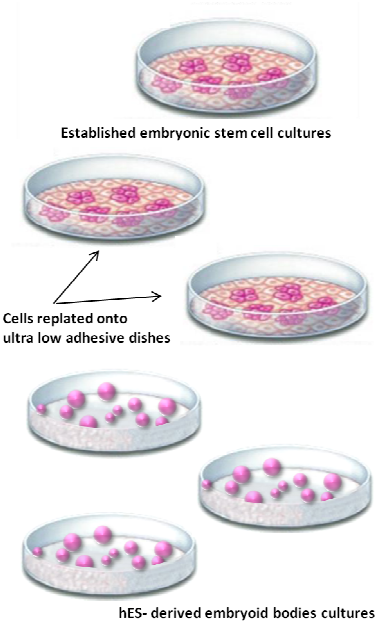

Figure 1.2 Techniques for generating hES-derived embryoid body cultures

In this sight, having the possibility of generating relevant numbers of defined cell populations may allow the development of novel cell therapies or, in parallel, to perform studies on specialized human cells that would permit new insights for the development of the necessary therapeutic strategies.

Nowadays, the number of hES cell lines that have been derived has notably increased. In order to assess the similarities and differences of hES cells and to identify a set of well-validated markers to establish hES cells identity of newly derived lines, the International Stem Cell Initiative (ISCI) [12] was asked by the International Stem Cell Forum to perform a comparative study of a large and diverse set of hES cell lines [13]. The study identified the most common markers of both undifferentiated and differentiated states to assess the properties of the cell line of interest.

The hES cell lines that have been used in this study were purchased from the WiCell Research Institute (Madison, Wisconsin, USA) in accordance with the Italian legislation.

1.2.1.1 Embryonic stem cell research in Italy

The Italian situation regarding the research on hES cells is presented since it constitutes a matter of discussion among scientists and politicians and represents a crucial aspect for Italian researchers.

The Italian legislation with regards to the use of stem cells is defined by the 40/2004 law (Appendix A). This legislation forbids any experimentation on human embryos. However, as it is forbidden to work on human embryos and directly derive new cell lines, it is allowed to perform experimentations on cell lines that have been derived in other countries. The confirmation of that came from the Italian *"Comitato Nazionale di Bioetica"* that expressed its approval within the *"European Centre for the Validation of Alternative Methods"*. The "parere" of the "Comitato Nazionale per la Bioetica su ricerche utilizzanti embrioni umani e cellule staminali" is reported in Appendix B.

Interested on these bioethical debates, the BioERA research group actively participated to the 3rd Italian National Congress of the Group of Italian Researchers on Embryonic Stem Cells (IES Group) in 2008. The leading theme was: "Financing for research on embryonic stem cells: The situation in Italy and its origins". Our report of the event has been published on the Italian journal "Notizie di Politeia. Rivista di Etica e Scelte Pubbliche" [14] and on the newsletter of ESTOOLS (the largest grouping of human embryonic stem cell researchers in Europe). The report is in Appendix C.

1.2.2 Adult stem cells

An adult stem cell is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ that can renew and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. Adult stem cells can be obtained from tissues of endodermal, mesodermal and ectodermal lineages.

The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found.

Unlike embryonic stem cells, which are defined by their origin (cells isolated from the preimplantation-stage embryo), the origin of adult stem cells in some mature tissues is still under investigation. Research on adult stem cells has generated a great deal of excitement. Until recently, it was thought that tissue-specific stem cells could only differentiate into cells of the tissue they belong to, however, recent studies suggested that tissue-specific stem cells can differentiate into lineages other than the tissue of origin [15].

1.2.3 Induced pluripotent stem cells

The discovery that somatic cells can be reprogrammed to a pluripotent state, has ushered in a new and exciting era in regenerative medicine.

The promise of treating human genetic and degenerative diseases through the application of tissue engineering and regenerative medicine approaches has become significantly closer to realization since the recent discovery that somatic mammalian cells can be epigenetically reprogrammed to a pluripotent state through the exogenous expression of the transcription factors OCT4, SOX2, KLF4, and c-MYC [16].

These first induced Pluripotent Stem (iPS) cells were closely related, but not identical, to ES cells. They only differed in their capacity for germline transmission since the iPS cells could contribute to chimerism when introduced into early mouse embryos but could not produce germ line transmission, in contrast with ES cells. Already in 2007, however, the groups of Yamanaka, Jaenisch and Hochedlinger produced iPS cells capable of germline transmission by modification of the selection criteria [17][18][19].

More recently, rare iPS cell lines have been identified that are even capable of generating "all-iPS cells" mice upon injection into tetraploid blastocysts [20][21][22][23], suggesting that at least some iPS cell clones have a developmental potency equivalent to ES cells.

Since iPS cells have been derived from different species including humans [7][24] [25], rats [26], and rhesus monkeys [27] by expression of the four Yamanaka factors, it is likely that the fundamental features governing pluripotency are conserved during evolution. Similarly, iPS cells have been derived from different somatic cell populations, such as keratinocytes [28][29], neural cells [30][31], stomach and liver cells [32], and melanocytes [33] as well as from genetically labeled pancreatic β cells [34] and terminally differentiated lymphocytes [35][36] further underscoring the universality of induced pluripotency. In order to use iPS cells for clinical applications, suitable and safe techniques of factor delivery have been attempted. While the first studies on iPS cells used retroviral vectors - that stably integrated into the host cell genome to introduce the four factors [16][18][19][29]), several other safer solutions have been chosen more recently.

Lentiviral vectors, whose expression can be controlled by the drug doxycycline, thus dicreasing the risk of continued transgene expression, have been used for factors delivery in two recent studies [37][38]. Inducible vector systems have been used to generate so-called "secondary" reprogramming systems. These systems entail differentiating "primary" iPS cell clones, generated with doxycyline-inducible lentiviral vectors or transposons, into

genetically homogeneous somatic cells using either *in vitro* differentiation [29][39] or blastocyst injection [40][41]. These somatic cells are then cultured in doxycycline-containing media, thus triggering the formation of "secondary" iPS cells at efficiencies which are generally orders of magnitude higher than those obtained after primary infection.
Three different approaches have been attempted for generating integration-free iPS cells: the use of vectors that do not integrate into the host cell genome, as adenoviral vectors [42][43], plasmids [44], Sendai virus [45], polycistronic minicircle vectors [46] and self-replicating selectable episomes [47]. The use of integrating vectors that can be subsequently removed from the genome [41][48][49][50][51]; the use of other factors delivery systems but nucleic acid-based vectors, as purified recombinant proteins [43] whole-cell extracts isolated from either ES cells [52] or genetically engineered HEK293 cells [53]. A more efficient and safer way of producing integration-free iPS cells may be the introduction of modified RNA molecules encoding for the reprogramming factors into somatic cells. This approach has been recently validated by Warren *et. al*. (2010).

1.3 The clinical potentialities of hematopoietic stem cells

A particularly promising cell-based therapy is hematopoietic stem cell (HSC) transplantation because of its wide range of applicability, for example in the treatment of blood cancers, hereditary blood disorders, or, in some cases, to replace cells damaged by chemotherapy [55].

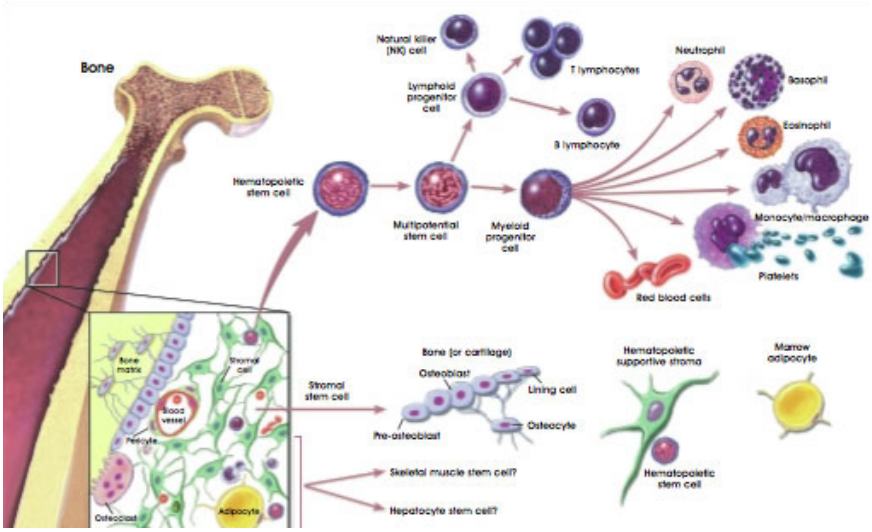

Figure 1.3 Hematopoietic and stromal cells differentiation

Hematopoietic stem and progenitor cells (HSPCs) are responsible for the renewal of all mature blood cells. Hematopoiesis is among the best-defined differentiation cascades in mammalian tissues due to the ease of access and morphologic distinctiveness of many of its members [56]. In adults, for example, the red blood cell pool of approximately 25 trillion cells is turned over every 120 days, necessitating the production of approximately 200 billion red blood cells per day to maintain homeostasis [57].

HSCs are defined operationally by their capacity to reconstitute the entire blood system of a recipient [58]. In adult mammals, the majority of hematopoietic progenitor cells reside in the bone marrow with a small number continually escaping into the circulation, then rehoming back into the BM [59][60]. HSCs and HSPCs proliferate and differentiate, producing the different classes of blood cells. In this process, different steps, which correspond to distinct intermediate cell phenotypes, are identified, as reported in Figure 1.3. While progenitors seem to play a role in the immediate period after transplantation, only HSCs are able to give rise to long-term bone marrow reconstitution [55].

Rapid advances in our understanding of hematopoiesis over the past decade has allowed the mimicking hematopoiesis *ex vivo*, thus enabling *in vitro* expansion of hematopoietic tissue for clinical applications purposes.

Bone marrow cells constitute a promising source of HSCs for therapeutic aims, but their real clinical use present serious limitations.

First, the bone marrow harvesting of cells is an invasive procedure. Second, an immunological response, called graft-versus-host disease (GVHD), is registered after transplantation (with the only exception for homozygous twins). Thus, a requirement for bone marrow transplantation is the human lymphocyte antigen (HLA) matching between donor and patient. However the high degree of HLA polymorphism makes a match between unrelated humans a rare possibility, and even between siblings the probability of a HLA match is 25%. All these medical implications result in very long times before a suitable donor can be found and transplantation performed.

Alternative source for HSCs transplantation is umbilical cord blood.

1.3.1 Recent perspectives for the obtainment of HSCs

The generation of HSCs, capable of long-term hematopoietic reconstitution, would open new doors for both disease correction and regenerative medicine.

Human induced pluripotent stem cells have been differentiated into a wide variety of specialized cells, including hematopoietic stem cells. Hematopoietic lineage specification from pluripotent cells has been obtained using either embryoid body formation in the presence of hematopoietic cytokines or co-culture with stromal cell lines. Several blood cell types have been generated from murine or human ES cells. These include mouse and human B- and T-lineage cells [61], megakaryocytes [62] and erythroid cells. However, the generation of HSCs conferring multilineage, hematopoietic reconstitution, appears to be more challenging. Several works reported the difficulty of producing HSCs capable of reconstituting adult, irradiated recipients [63][64]. Intravenous injection of murine or human ES- and iPS cell-derived hematopoietic cells resulted in fact in little or no engraftment [65][66][67][68][69][70]. Recent studies have gained more encouraging results. In particular, culture of human ES and iPS cells with medium conditioned by HepG2 cells, a human hepatocarcinoma cell line, was shown to enhance the generation of mesodermal derivatives, including hematopoietic cells [71].
However, as just reviewed, there are significant obstacles to the generation of these cells and further studies are now required to compare the hematopoietic potential of human iPS cells with that of hES cells [72][73][74].

1.3.2 Umbilical cord blood: a promising source of HSCs for transplantation

Evidence supporting the efficacy of umbilical cord blood (UCB) transplantation in adults has significantly increased over the past years, as it now becomes a standard alternative to bone marrow transplantation in some hematopoietic stem cell transplantation centers.
The use of UCB-derived stem cells is expanding in the medical field owing to the facts that UCB is easy to procure from waste products without risk to the donor, and the cells are "younger" than those obtained from adult BM and more tolerant to human leukocyte antigen (HLA) mismatches for lowering risk of graft-versus-host disease [75].
Disadvantages of UCB, include the low, and sometimes limiting, number of cells collected in single donor units which can be less than optimal for engraftment of many adults and higher weight children, and the relatively slower speed to engraftment of neutrophils and platelets.
As far as the number of cells which can be collected from a single donor unit, considering that the minimum number of cells required for transplantation is $2.5 \cdot 10^7$ cryopreserved

nucleated cells/kg of patient, the UCB use is mainly limited to pediatric patients [76]. The possibility to compensate the low number of mononucleated cells with the use of multiple cord blood units may be associated with increased GVHD and does not meaningfully reduce the time to neutrophil and platelet engraftment [77]. Thus, means to enhance numbers and/or potency of collected cells and their engrafting capability through *ex vivo* and/or *in vivo* maneuvers would likely enhance the efficacy and applicability of CB transplantation.

1.3.3 HSCs in vitro expansion

In vivo HSC proliferation is regulated by a complex milieu of chemical, mechanical, and electrical signals from the local surrounding environment, the stem cell niche.
In vitro HSCs are conventionally cultured in Petri dishes where cells grow in suspension.
It is well accepted that *in vitro* culture systems present substantial differences from the *in vivo* environment. *In vivo* cells reside in the specific microenvironment of the tissue they belong to and interact with several factors, including the extracellular matrix (ECM), neighbor cells and various soluble factors, whereas isolated cells cultured in standard cell culture dishes are exposed to a bulk environment which is very different from the environment *in vivo*.
Because of these deep differences between *in vivo* and *in vitro* cell niche, the protocols for *in vitro* HSC expansion need to span a wide range of experimental conditions, in terms of type, timing and dose of soluble growth factors to reproduce *in vivo* stem cell niche signals (Verfaillie, 1992). Furthermore, the characterization of cells cultured *in vitro* is important to understand different aspects of the expansion process, such as repeatability and heterogeneity inside the HSC population, to increase our understanding of cell behavior, and to detect the retain of multipotency after the expansion process.
Among the different signals involved in the hematopoietic stem cells niche, oxygen concentration is known to play an important role.
During my PhD, a technological apparatus has been developed for studying the effects of oxygen concentration on UCB-derived CD34+ cells expansion.

1.4 Tissue engineering of cardiac tissue

Heart failure (HF), hypertension, and myocardial infarction are common causes of death in Western world. In Europe, the estimated frequency of HF patients is 0.4–2%, and

the incidence increases in the elderly, reaching a range between 6–10% in the >65-year-old population [78]. HF is responsible for a large number of prolonged and recurrent hospitalizations, representing 1–2% of global health care costs [79].

The onset and evolution of cardiac failure depends on the accumulation of old, poorly contracting cells and the subsequent formation of scar tissue.

Heart failure after a myocardial infarction is often progressive. When heart muscle is damaged by injury such as a heart attack, cardiomyocytes die. Macrophages, monocytes, and neutrophils migrate into the infarct area, initiating the inflammatory response [80]. Infarct expansion then begins to occur because of the activation of matrix metalloproteases, which degrade the extracellular matrix and result in myocyte slippage. This weakening of the collagen scaffold results in wall thinning and ventricular dilation. After the initial inflammatory phase, there is an increase in fibrillar, cross-linked collagen deposition, which resists deformation and rupture [81] Thus, during this process of ventricular remodeling, functional contracting tissue is replaced with nonfunctional scar tissues diminishing heart pumping ability.

Current therapeutic strategies to treat heart failure are limited to pharmacological therapies [82], mechanical cardiac support [83] and surgical transplantation [84].

The pharmacological therapies with diuretics, inotropes, vasolidators, ACE inhibitors, β-blockers, and antiarhythmic drugs have demonstrated to reduce the patients mortality, relieving and stabilizing symptoms and preventing the progression of myocardial dysfunction [85]. However, poor prognosis and shorter life expectancy of heart disease patients clearly indicate the need for additional or alternative solutions to complement these approaches.

Mechanical circulatory support devices, such as left ventricular assist devices (LVADs), are currently used as a transitory option to cardiac transplantation. Current limitations of these support devices include thromobogenicity, infections, and complications due to size and weight of the device, and power transmission [86].

Currently, heart transplantation is the only successful and definitive treatment for heart failure, however it is hampered by a severe shortage of organs donors and rejection episodes [83].

Recently, cellular transplantation and tissue engineering approaches have emerged as promising alternatives to heart transplantation.

There is growing evidence that heart muscle has the ability to regenerate through the activation of resident cardiac stem cells or through recruitment of a stem cell population

from other tissues [87][88] but the regenerative capability of the heart cannot replace large-scale tissue loss [97][89]. Consequently, two strategies have been taken for delivering cells and assist cardiac repair: the infarcted myocardium can be repaired using cells, biomaterials and regulative factors (cardiac tissue engineering) or cells may be delivered directly into the infracted myocardium (*in situ* cellular cardiomioplasty).

The first approach, which provides alternatives to the direct cell delivery into the myocardium, can be subdivided into three approaches: *in situ* tissue engineering, where cells are combined with injectable biomaterial and injected into the infracted myocardium; *in vitro* tissue engineering, where cardiomyocytes are seeded into 3D constructs to develop functional tissue constructs or stem cells are loaded into tissue constructs and implanted for *in vivo* regeneration of myocardium; scaffold free tissue engineering where cells and aggregates are used for cardiac regeneration.

1.4.1 In situ cardiac tissue engineering

This approach combines the injectable biomaterial with cells, which will be then delivered into the infarcted myocardium. The presence of the biomaterial can improve cell retention, cell survival [90] and can serve as a vesicle for the delivery of pharmaceutical molecules [91][92][93]. This approach is attractive since it may be performed by minimally invasive surgery [94]. Injectable biomaterials include fibrin [91][92][93][94] [95][96][97][98][99], collagen [100], Matrigel [101][102], self-assembling peptides [103][104], chitosan [105] and alginate [106][107]. Cell types include skeletal myoblasts [97], endothelial cells [103], bone marrow mononuclear cells [95][99], MSCs [98], embryonic stem cells (ESCs) [102] and neonatal cardiomyocytes [103][105].

1.4.2 In vitro engineering of cardiac tissue constructs

This approach involves engineering of cellularized 3D tissue constructs to be implanted on the infarcted myocardium. Cardiac function can be improved through cell participated remodeling [108][109], and providing mechanical support to the myocardium. This approach may result in a better engraftment efficacy than the traditional direct cell delivery because cells are uniformly distributed in the construct [110][111].

1.4.3 Scaffold-free cardiac tissue engineering

Cell sheet engineering is a scaffold free approach that stacks monolayer cell sheets to build a 3D tissue and implant it to cover the infarcted myocardium [112][113][114][115].

1.4.4 In situ cellular cardiomioplasty

Selecting the type of cells and creating a suitable environment in which cells can grow is a technically and biologically important aspect. Numerous studies have experimentally addressed the potential of different types of stem cells to differentiate in contractile cells. Stem cells, after differentiation, should integrate both functionally and structurally into the surrounding viable myocardium and develop a network of capillaries and larger size blood vessels for supply of oxygen and nutrients to the injured region.

The most promising cell sources for regenerative medicine are human stem cells capable of differentiation toward cardiac lineage, such as: human embryonic stem cells [116][117], induced Pluripotent Stem Cells [118][119][120], Cardiac Stem Cell [121][122], Bone Marrow Stem Cells, Fetal Amniotic Stem Cells, Adipose tissue derived stem cells [123], multipotent adult progenitors [124], mesoangioblasts [125] and muscle derived stem cells [126].

Mesenchimal Stem cells seem to be an exciting source for cell therapy, because they can differentiate *in vitro* into nerve cells, skeletal muscle cells, vascular endothelial cells [127], and into cells with cardiomyocyte features [128]. Furthermore, after differentiation, these cells are positive for specific cardiac protein, such as β-myosin heavy chain, cardiac troponin T, α-cardiac actin and desmin, they prove functionality with Ca^{2+} transients [129], and respond to α and β adrenergic stimulation with an increase in contractility [130]. Although some improvements in cardiac performance after cell transplantation were observed, the cardiomyogenic efficiency of these cells is still very low (0.02%) [131].

Embryonic stem (ES) cells, derived from the inner cell mass of the blastocyst [132] and Cardiac Stem Cells, harvested from endomyocardial biopsy of patients [133], seem to be the best promising sources for cardiac regeneration therapy because they differentiate into beating cells with a cardiomyocyte phenotype and they can also be expanded *in vitro* to generate large quantities of cells. In particular, ES cells can spontaneously organize, after differentiation, a functional syncitium with action potential propagation [134][135].

In this scenario become of undoubted interest attempts to control environmental factors which may influence cell proliferation and cardiac differentiation. In particular

during my PhD research, I did focus on the comprehension of the effects of secreted soluble factors in the cell microenvironment for the obtainment of homogenous population of cardiac cells from human embryonic stem cells.

1.5 From discovery to clinical use

Moving from the bench to the bedside is an expensive and arduous journey with a high risk of failure. One roadblock on the path of translational medicine is the paucity of high quality cell populations for clinical applications. The possibility to couple technologies to the conventional biological approaches, guarantees controlled and defined cell culture conditions, reproducible and predictable outputs. From the discovery of stem cells to their use for a specific clinical application, the resolution of many interwoven issues, knowledge and expertise from very diverse areas are required. Thus, the road map that brings stem cells to the clinics is a long process which involves researchers with different attitudes and skills, like biotecnologists, engineers, material scientists. Producing a cell therapy product for clinical use, introduces many regulatory and engineering challenges not usually dealt with scientists. Innovative technologies should be integrated into cell production processes to control the products identity and reproducibility.

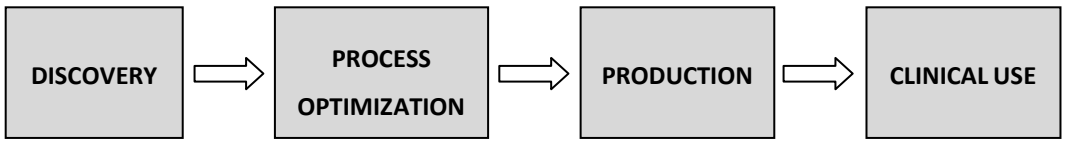

Figure 1.4 Schematic representation of the road map from hematopoietic stem cell discovery to their clinical use.

The schematic in Figure 1.4 summarizes the road map from the discovery of stem cells to their clinical use. The first step is discovery, which constitutes the basis for a feasible clinical application. It involves the full characterization of the system and the collection of the biological and technical information needed for the further steps.

Process optimization implies the understanding of the relative importance of the different parameters involved in the processes in order to find the proper conditions for an efficient and reproducible stem cell expansion. Screening of wide range of experimental conditions occurs at this stage along with the development of reliable cell quality detection tools. The

cell quality after *in vitro* culture is the main goal of this step. This step occurs on a small scale to provide increased insight and understanding of the system holding down the costs.
Once the key parameters for stem cell expansion are defined, the process needs to be implemented for production at the clinical scale. At this phase, other variables come into play, among them the development of a cost-effective process, robustness and reproducibility in the methodology, identification of the opportune measurements for cell quality control and process profile monitoring.
The last step is the clinical use of stem cells.

1.6 Aim of the thesis

The aim of this PhD thesis is the development and optimization of technologies for human stem cell culture under defined and controlled conditions.
In particular new methodologies, that couple conventional cell expansion systems with biomaterials and microtechnology, are developed in order to improve the efficiency and the quality of stem cell products. The need to have a sufficient number of cells that maintain the appropriate phenotype and perform the required specific biological functions in a robust and cost-effective way is the main prerequisite for clinical applications. In this scenario, technologies are fundamental tools for reaching this goal.

Two applications, will be presented: 1) a stirred bioeractor with a working volume of 10ml/well for culturing of human UCB-derived hematopoietic stem cells; 2) a micro-structured hydrogel for controlling cardiac differentiation of human embryonic stem cell-derived embryoid bodies.

A general aim of the research is to yield the small working volumes of the proposed systems, for spanning simultaneously a wide range of cell culture conditions, and thus placing this research in the critical phase of process optimization, where cost reduction and product quality control are needed.

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Chapter 2
Design of a stirred bioreactor for human stem cell expansion

This chapter presents the design and development of a six-well stirred bioreactor, suitable to perform dynamic cell cultures in suspension in a working volume of few milliliters. In § 2.1 a general overview regarding conventional and innovative *in vitro* stem cell culture methods is reported, whereas § 2.2 focuses on *in vitro* expansion methods for HSCs. § 2.3 presents the developed bioreactor and special attention is directed to the stirring system. § 2.4 discusses the rational approach and § 2.5 reports the experimental plan. In § 2.6 the results of the oxygen solubility studies are reported and discussed. The chapter ends with the discussion of the results from preliminary biological experiment coupling the bioreactor and HSCs and hES cells (§ 2.7) and the conclusive remarks (§ 2.8). The cited literature is reported in § 2.9.

2.1 *In vitro* stem cell culture methods

2.1.1 Static cell culture

Conventional static culture systems, such as polystyrene petri dishes or flasks, are the most widely used devices for culturing cells growing in monolayer or in suspension. These containers are user-friendly, sterile and inexpensive. Temperature and pH are controlled in the biological incubators: temperature is usually set at 37°C, whereas pH control is performed by maintaining an atmosphere at 5% CO_2 concentration, which corresponds to the physiological pH of 7.2. However, conventional culture systems present several limitations, including: lack of mixing, resulting in critical concentration gradients for pH, dissolved oxygen, cytokines and metabolites; difficult or even impossible online monitoring and control; low process reproducibility; repeated handling required to feed

cultures or obtain data on culture performance [1][2]. Thus culture methods are in need of both optimized protocols and *ad hoc* technologies.

2.1.2 Dynamic cell culture: the bioreactors

The use of bioreactor systems is an alternative approach to standard petri dishes and flask cultures of cells *in vitro.*

Bioreactors are defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (*e.g.* pH, temperature, pressure, nutrient supply and waste removal) [3].

Advanced bioreactors are required when a large number of cells is needed, accessory cells are used or high cell densities are desired, A deeper and broader insight into the influence of exogenous input on cell expansion and commitment is only achievable in defined and controlled cell culture conditions.

A few studies have been performed with different types of bioreactors for the *ex vivo* expansion of cells, involving perfusion chambers, stirred reactors, hollow fiber, rotating and packed bed reactors. Stirred bioreactors, suitable for cell culture in suspension, provide a homogeneous environment, allowing sampling, monitoring and control of culture conditions (Figure 2.1). Typical operating modes include batch, fed-batch and perfusion mode [4].

In the road map from stem cell discovery to clinical use (§ 1.5), bioreactors small-scale can be useful in the phases of process optimization and production, where cost reduction and product quality are required [5].

The adaptation of systems which are classically used in industrial fermentation processing, wastewater treatment, food processing and production of pharmaceuticals and recombinant proteins (e.g. antibodies, growth factors, vaccines and antibiotics), to stem cell culture, requires a careful choice of biocompatible materials, and a minimization of the shear stress, caused by the stirring.

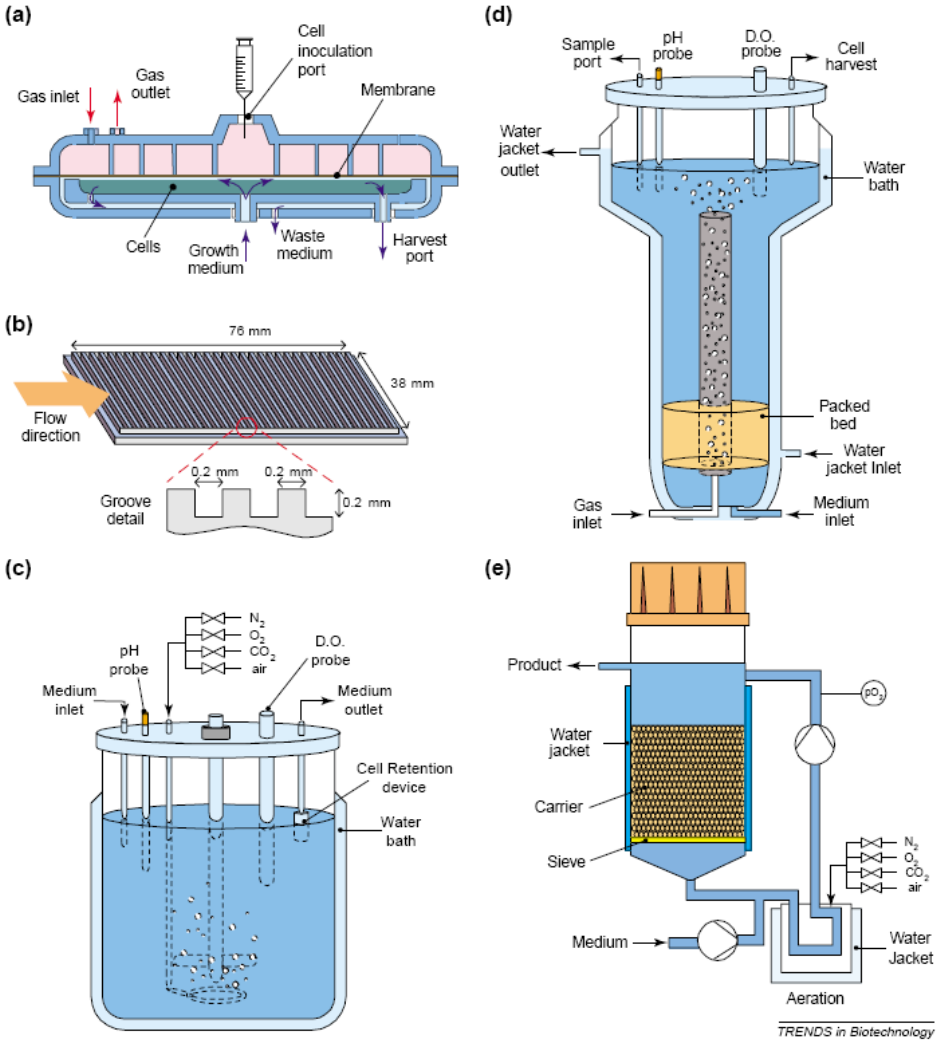

Figure 2.1 Bioreactors more frequently used for the culture of HSCs: (a) perfusion chamber; (b) grooved surface of perfused chamber; (c) stirred tank; (d) airlift fixed-bed; and (e) fixed bed or fluidized bed bioreactor [4].

A major challenge to successfully scaling-up animal cell culture is to achieve sufficient oxygen supply and mixing without creating mechanical stresses on the cells [6], as animal cells are known to be extremely sensitive to fluid mechanical stresses due to their lack of a protective cell wall. In fact, in suspension culture systems, both mechanical agitation and sparging aeration can cause animal cell death [7]. At present, research is performed in bioreactors with volumes in the range of about 100-1000ml to culture embryonic, mesenchymal, neural [8][5] and HSC cells [9][10][11].

In a bioreactor for animal cells expansion the following parameters are usually controlled:

- stirring speed
- pH (which can be modified by the introduction of CO_2)

- temperature (which can be modified by a water jacket)
- oxygen partial pressure (which can be modified by the introduction of a oxygen/nitrogen mixture [12]

In the design of a bioreactor, other fundamental parameters should be taken into account. The type of material used to construct a device plays a major role in the reproducibility and reliability of the expansion process and other factors such as cleaning, sterilization, and re-use can also affect the advantages in the use of a specific material [13].

2.2 *In vitro* hematopoietic stem cells expansion

Hematopoietic cells are most commonly expanded in static culture systems such as well-plates, T-flasks or gas-permeable culture bags, especially when co-cultured with stromal cells [4][1][9][14] However, these methods present unsolvable limitations due to their non-homogenous nature in terms of concentration gradients of pH, dissolved gas, nutrients, cytokines and metabolites [15] [1] [11]. Further draw-backs of static systems for *in vitro* expansion applications lie in lower process reproducibility, reduced possibilities of on-line monitoring and control, and the limitations of available surface area which can restrict productivity, and eventually limit the possibility of developing a suitable process that can be approved by FDA [4][1]. Several studies, performed on the use of different types of bioreactors for the expansion of haematopoietic stem cells, demonstrate that hydrodynamic forces present in agitated bioreactors are known to affect growth, viability, metabolism, cell cycle, cell size and surface marker expression[16][17][18][19][20][21]. HSCs do not require surface attachment to grow and have been successfully cultured in stirred bioreactors with improved performance, as mixing overcomes diffusion limitations of static culture systems. Recent studies demonstrate that stirred systems could improve HSCs expansion *in vitro* compared to static ones [22][23].

In order to study the effects of hypoxia on UCB-derived CD34+ cells, a six well stirred bioreactor has been developed. The bioreactor presented is a promising tool to perform accessible stem cell cultures under dynamic conditions, and to investigate the effect of convective mass transport on cell interaction with the local surrounding environment.

2.3 The six-well stirred bioreactor

The bioreactor system developed in this work is composed by six 10-ml volume stirred wells. The involved volumes make the system suitable both for process optimization and for stem cell production. In particular it may be a promising tool in view of a personalized cell-based therapy, where stem cells are expanded *ad hoc* for a single patient.
Because most of the currently available hematopoietic cell culture protocols are developed in conventional cell culture systems, such as multiwells or flasks, a bioreactor that has a high degree of continuity with those systems makes straightforward the adaptation of these protocols. Besides, it helps understanding the effective role of mixing in stem cell culture, without the interference of other culture variables.
The milli-liter-scale bioreactor is constituted by a lid with an integrated mechanical stirring system which can be coupled to a standard six-well stirred suspension bioreactor disposable six-well plate. The system can be placed inside a biological incubator, thus taking the advantage of incubator temperature and pH control and avoiding complex systems to control these parameters. Furthermore, it allows up to six experiments to be performed in parallel under stirred suspension conditions.
Considering the interdisciplinary of stem cell field, where researchers with different background work together on *in vitro* stem cell culture, it is important to develop a robust device that can be handled even by users without a strong technical background, for a wide-spread use, and repeatability of the results obtained. Thus simplicity in a bioreactor for stem cell culture is not a secondary aspect.

2.3.1 The bioreactor design

The bioreactor is designed to keep cells suspended in conditions that respond to the essential requirements for a stem cell culture: biocompatibility of materials, maintenance of sterility, and low shear stress.

The bioreactor device (Figure 2.2) is composed of three main components: a conventional polystyrene six-well plate, a lid coupled to an integrated stirring system and a gas inlet system.

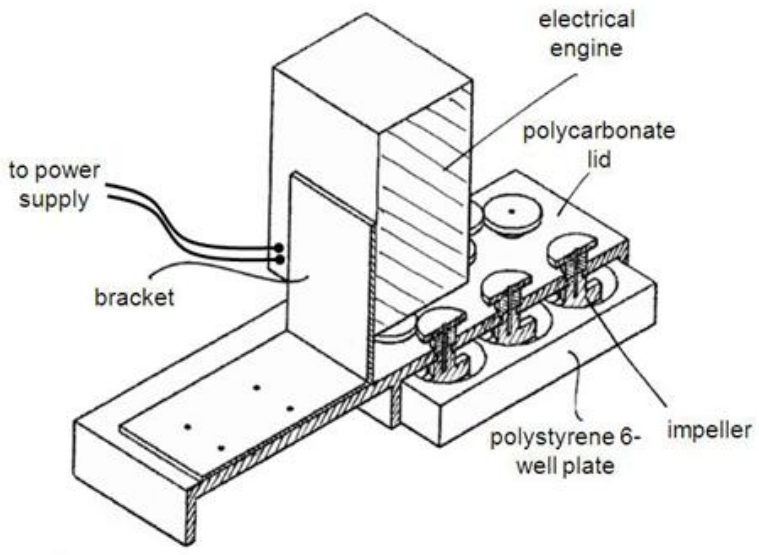

Figure 2.2 Section of the bioreactor system coupled to a six-well plate

The polycarbonate lid fits the multiwell as a cover and presents a part which sustains an electrical engine (RS Components, Milan, Italy).

The lid presents six openings, in correspondence to the centre of each well of the plate for the passage of the stirred shafts. A detailed view of the coupling between the opening and the stirrer is presented in Figure 2.3A.

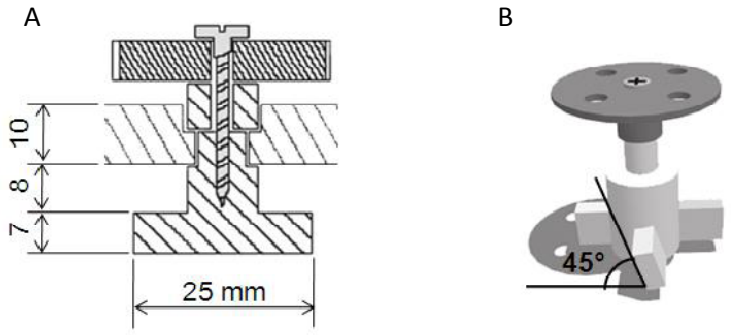

Figure 2.3 Section of the impeller inserted in the lid (A). Three-dimensional representation of the impeller, shaped as a turbine with 45° pitched blades (B).

All the materials either in contact or in proximity to the cell culture are biocompatible: polystyrene from the 6-well plate, PTFE from the impellers, and polycarbonate from the lid [13]. The system ensures cell culture sterility as the six-well plate is disposable, and the lid, after engine removal, is autoclavable at 121°C. Furthermore, as shown in Figure 2.3A, the

openings on the lid for the stirrer shaft passage are shaped so that entering gases pass through a tortuous path that blocks the suspended solids responsible of carrying contamination agents. The impeller is shaped as a turbine with 45°- pitched blades to produce an axial flow pattern that favors cell suspension from the bottom of the well, where cells otherwise settle.

The impeller Reynolds number, *Re*, is defined as:

$$Re = D^2 N\rho/\mu \tag{1}$$

where

- D is the impeller diameter (25 mm)
- N is the impeller rotational speed (rounds per minute)
- ρ is the medium density
- μ is the medium viscosity

A stirring rate of 15 rpm has been chosen for the experiments, to avoid dead zones in the well and to keep the shear on the cells at low values. In this condition, approximating medium properties with those of water, $Re \approx 10^2$ and the flow is in the transitional regime between laminar and turbulent, the approximate maximum shear stress in the well is 3.6×10^{-3} Pa, which is orders of magnitude lower than the critical shear stress that is harmful for the cells [24]. The bioreactor is also implemented to perform hypoxia cell cultures. All the components of the bioreactor are sealed to avoid gas leak, and the lid is provided with an inlet and an outlet. The system is then fed with a gas mixture of 5% O_2, 5% CO_2, and N_2, after a humidification step.

2.3.1.1 The stirring system

The polytetrafluoroethylene (PTFE) impeller is shaped as a turbine with 45° pitched blades (Figure 2.3B) and is connected to a gear (RS Components) by a screw of stainless steel. The motion is transmitted from the electrical engine to the impellers by a gear train, and the driving gear is directly fixed to the engine shaft. The engine is protected by a plastic box from the humidity of biological incubators and removably fixed to the lid by a bracket. Electrical wires connect the engine to a power supply (Kert, KAT4VD) which is placed outside of the incubator.

The system has been designed according to the guidelines in Brodkey *et al.*, 1988.
The main purpose of mixing is to reduce concentration gradients in the culture system, thus medium temperature, gas concentration, nutrients (supply of glucose, glutamine, amino acids) and products from cellular metabolism (such as ammonia and lactate) are homogeneously distributed in the medium [26].

In static conditions, the mass transport in the culture medium is controlled by diffusion and is described by Fick's law:

$$N = -D\frac{\partial C}{\partial z} \tag{2}$$

where

- D is the diffusion coefficient, [m^2/s]
- C is the concentration of substance [Kg/m^3],
- N is the diffusion flux, [Kg/m^2s]

The stirring induces convective motion, which becomes predominant for the mass transport, because orders of magnitude faster than diffusion.
In these conditions, the mass flux is expressed as sum of two contributions, a diffusive and a convective flux:

$$N = -D\frac{\partial C}{\partial z} + VC \tag{3}$$

where V is convective velocity.
On the other hand, acceptable levels of shear stress are critical characteristics to maintain when culturing cells in suspension. These characteristics are influenced by vessel geometry, impeller design, and addition of equipment such as sparging devices and measurement probes, as well as the cell culture medium properties. Shear stress conditions can be estimated through calculating and measuring (where possible) the maximum shear stress t_{max} values [27].

To verify the maximum shear stress on the cells at a defined impeller rate, an approximate calculation of the shear, τ, at well bottom is performed by:

$$\tau = \mu\frac{\partial v_x}{\partial \gamma} \tag{4}$$

where

- μ is the dynamic viscosity of the medium [Pa·s]
- v is the velocity of the impeller tip [m/s]
- y is the spatial coordinate [m]

Foaming is another disadvantage of stirred system. In case of foaming, surface-active anti-foams can minimize cell damage by decreasing cell-bubble attachment but must be evaluated for stem cell toxicity [8].

2.3.1.2 The gas inlet system

In the gas inlet system, gas is first saturated with water and then fed inside a hermetic box containing the bioreactor. A controlled-composition gas inlet obtained by mixing pure (> 99.999%) compressed O_2, N_2 and CO_2 gases by a system of digital thermal mass flow meters.

2.3.2 Study of oxygen solubility

The stirring promote the homogenization of the gases, leading to the achievement of an equilibrium between their concentration in the gas phase and the liquid phase.
The thermodynamic equilibrium of gases is described by Henry's law:

$$pO_2 = y_{O_2} \cdot P = H \cdot x_{O_2} \quad (5)$$

where

- H is the Henry's constant, [Pa]
- P is the pressure of the solute in the gas above the solution [Pa]
- p_{O_2} is the oxygen partial pressure [Pa]
- x_{O_2} is the oxygen mole fraction in the liquid phase
- y_{O_2} is the oxygen mole fraction in the gas phase

2.4 Rational approach

Since oxygen tension plays a key role in regulating hematopoietic stem cell behavior, one of the aspects to investigate when planning the design of a bioreactor for the expansion of hematopoietic stem cells, is the oxygen concentration in the cell culture medium, particularly when experiments are conducted in hypoxic conditions.

Oxygen, due to its low solubility in cell culture media, and its high rate of consumption by exponentially growing cells, is one of the most difficult requirements to satisfy in mammalian cell cultures.

Thus, a study of oxygen solubility in different types of media, with and without serum, has been performed.

2.5 Experimental plan and set up

Accurate measurements of oxygen solubility in different media are performed.

The main components of the experimental setup are a system to produce definite-composition mixtures, a system to ensure temperature control, and the system of detection itself.

A 25-ml Schott Duran bottle (Zetalab Srl, Padua, Italy) is filled with 5 ml of medium. A definite-composition gas mixture is fed on medium surface with a flow rate of 100 ml/min. A fluorescent probe is put inside the medium in proximity of the bottle bottom. The bottle top has a silicon part that is perforated for the probe, and the inlet and outlet gas tubing to pass through.

Compressed nitrogen and oxygen cylinders with a purity of 99.999% (Air Liquide, Milan, Italy) are employed. Thermal mass flow meters EL-FLOW Series F-201C (Precision Fluid Controls Srl, Milan, Italy), equipped with HAM-LET filters of 7 μm (Precision Fluid Controls Srl), are used to produce mixtures with definite composition. Gases from flow meters are mixed in a 3-way coupling. All the connections are non-porous polyamide Rilsan tubing of 4-mm internal diameter, except a 250-μm diameter tube used to feed the gas mixture at medium surface.

Temperature control is accomplished by putting the 25-ml bottle, containing the medium, in a thermostatic bath, where temperature is controlled by PID thermoregulation with GTR2000

Circulator (Isco, Milan, Italy). Fiber Optic Oxygen Sensor System (GHT Photonics, Padua, Italy) is used to detect oxygen solubility by fluorescence measurements.

2.5.1 Oxygen probe calibration

The oxygen probe is calibrated with water at three different temperatures (27, 37, 47°C). For each temperature, 100% nitrogen is fed until equilibrium is reached. Then mixtures of nitrogen with oxygen at different partial pressures (0.04, 0.08, 0.12, 0.16, 0.20 atm) are used, allowing the system to reach equilibrium. All measurements are repeated three times. Calibration is obtained by comparing the data to those Benson and Krause (1976) and converting fluorescence intensity values to oxygen solubilities.

2.5.2 Oxygen solubility measurements

Oxygen solubility in Iscove's Modified Dulbecco's Medium (IMDM), with and without 10% Fetal Bovine Serum (FBS), is experimentally measured at three different temperatures (27, 37, 47°C) and partial pressures (0.04, 0.08, 0.12, 0.16, 0.20 atm), following the same procedure used for calibration with water. Analogous measurements are performed also for Dulbecco's Modified Eagle's Medium (DMEM), which is commonly used for hematopoietic and other types of stem cells [29] with and without 10% FBS.

2.5.3 Oxygen transport

In order to analyze the profile of oxygen concentration in water in correspondence to a modification of oxygen partial pressure of the atmosphere in static and dynamic conditions, the following apparatus is used. A polystyrene six-well plate is used and filled with 10ml water/well. In one condition water is static, in the other it is stirred at 40 rpm, using the stirring device described below. At time 0, both systems are at equilibrium with a nitrogen – oxygen mixture of 21% oxygen. A mixture of 10% oxygen is then fed on water surface at a flow rate of 100ml/min. The apparatus for the production of gas mixtures of definite compositions and the probe system are the same as illustrated in the previous paragraph. Measurements are taken placing the probe at the well bottom at 1-cm depth and at room temperature (22°C).

2.6 Results and discussion

2.6.1 Oxygen solubility

Accurate measurements of oxygen dissolved in different media, at thermodynamic equilibrium, are performed at 27, 37, and 47°C. Results for experiments at 37°C are shown in Figure 2.4 for water, DMEM, DMEM supplemented with 10% FBS, IMDM, and IMDM with 10% FBS. A linear fitting, according to Henry's law, is in excellent agreement with the experimental data in all cases. From these data Henry's constants at 37°C are determined, which are reported in Table 2.1, along with the constants at 27 and 47°C.

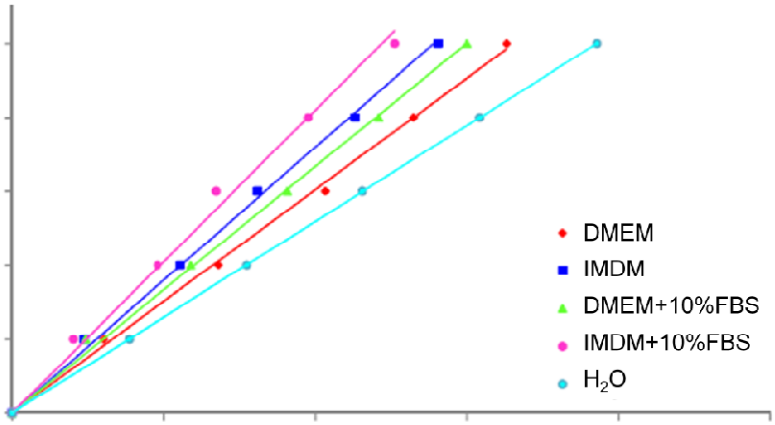

Figure 2.4 Thermodynamic data of oxygen solubility at 37°C. Experimental data and linear fitting of oxygen partial pressure as a function of molar fraction in the liquid phase, in different media (see legend).

As shown in Table 2.1, Henry's constant increases from water to DMEM, and from DMEM to IMDM. Moreover, the presence of serum, because of its high protein content, affects the solubility. At 0.2 bar of oxygen partial pressure, DMEM and IMDM solubility values were diminished by 9.4%, and by 12.2% respectively, when supplemented with 10% FBS. The effects of serum on hematopoietic cell cultures involve many complex interactions [30], and it is often difficult to discern which serum component contributes to a particular effect. As oxygen is known to have a significant influence on hematopoietic cell behavior [31], the differences in HSC expansion may be partly attributed to oxygen availability to the cells.

Table 2.1 Henry's constant. Experimentally-derived Henry's constants for different media at the temperature indicated.

H_{O_2}(bar)	T = 27°C	T = 37°C	T = 47°C
H_20	45071	51848	57835
DMEM	52401	60411	64148
DMEM + 10% FBS	56788	66675	72533
IMDM	60048	71807	76991
IMDM + 10% FBS	67446	81732	84482

2.6.2 Transient of oxygen concentration

The transient of oxygen concentration in water at 22°C after the nitrogen-oxygen mixture in the atmosphere is modified from 21% to 10% oxygen partial pressure. In static cell culture conditions, medium equilibration with a hypoxic atmosphere requires many hours, as verified in water. After 10 hours, in fact, only about 70% of the equilibrium is reached (Figure 2.5). Thus, the effective O_2 concentration in the cell microenvironment is affected by gradients and difficult to be determined. In stirred conditions, mass transport is highly enhanced, as the time required to reach the new equilibrium is about 1h.

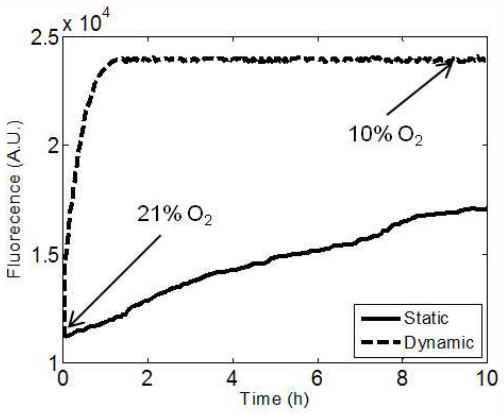

Figure 2.5 Comparison of oxygen transient in water in static vs stirred conditions. At time O, both the systems are at equilibrium wih a nitrogen-oxygen mixture of 21% oxygen partial pressure and a mixture of nitrogen-oxygen at 10% oxygen partial pressure is fed at the water surface. Measurements are taken online using a fluorescent probe placed at 1-cm depth. The stirring is fixed at 40 rpm and the measurements are taken at 22°C.

2.7 Preliminary biological experiments

Preliminary experiments, whose results are not shown here, have been performed to optimize the system. For instance, impeller geometry, and operative conditions, such as working volume and mixing rate, have been defined. The bioreactor has been then used for culturing both hematopoietic stem cells and hES-EBs. First of all, the results show that the bioreactor configuration ensures high viability up to 20 days of culture. However, issues related to cell aggregation phenomena were observed when culturing hES-EBs. While the system is suitable for hematopoietic stem cell cultures with performances comparable to conventional culture systems, in my hands, the dynamic conditions lead to an increased hES-EBs aggregation compared to cells cultured in static conditions. Aggregation of EBs should be avoided since it increases heterogeneity in the cell population. Because of this technical issue, alternative solutions have been considered.

Such limitation has been overcome by using biomaterials as tools to prevent cell aggregation and control stem cell fate. In particular, a microstructured hydrogel, which permits to obtain physically separated hES-EBs, has been realized. The innovative cell culture system will be presented in Chapter 4.

2.8 Conclusions

In this work a stirred bioreactor system has been developed for stem cell culture ins suspension under controlled conditions.

The system is formed by a cover with an integrated stirring system that has dimensions compatible with conventional disposable six-well plates. The bioreactor has been designed as simple, user-friendly, and as robust as possible in order to be handled even by researchers without a strong technical background. Only essential features for stem cell culture are included, such as biocompatibility of materials, sterility, and low-shear mixing. System complexity is kept down by the possibility to exploit temperature and pH control of the biological incubator, where the bioreactor is placed during cell culture.

In this device cell culture is performed under mixing conditions, which is the only difference respect to conventional culture systems, where medium is stagnant. Thus it allows the use of already developed protocols of hematopoietic stem cell culture, looking exclusively at the

effect of mixing. Besides, the bioreactor allows up to six parallel experiments under identical conditions.

In this work, the oxygen solubility in different types of media has been studied, in order to understand the effective oxygen concentration in the cell microenvironment during cell culture. The data show that the mixing provided by the stirring system of the bioreactor, allow for reaching oxygen equilibrium in the medium in a defined time, whereas in static cultures oxygen concentration is difficult to be determined and is affected by gradients. On the wake of these results, a slightly more complex version of the bioreactor allows for culturing stem cells under hypoxic conditions. This aspect is particularly important since oxygen tension is recognized to play a key role in regulating stem cell behavior.

Preliminary experiments have been performed culturing both HSCs and hES-EBs. The bioreactor was suitable for stem cell culture ensuring high viability up to 20 days, but issues related to hES-EBs aggregation were observed. Because of this severe technical limitation, alternative solutions for culturing hES-EBs have been considered. The possibility to use biomaterials as substrates for preventing cell aggregation and controlling cell fate will be discussed in Chapter 4 together with the developed microstructured hydrogel.

To conclude, this bioreactor may be a promising tool to perform stem cell culture under dynamic conditions and in view of a personalized cell-based therapy, where stem cells are expanded *ad hoc* for a single patient.

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Chapter 3
Application of the bioreactor for HSC expansion in hypoxic conditions

The bioreactor presented in § 2.3 has been successfully used for studying the role of hypoxia on UCB-derived CD34+cells c-kit expression. In this chapter, § 3.1 and § 3.2 reviews the concepts of stem cell niche and the importance of oxygen concentration for stem cell undifferentiated state maintenance, with special regards to hematopoietic stem cells. The rational and experimental approaches, together with the results of UCB-derived CD34+ cells cultures under hypoxic conditions are described in § 3.3 and § 3.4 respectively. § 3.5 drafts the final remarks of the present studies and § 3.6 reports the cited literature.
The work will underlie how the use of technology can provide the basis to investigate a class of cell biology questions that would be difficult to explore in traditional culture systems.

3.1 The stem cell niche and oxygen concentration

Hypoxia is commonly associated with pathologies, such as tissue ischaemia and inflammation and tumors [1]. Hypoxic environment, however, are also present in developing embryos and in cell niche in the adult. The niche refer to a defined anatomical compartment that includes cellular and acellular components that integrate both systemic and local cues to regulate the biology of stem cells [2][3][4][5]. Cells, blood vessels, matrix glycoproteins, and the immediate space around the cell provide a highly specialized microenvironment for a stem cell [4]. Contact and communication between these elements is critical for stem cell self-renewal and multipotency. While much interest has focused on identifying the structural and humoral factors that mediate this biology, an underexplored field is the metabolic milieu of this highly specialized microenvironment [4].

Currently, hypoxia is known to promote an undifferentiated state in several stem and precursor cell populations; the signaling cascade and transcriptional program activated in hypoxic conditions appear to be shared by many stem cells. The early *in vitro* tissue and cell cultures were performed paying high attention to the balance of nutrients, growth factors, and pH buffers used to grow cells *in vitro* [6], while very little interest was addressed to the oxygen concentration present in culture media, as it was assumed that ambient air was adapt for cell culture [7]. In contrast, direct measurements of tissue oxygen tensions in developing embryos revealed that these tissues demonstrated that oxygen tensions was lower than had been supposed [8]. Oxygen measurements of tissues known to host stem cells revealed even lower oxygen tensions, thus raised the question of whether those environmental conditions were necessary for the niche to maintain stem cells [9][10][11].

Recent experiments performed under controlled oxygen concentrations showed that lower oxygen tensions greatly influenced both embryonic and adult stem cell behavior [12][13][14].

Adult tissues experience a wide range of oxygen tensions, considerably different from the inhaled oxygen tensions of the air at 160 mmHg (21 %). The partial pressure oxygen (pO_2) of inspired air progressively decreases after it enters the lungs and as it goes throughout the body. By the time it reaches organs and tissues, pO_2 levels have decreased to 14–65 mm Hg (2%-9%) [15]. This pressure represents an important change from the oxygen tensions that are typically considered 'normoxic' by conventional standards of cell-culture practice.

These observations, coupled with the direct oxygen measurements in different tissues and the experiments under controlled oxygen concentrations, gave birth to the hypothesis that low oxygen tensions were indeed critical to the metabolic milieu of stem cell niches.

It has been hypothesized that cells that undergo aerobic metabolism are subject to oxidative stress through the generation of reactive oxygen species that can damage DNA. This risk is supported by the observation that mouse embryonic fibroblasts accumulate more mutations and senesce faster when cultured under 20% than O_2 cells cultured under 3% O_2 [16]. By residing in microenvironments under relatively low oxygen tensions (in the range of 1%–9%), stem cells may escape this damage.

3.2 The role of oxygen in the maintenance of HSCs

One of the best characterized stem cell niches is the hematopoietic stem cell one [5]. Tracer studies of transplanted HSCs reveal that they most likely reside in bone cavities

specifically adjacent to endosteal bone lined by osteoblast cells in hypoxic ocnditions [2][3]. HSCs share an important relationship with osteablasts and other stromal elements of the bone marrow niche critical to their maintenance and protection [2][3][17][18]. However, in several papers published in the1990s, it was postulated that the bone marrow niche was hypoxic relative to other tissues [10]. This model was based on the observation that quiescent HSCs localized to regions of the bone marrow that were several cells away from blood vessels. With several stromal cells and progenitor cells observed to physically reside between the HSCs and the closest blood vessel, it was hyphotized that this niche was relatively hypoxic when compared to other tissues, as these cells competed for the already scarce nutrient and oxygen supply. Mathematical models based on animal data supported this hypothesis and predicted oxygen tensions to be as low as 1% [19]. It is now widely accepted that gradients of oxygen from below 1% in hypoxic niches to 6% in the sinusoidal cavity exist within the human bone marrow [14].

HSCs present in the hypoxic niche express higher levels of Notch-1, telomerase, and the cell-cycle inhibitor p21 than cells closer to the vasculature [20]. This observation was recently supported when work on xenotransplanted CD34+/CD38- human cord blood cells cultured under 1% O_2 demonstrated engraftment with significant reduction in proliferation and upregulation of the cell-cycle inhibitor p21 [21]. Remarkably, extremely low oxygen tensions (0.1%) push CD34+ cells into an essentially quiescent state [22].Collectively, this evidence, as outlined by several reviews, suggests that hypoxia is a critical component of the HSC niche, and exposure of HSC to elevated oxygen tensions negatively affects their self-renewal and promotes cell-cycle entry.

One of the advantages of residing in a hypoxic niche is that stem cells can maintain slow-cycling proliferation rates while avoiding the oxidative stress associated with more well-oxygenated tissue [16][10][14][23]. The Hypoxia-Induced factor 1 (HIF-1) has emerged as a key regulator of this mechanism. In fact, several groups have demonstrated that HIF can mediate cell-cycle arrest in several cell lines [24].

The heterodimeric transcription factor hypoxia-inducible factor (HIF)-1 constitutes the major molecular response to hypoxia. It consists of two basic helix-loop-helix proteins: an oxygen-sensitive HIF-1α subunit and an oxygen-insensitive HIF-1β subunit (also called aryl hydrocarbon receptor nuclear translocator, Arnt). Three regulatory HIF subunits have been characterized: HIF-1α, HIF-2α, and HIF-3α. HIF-1α is expressed ubiquitously, whereas HIF-2α and HIF-3α expression appear to be restricted to certain tissues [25]. Regulation of HIF-1 activity is mediated by post translational modification of the oxygen-dependent

degradation domain (ODD) in the α subunit. At oxygen levels above 5%, hydroxylation of the proline residues 402 and 564 in the ODD of HIF-1a enables binding of the ubiquitination ligase von Hippel-Lindau tumor suppressor protein, which leads to degradation of HIF-1α by the proteosome [26] In contrast, at oxygen level below 5%, hydroxylation is inhibited leading to stabilization of HIF-1α. Mice with HIF mutations develop extensive hematopoietic pathologies. Embryos lacking Arnt have defects in yolk sac vascularization, suggesting a defect in primitive hematopoiesis.

Measurements of oxygen tension in the cord blood immediately after delivery of the infant [27] [28] show that CD34+ cells isolated from this source reside in hypoxic conditions too. The fact that the developmental state of multiple stem or progenitor cells populations is influenced by oxygen levels, does implicate the existence of O_2-sensitive pathways involved in the regulation of stem cells fate.

Recent publications demonstrated that performing HSC cultures in hypoxia seems to favor survival and stem cell self-renewal, as opposed to differentiation [10][29][30], thus, the use of low O_2 levels during *in vitro* UCB HSCs culture may represents an important advance and could have valuable clinical implications.

Using UCB-derived CD34+ cells, the role of hypoxia on c-kit expression of is investigated in this work.

3.3 Rational approach

In order to study the effects of hypoxia on c-kit expression in UCB-derived CD34+ during *in vitro* expansion, the six-well bioreactor (described in chapter 2), which guarantees a defined and controlled oxygen concentration in the medium, is employed to perform biological experiments under defined hypoxic conditions. This kind of study would not be possible culturing cells in conventional cell culture systems, because of the slow rate of oxygen exchange at the gas-liquid interface in static conditions as described in the preliminary data reported in the previous chapter.

3.4 Experimental plan

At the beginning of each culture experiment, cells are selected for CD34, cultured and fully characterized for the expression of differentiation markers, to guarantee a repeatable starting point, in the limit of biological variability.

CD34+ is a heavily glycosylated surface antigen which is preferentially expressed on hematopoietic stem/progenitor cells and progressively lost as cells differentiate. It appears to be involved in signal transduction to regulate the expression of other hematopoiesis - associated genes and is clinically used for cell selection [31][32].

Viability and proliferation rate of CD34+ cells isolated by either a single- or a double-step magnetic separation is then investigated.

In order to verify the biocompatibility of the bioreactor and exclude any negative effect to the cells due to mixing, cells are cultured for up to 13 days in dynamic or static conditions (for negative control) and six days after seeding, cell viability (by 7-AAD staining), proliferation (by CFDA-SE staining and hematocytometer counting) and morphology (by optical microscopy) are analyzed. Analysis of differentiation markers by flow cytometry and analysis of cells ability to differentiate by colony forming assay will complete the analyses.

In order to study the effects of hypoxia on c-kit expression, cells are then cultured in dynamic conditions under hypoxic or normoxic (for negative control) conditions. Cell morphology and proliferation are analyzed by optical microscopy and hematocytometer counting respectively and c-kit expression is quantified by flow cytometry.

3.4.1 Umbilical Cord Blood derived CD34+ culture

3.4.1.1 Cell selection

Cord blood (CB) units (Figure 3.1A) are obtained from Bone Marrow Stem Cell Transplant Laboratory (Pediatric Oncohaematology Department, Padua, Italy), after informed consent. The blood from five or six units is used for each experiment.

Cord blood is eluted with 20 ml phosphate buffer saline (PBS) 1X (Gibco, Invitrogen, Milan, Italy). Mononuclear cells are isolated by density-gradient centrifugation (2000 rpm, 30 min), layering on equal volume of Ficoll (GE Healthcare). Mononuclear cells are counted by hematocytometer, and analyzed by flow cytometry as a negative control.

CD34+ cells are isolated by superparamagnetic microbeads (CD34 antibody, QBEND-10, Abcam, Cambridge, UK) selection using high-gradient magnetic field and mini-MACS

columns (Miltenyi Biotech, Glodbach, Germany). The selection of the cells is verified by flow cytometry (FACS Calibur, Becton Dickinson) after counterstaining with CD34-phycoerythrin antibody (AC136 clone, Miltenyi Biotec).

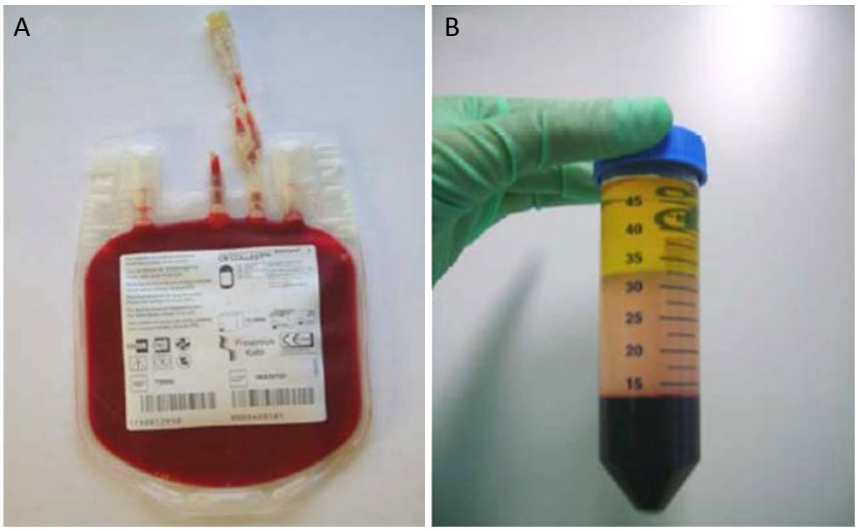


Figure 3.1 A cord blood unit (A). Cord blood after density-gradient centrifugation. Mononuclear cells and HSC cells are in the middle part of the surnatant (B).

3.4.1.2 In vitro cell culture

The selected cells are suspended in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco), 10% scomplemented FBS (56°C, 30 min), with 10% FBS (Gibco), 1% Penicillin/Streptomycin (Gibco), and supplemented with the following human citokynes (PeproTech Inc, Rocky Hill, NJ): hSCF (50 ng/ml), hTPO (10 ng/ml), hIL-6 (10 ng/ml), hFL (50 ng/ml), according to the protocol in Gunetti *et al*., 2008.

CD34+ cells are seeded in a 6-well plate (Becton Dickinson) at a density of 3600 cells/ml. Each well is filled with 10 ml of medium. Cells are cultured for 6 days both under normal oxygen conditions and in hypoxia. Hypoxia cultures are performed by feeding to the system a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. Dynamic cultures are performed at an impeller speed of 15 rpm.

3.4.1.3 Cell characterization

Cell count and viability was detected by hematocytometer. After suspension in 40 µl PBS 1X,cells were stained by incubation at 4°C for 15 min with a panel of monoclonal antibodies: PE-conjugated CD34 (AC136 clone, Miltenyi Biotec), APC-conjugated CD117 (104D2 clone, Biolegend, Milano, Italy), FITC-conjugated CD38 (HIT2 clone, Biolegend),

CD41 (HIP 8 clone, Biolegend), CD61 (monoclonal antibody, Immunotech, Czech Republic).

Cells were also incubated with 7-amino-actinomicyn D (7-AAD, Becton Dickinson), to analyze viability. Staining with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Gibco) is performed before seeding, according to the protocol in Lyons *et al.*, 2004. Labeled cells are suspended in 1 ml PBS 1X, centrifuged (1200 rpm, 5 min), and suspended in 200 µl PBS 1X. Cells are then analyzed by flow cytometry acquiring 10^4 events.

Colony-forming cell (CFC) assays are performed seeding 1000 cells from each well in a 24-well plate and grown in a semisolid medium (400 µl of Methylcellulose-MethoCult® GF H4434,

StemCell Technologies, Grenoble, France) for 14 days. The morphology of the hematopoietic progenitor cell colonies:

- burst forming unit-erythroid (BFU-E)
- colony forming unitgranulocyte-macrophage (CFU-GM)
- colony forming unit-granulocyte-erythroidmacrophage-megakaryocyte (CFU-GEMM)

is then observed by optical microscopy.

3.4.2 Experiments at 20% oxygen partial pressure

Two experiments are performed to compare the different proliferation rate of $CD34^+$ cells isolated by either a single- or a double-step magnetic separation. The fraction of $CD34^+$ cells is higher than 35±3% in the first case and 83±2% in the second one, as revealed by flow cytometry measurements immediately after selection (Figure 3.2).

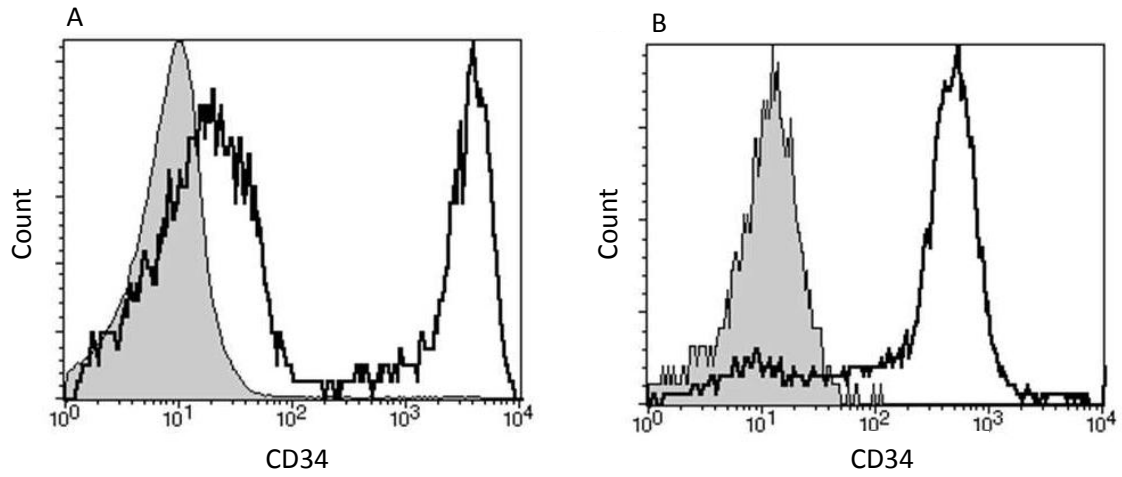


Figure 3.2 Flow cytometry analysis of umbilical cord blood mononuclear cells after isolation of CD34+ cells by one (A) or two (B) selection steps using high-gradient magnetic field and mini-MACS® columns.

Isolated cells are then cultured in the stirred bioreactor and under static conditions as a control. The two experiments are performed at different initial cell densities because of the limited number of double-selected cells available. Initial cell density is 54000 cell/ml for single- selected cells, and 10000 cell/ml for double-selected cells).

3.4.2.1 Results and discussion

Results of CD34, CD38, CD41, and CD117 expression performed immediately after the selection by flow cytometry are reported in Table 3.1 Flow cytometry protein expression characterization of cells double-selected for CD34, used in culture experiments. Data are shown as mean ± standard deviation. Viability is detected by flow cytometry after 7-AAD staining. The fraction of dead cells is always lower than 4% (data not shown).

Table 3.1 Flow cytometry protein expression characterization of cells double-selected for CD34, used in culture experiments. Data are shown as mean ± standard deviation.

	Percentage of cells
7-AAD⁻	(98 ± 2) %
CD34⁺ [a]	(82 ± 2) %
CD38⁻ [a]	(99 ± 1) %
CD41⁻ [a]	(98 ± 2) %
CD117⁺ [a]	(72 ± 2) %

In Figure 3.3, fold expansion of cells in both the conditions for two experiments is shown.

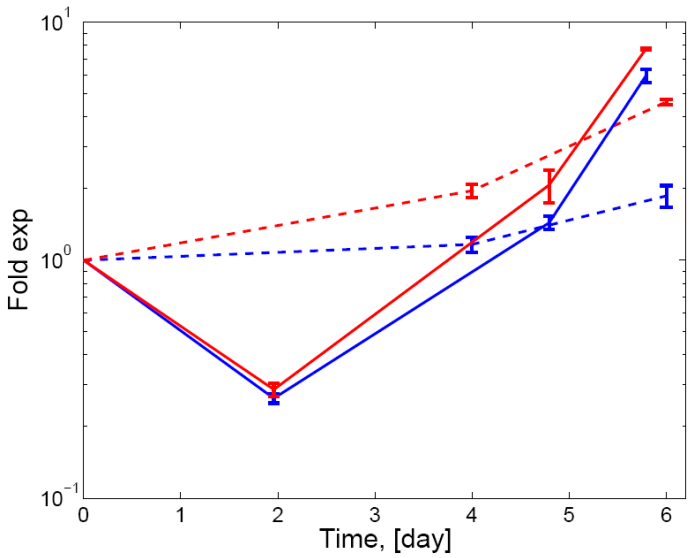


Figure 3.3 Growth curve of cells cultured under static and dynamic conditions at pO2=20%. Hematocytometer Measurements of fold expansion of cells isolated by one (dashed), and by two magnetic separation steps (solid) cultured under static (blue) and dynamic (red) conditions. Initial cell density is 54000 cell/ml for single-step selected cells, and 10000 cell/ml for double selected cells. Mean and standard deviation data for three repeated experiments for single-selected cells, and two repeated experiments for double- selected cells.

The initial drop in cell concentration for the double-selection case is due to the adaptation phase of the cell culture to the environment. In both, single- and double-selection, stirring increases the proliferation rate. This effect is enhanced in the single-step selected cell culture, because of a higher seeding density that makes material transport a more relevant concern. Even if starting from a lower cell density, double-selected cells present a higher division rate. This confirms that cells mainly contributing to the proliferating population belong to the $CD34^+$ cell fraction, thus next experiments will be performed using double-selected cells.

Before performing experiments in hypoxic conditions, the bioreactor is tested in normoxia for biocompatibility and other possible negative effects on cells. Cells, seeded at a density of 3600 cells/ml are cultured in normoxia for 13 days in parallel under static and dynamic conditions. For dynamic conditions the stirring rate is fixed at 15 rpm. The approximate maximum shear stress in the well is 3.6 10^{-3}Pa, which is orders of magnitude lower than the critical shear stress harmful for cells [35].

The analyses of cell viability by 7-AAD (Figure 3.4), of cell proliferation by CFDA-SE (Figure 3.4B) and the count by hematocytometer (Figure 3.4C) after 6 days of culture are presented.

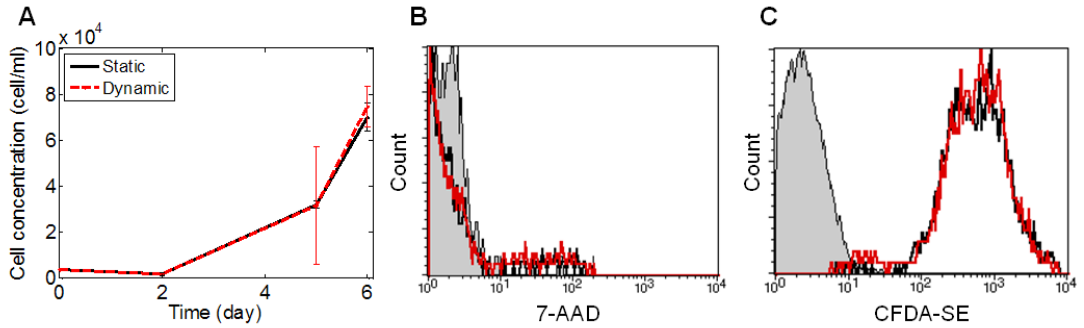


Figure 3.4 Comparison of cells cultured under static and dynamic conditions, at 21% oxygen partial pressure, after 6 days. (A) Cell count by hematocytometer, data are shown as mean ± standard deviation of three repeated experiments. (B) Cell viability detected by 7-AAD, the histogram shown is representative of two repeated experiments. (C) Cell proliferation detected by CFDA-SE, cells are stained at the time of seeding, the histogram shown is representative of two repeated experiments.

Comparable results are obtained in the two conditions.

Cell morphology is observed after seeding, 6 days and 13 days of culture by optical microscopy, as presented in Figure 3.5.

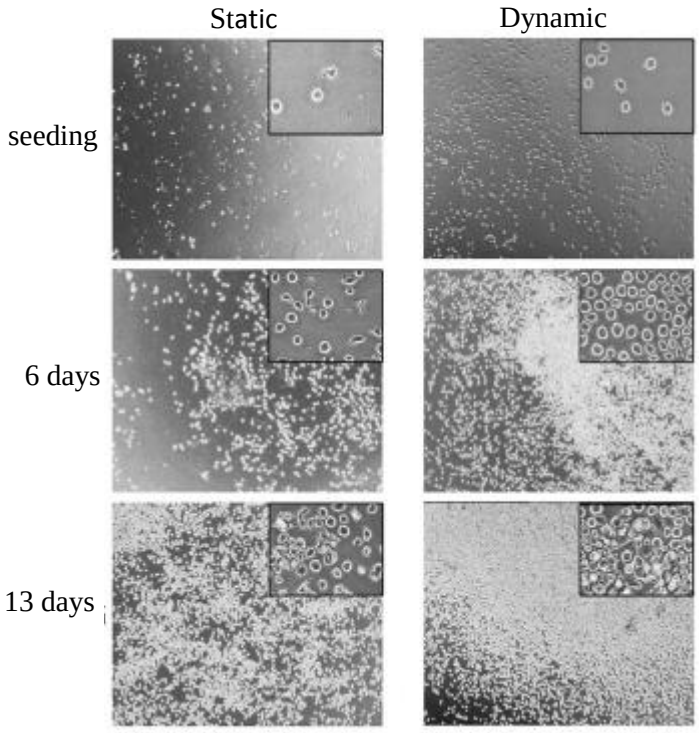


Figure 3.5 Morphology detected by optical microscopy at 40X and 200X (enlargements) for the culture under static and dynamic conditions after seeding, 6 days and 13 days of culture at 21% oxygen partial pressure.

No evident morphological differences are visible between the two conditions, as presented in Figure 3.5 The apparently higher number of cells in Figure 3.5A is due to the accumulation of the cells at the center of the well when stirring is stopped.

Figure 3.6 shows the results from flow cytometry analyses taken after 6 and 13 days of static cell culture. Markers CD34, CD38, CD41, CD61, and CD117 are analyzed.

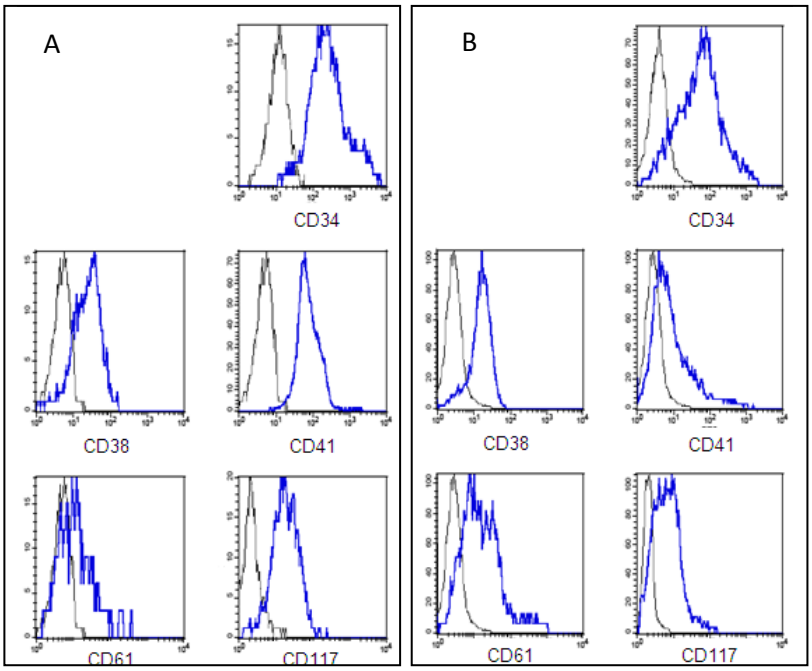


Figure 3.6 Flow cytometry measurements of cells cultured under static conditions at pO2=20% at an initial cell density of 3600 cells/ml. Profile of markers CD34, CD38, CD41, CD61, and CD117 in the cell population cultured in static conditions (blue) at day 6 (A) and 13 (B). Black curves refer to the negative control without fluorescent staining.

HSCs are $CD34^{+}$, $CD117^{+}$, $CD38^{-}$, $CD41^{-}$, $CD61^{-}$ [36], but during *in vitro* culture they may change their markers expression profile. For example, as shown in Figure 3.6, cells become more $CD34^{-}$ and $CD38^{+}$. This change may be related either to cell differentiation or to aberrant modifications that occur because of inadequate *in vitro* cell culture conditions. A proper protocol to maintain cell phenotype stable during culture is currently not available, and research is in progress in this direction.

Flow cytometry analyses taken after 6 and 13 days of cell culture in bioreactor did not show remarkable differences with respect to results in Figure 3.6 reported for static culture.

The multipotent ability to differentiate is verified by the colony-forming cell assay, after 6 days of culture in the two conditions, as shown in the images of the hematopoietic progenitor cell colonies (Figure 3.7). BFU-E (Figure 3.7A), CFU-GM (Figure 3.7B), CFU-GEMM (Figure 3.7C) progenitor colonies derived from cells culture under static or dynamic conditions are shown.

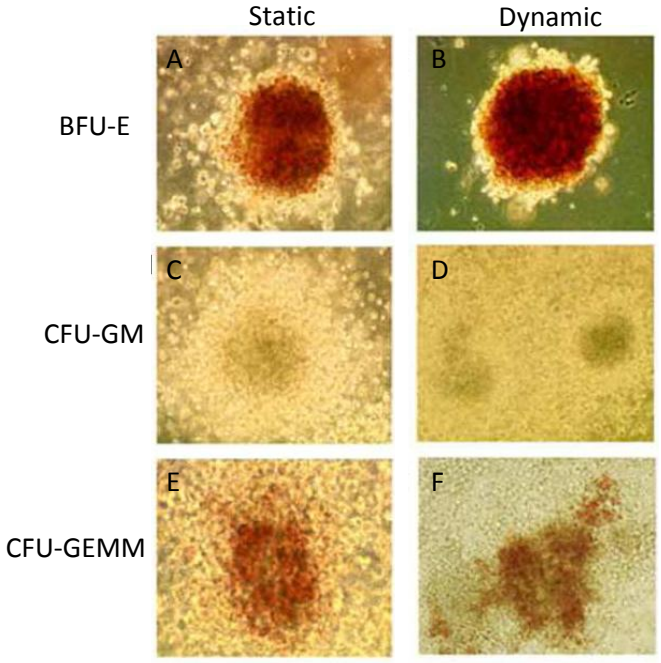


Figure 3.7 Hematopoietic progenitor cell colonies morphology detected by optical microscopy at 40X: BFU-E (A, B), CFU-GM (C, D), and CFU-GEMM (E, F), derived from cells cultured in static conditions (A, C, E) and in bioreactor (B, D, F) after 6 days of in vitro culture and then grown in a semisolid medium for 14 days.

No evident morphological differences are visible in the progenitor cell colonies between cells cultured in static or dynamic conditions.

To conclude, data obtained from preliminary experiments show that dynamic culture does not alter cell viability, morphology, proliferation and differentiation potential. Thus, we consider the low shear stress of mixing is not affecting cell bahaviour the bioreactor may be used to evaluate the role of oxygen on UCB-CD34+ cells expansion under hypoxic conditions.

3.4.3 Experiments at 5% oxygen partial pressure

In order to verify the maintenance of cell morphology when cells are cultured in hypoxic conditions, 3600 CD34+ cells/ml are first seeded both static and dynamic conditions for 13 days at 5% oxygen partial pressure.

3.4.3.1 Results and discussion

Cell morphology is observed after seeding, 6 days and 13 days of culture by optical microscopy, as presented in Figure 3.8.

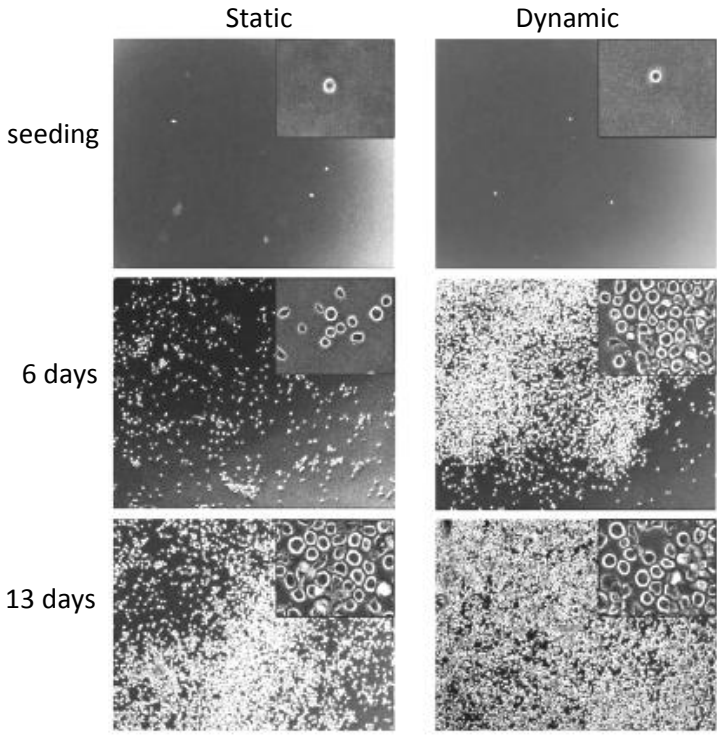


Figure 3.8 Morphology detected by optical microscopy at 40X and 200X (enlargements) for the culture under static or dynamic conditions at 5% oxygen partial pressure after seeding, 6 days and 13 days of culture.

Since no evident morphological differences are visible between the two conditions, cells are cultured at an initial cell density of 3600 cells/ml in the bioreactor and kept at 5% or 21% oxygen partial pressure for studying the effects of hypoxia on CD34+ cells expansion. Cell analyses are performed at days 5 and 6 days, when cultures are stopped. In Figure 3.9 the growth curve of the cells cultured in dynamic conditions at 5% or 21% oxygen partial pressure.

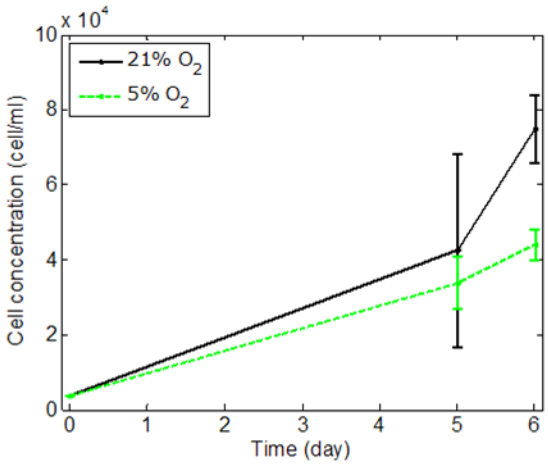
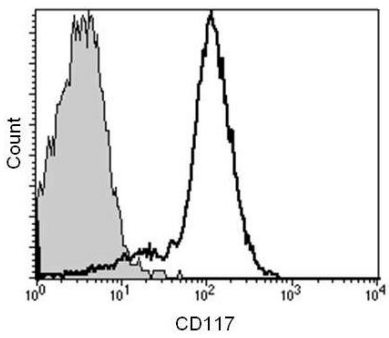


Figure 3.9 Cell count by hematocytometer at days 5 and 6 after seeding. Black line refers to cells cultured in normoxic conditions, green line to those cultured in hypoxic conditions.

The proliferation rate is lower in hypoxic conditions. These data are confirmed by other studies [10][22]. The low cell-cycle activity in hypoxia conditions has been associated with an increase in the proportion of long-term reconstituting hematopoietic stem cells [37].
Analysis of c-kit expression by flow cytometry at day 6 of culture is presented in Figure 3.10A.

A



B

	Percentage of cells	
	21% O_2	**5% O_2**
CD117⁺	(78 ± 2) %	(84 ± 1) %

Figure 3.10 Flow cytometry measurement of CD117 expression at day 0 after CD34 selection (A). Percentages of CD117+ cells obtained by flow cytometry data analysis (B).

The results show 78% and 84% respectively of c-kit+ cells in normoxic and hypoxic conditions (Figure 3.10B), suggesting a relationship of dependence between c-kit expression and oxygen levels. To the best of our knowledge this was not reported before.

In both the cases, the population increases its heterogeneity during culture and three subpopulations are visible, according to c-kit fluorescence (Figure 3.11).

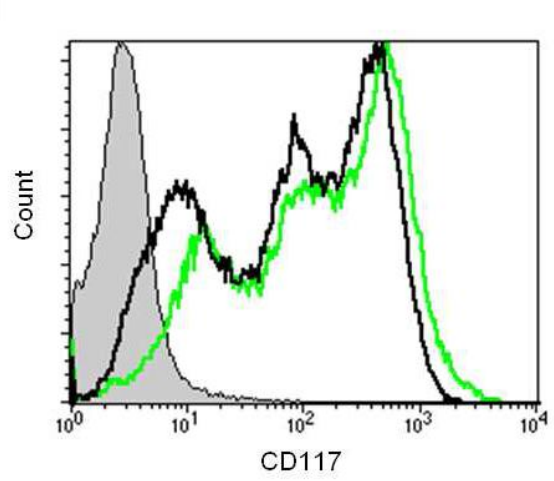


Figure 3.11 Flow cytometry measurement of CD117 expression at day 6 of culture.

3.5 Conclusions

As discussed in § 3.1, hypoxia is known to promote an undifferentiated state in several stem and precursor cell populations and hypoxic environment are present in developing embryos and in cell niche in the adult. On the other hand, *in vitro* tissue and cell cultures are performed paying high attention to the balance of nutrients, growth factors, and pH buffers used to grow cells *in vitro* and very little interest to oxygen concentration present in culture media, as has been assumed for long that ambient air is adapt for cell culture. When oxygen measurements of tissues known to host stem cells revealed even hypoxic environment, thus raised the question of whether those conditions were necessary for the niche to maintain stem cells. Recent experiments performed under controlled oxygen concentrations showed that lower oxygen tensions greatly influenced both embryonic and adult stem cell behavior [12][13][14].

One of the best characterized stem cell niches is the hematopoietic stem cell one and, as discussed in § 3.2, several publications demonstrate that oxygen concentration gradient in the hematopoietic cell niche *in vivo* play a fundamental role in the maintenance of cell pluripotency or multipotency. Thus, the use of controlled oxygen levels during *in vitro* UCB-derived HSCs culture would represent an important advance and might have valuable clinical implications. In order to address this challenge, the the stirred bioreactor (presented

in § 2.3), designed and developed to guarantee a highly controlled microenvironment in terms of oxygen concentration was used for culturing UCB-CD34+under defined conditions. The innovative culture system was first tested under normoxic conditions, to exclude any negative effects respect to cell viability, morphology, proliferation and differentiation potential. Once verified these fundamental aspects, the bioreactor has been used for culturing cells under hypoxic conditions, using those in normoxic as negative control. After 6 days of culture, lower proliferation was detected in hypoxic conditions compared to normoxic ones. These data are confirmed by other studies [10][22] The low cell-cycle activity in hypoxia conditions has been associated with an increase in the proportion of long-term reconstituting hematopoietic stem cells [37].

Moreover, by flow cytometric analyses, different profiles of c-kit expression between cells cultured in hypoxic conditions and to those cultured in normoxic atmosphere were obtained.

Specifically, in hypoxia we found an over-expression of c-kit respect to the 21% oxygen condition. It is known that c-kit (or CD117), the Stem Cell Factor (SCF) receptor, belongs to the family of the tyrosine kinase receptors [38] and is expressed in most of the hematopoietic stem and progenitor cells and has an important role in hematopoiesis [39]. The biological answer of HSCs to hypoxia is mediated by gene expression modifications and involves specific transcription factors. Among the recruited factors, the HIF-1 plays a fundamental role. Targets of HIF-1 are hematopoietic growth factors, and many other factors involved in cell survival, growth and motility. These data support a correlation among c-kit, SCF, HIF-1, hypoxia in HSCs.

The data obtained in the work provide the basis for further investigations in order to understand the molecular mechanisms underneath oxygen concentration and c-kit expression, since the study of c-kit pathway also involves an autocrine loop due to the cell secretion of SCF under hypoxic condition when HIF-1 is up-regulated [40].

These exciting findings underline the importance of oxygen tension as a metabolic regulator of stem cell biology and represent an added dimension of stem cell control that allows cells to maintain self-renewal and multilineage differentiation potential. Because of these evidences it is also supposable that gaseous messengers other than oxygen may be novel regulators of stem cells, thus highlights the importance of investigating the metabolic niche more closely.

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Chapter 4
Biomaterials for human stem cell culture

As reported in § 2.7, the culture of stem cells in the stirred bioreactor could be not straightforward because aggregation of stem cells can take place leading to the formation of heterogeneous cell clusters. This behavior was, for instance, observed for hES-EBs. In this perspective, one of the strategies that could be used to prevent random cell aggregation is the use of physical barriers in order to form separate compartments. The present chapter overviews two different biomaterial-based strategies that have been attempted in order to address this challenge: the single cell dispersion and the microwell confined cell cluster cultures.

After a brief introduction on the importance of three-dimensional scaffolds for *in vitro* cell culture (§ 4.1), hydrogels are introduced and described in § 4.2. Following, the potentialities and the limitations of a photopolymerizable hyaluronic acid based hydrogel are presented in § 4.3. The new opportunites offered by a polyacrylamide hydrogel are illustrated in § 4.4 together with the results of preliminary biological experiments. § 4.5 drafts the conclusions of the chapter and § 4.6 reports the cited literature.

4.1 Introduction

The broad goal of cell therapy is obtaining functional cells in sufficient quantity and proper quality for organ or tissue repair. In order to obtain homogeneous population of the specialized cells of interest, *in vitro* cell culture procedures should be performed under defined and controlled conditions. In addition, *in vitro* platforms for cell cultures are increasingly needed to study cell and tissue physiology and pathophysiology outside the organism.

The recreation of *in vivo* cellular microenvironments becomes a prerequisite to answer questions regarding cell behavior and to guide cell differentiation. As a result, biologists and bioengineers have investigated many three-dimensional scaffolds that recapitulate aspects of the cellular microenvironment for *in vitro* cell culture. In this scenario, biomaterials are revolutionizing many aspects of the biology and are essential for the interdisciplinarity of regenerative medicine. Among these, hydrogel-crosslinked networks possessing the physico-chemical properties to allow mass transport for ensuring cell viability, are good candidates for *in vitro* long-term stem cell culture.

4.2 Hydrogels

Hydrogels, crosslinked hydrophilic polymers, represent an important class of biomaterials in biotechnology and medicine because of their biocompatibility, causing minimal inflammatory responses and thrombosis *in vivo* [1][2], their ability to swell large quantities of water without polymer dissolution, their tunable permeability to oxygen, nutrients, and other water-soluble metabolites, thus giving them physical characteristics similar to soft tissues.

Over the past three decades, a number of hydrogels differing in structure, composition, and properties have been developed. Hydrogel materials have been used extensively in medicine for applications such as contact lenses, biosensors, linings for artificial implants, and drug delivery devices [3]. Cross-linking density and response to environmental stimuli (*e.g.* heat, light, electrical potential, chemicals and biological agents) may be manipulated, making these biomaterials ideal for producing tailored 3D cellular microenvironments. Besides, hydrogel transparency is a key issue for cell culture because it allows the visualization of living cells, optical image analyses and studies on the dynamics of interfacial processes, such as protein-surface interactions, using inverted microscopes [4]. Some types of hydrogels can be photopolymerized *in vivo* and *in vitro* in the presence of photoinitiators using visible or ultraviolet light. Photopolymerization is used to convert a liquid monomer or macromer to a hydrogel by free radical polymerization in a fast and controllable manner under ambient or physiological conditions. Photopolymerized hydrogels have been investigated for a number of biomedical applications including prevention of thrombosis [5][6]; post-operative adhesion formation [7][8][9]; drug delivery [10][11][12][13]; and for cell transplantation [14][15]. Here, we describe two highly modulable hydrogel-based substrates for human stem cell culture.

Two photopolymerizable hydrogels have been designed, developed and tested for human stem cell culture in suspension. The first is a hydrogel suitable for single cell dispersion culture; the second a hydrogel for confined cell clusters culture.

4.2.1 Photopolymerization

The main advantage of photopolymerization is the temporal and spatial control of this polymerization process affords, which leads to the control over polymerization exotherms and time of gelation and can be used for the fabrication of complex structures (via systems of lasers or masks). Photocrosslinkable hydrogels are placed underneath a mask that controls the exposure of light to particular regions of a film of hydrogel precursors. Where light is exposed, the photo-crosslinkable hydrogel will crosslink to generate structures that are in the shape of the mask.

4.3 Biomateral for single cell dispersion culture

Hyaluronic acid (HA) is one of the major components of the extracellular matrix and is found in all connective tissues. It is a naturally derived high-molecularweight (up to 1–2 million Da), viscoelastic compound, and structurally an unbranched glycosaminoglycan copolymer of D-glucuronic acid and N-acetyl-D-glucosamine [16]. HA is involved in important biological processes such as cell motility and proliferation. Cellular interactions with HA influence processes such as morphogenesis, wound repair, inflammation, and metastasis [17][18][19][20]. The biocompatibility and the lack of toxicity of its ester derivatives have been extensively investigated [21][22]. Moreover, these materials have proven to be adequate for production of different morphologies such as microspheres, threads, fibres, networks, and sponges [23]. Due to its high biocompatibility and low immunogenicity, HA is gaining popularity as a biomaterial for tissue engineering and tissue regeneration [13][24][25] [26]. Functional groups, such as thiols, acrylates, amines, can be conjugated to the HA backbone through mild chemistries and further used as crosslinking sites to form hydrogels as scaffolds for cells for *in vitro* and *in vivo* applications [27][28]. Several studies demonstrated that HA-based hydrogels are good candidates for culturing stem cells [29][30] and Gerecht *et.al.* (2007) demonstrated that a HA-based hydrogel was suitable for supporting hES culture. Because of the described properties and the data from

the cited publications, the HA-based hydrogel has been considered the best candidate for culturing hES single cell dispersion and for the further control of cell differentiation.

4.3.1 Hyaluronic acid based hydrogel preparation

In this paragraph, the photopolymerizable hydrogel (HYAFF120®) based on pothoinitiator-HA conjugate preparation is presented. The (Hyaff120®) is an esterified derivative of HA obtained by chemical reaction between HA with an alcohol bromide, which behaves as photoinitiator during the hydrogel curing. HA (200 kDa) has been provided by Fidia S.p.a (Abano Terme, Italy) and was produced by fermentative process. The Hyaff120® hydrogel is easily obtained by solubilising the polymer Hyaff120® in water and by curing the aqueous solution under UV light. The main advantage of this method respect to others involving HA, is the elimination of double bonds C=C in the polymer for the photocrosslinking reaction.

The process of Hyaff120® synthesis is schematically illustrated in Figure 4.1. During the esterification reaction the carboxylic group of the polysaccharide is substituted by bromide group using ion of an ammonium salt as intermediate of reaction. By increasing the bromide concentration, the percentages of esterification rise determining the increasing of its hydrophobic feature and the reduction of the UV-curing time. Low percentages of esterification (20-30%) allowed good solubility in water but it requires long time for the hydrogel formation (30 min at 4mW/cm^2). Higher degree of substitution (75 % esterification) allowed a faster cross-link (2-3 min at 4mW/cm^2) even if the concentration of ester is low (15 mg/ml), but the product is no more soluble in water because of its hydrophobic nature. The best compromise is achieved by producing a hydrogel with 50% degree of esterification in term of good water solubility and relatively moderate time in the UV processing of hydrogel preparation.

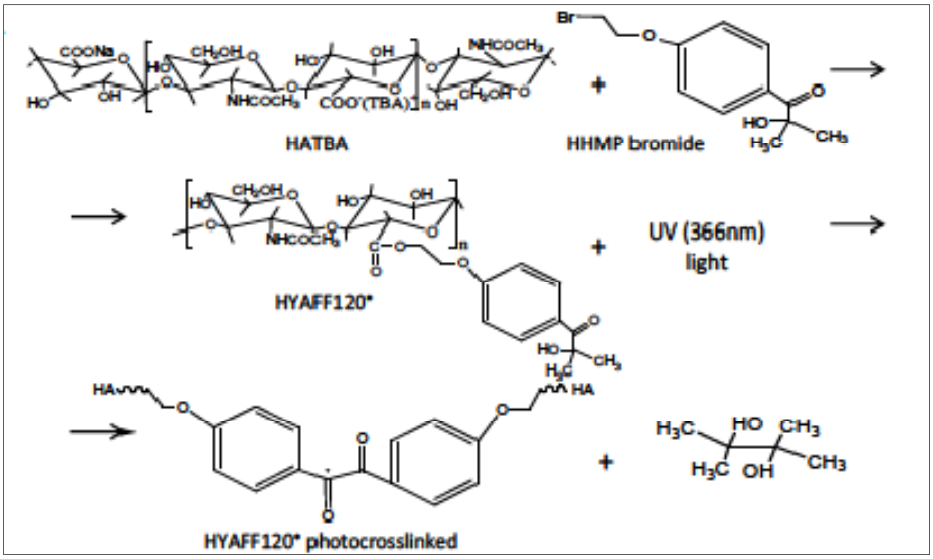

Figure 4.1 Schematic illustration of Hyaff120® synthesis

4.3.2 Analysis of hydrogel stability

Hydrogel stability is one of the fundamental parameters to be evaluated for a robust and reproducible *in vitro* cell culture system.

In particular, the effect of pH on the degradation rate are investigated.

A defined volume of Hyaff120 polymer is photopolymerized using 366 nm UV source. The distance of the UV source is 4 cm, the exposition time 55 sec.

The hydrogels are weighed after photo-polymerization and soaked at room temperature in buffers at different pH:

- borate buffer (pH ~ 8.6)
- PBS buffer (pH ~ 7.3)
- physiological solution (pH ~ 6)
- citric buffer (pH ~ 4)

The hydrogels are weighed at different time points, pH measured and data collected.

4.3.2.1 Results and discussion

The variation (%) of the measured weights compared with the initial one is reported in Figure 4.2.

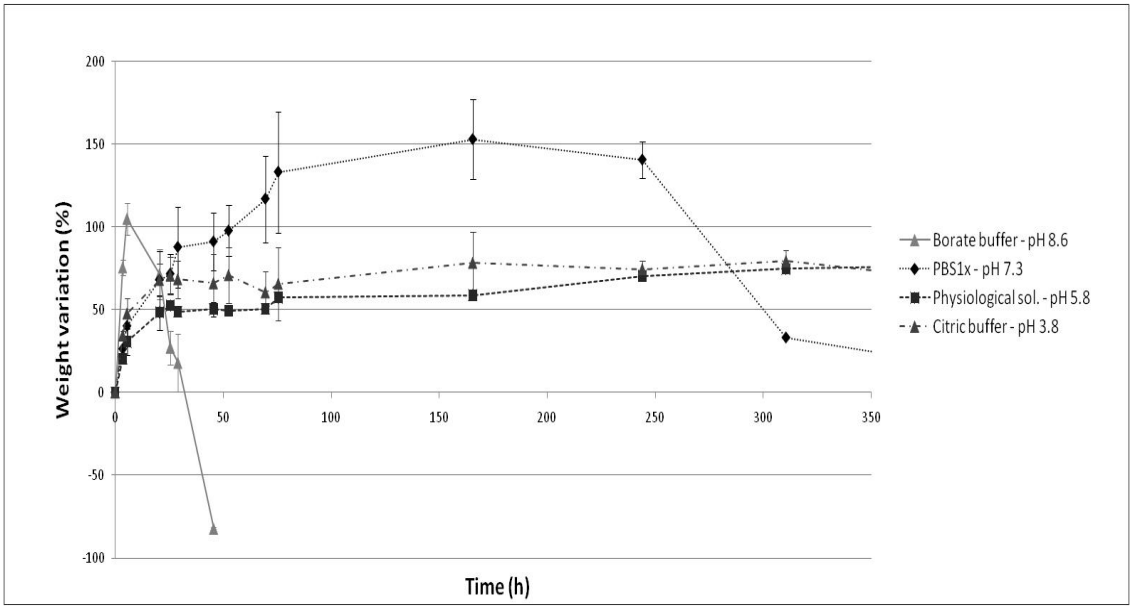

Figure 4.2 Influence of pH on hydrogel weight variation (%) vs time: curves for pH ~ 8.6 (borate buffer), pH ~ 7.3 (PBS), pH ~ 5.8 (physiological solution) and pH ~ 3.8 (citric buffer).

This analysis shows the drastic effect an enhancement of pH can have on hydrogel stability. The hydrogels maintained at pH 8.6 swell rapidly reaching the maximum of their weight after ~5 h but at the same way they degrade faster than all the other hydrogels going back to original weight after only 30 h.

Hydrogels at pH 7.3 swell and degrade at slower rate. They reach the maximum after ~200 h and go back to the original weight after ~500 h (data not shown). These hydrogels show the highest increment in weight.

A further reduction of pH translates both the phenomena (swelling and degradation) at longer time: at pH 5.8 the maximum weight is reached after ~700 h while 1100 h are necessary for the degradation to the original weight; at pH 4 the hydrogels are still increasing their weight after 1200 h (data not shown).

Analogue analysis is performed using DMEM which has a pH that changes from 7.5 to 8.7 under atmospheric conditions. The hydrogels weight variations are shown in Figure 4.3.

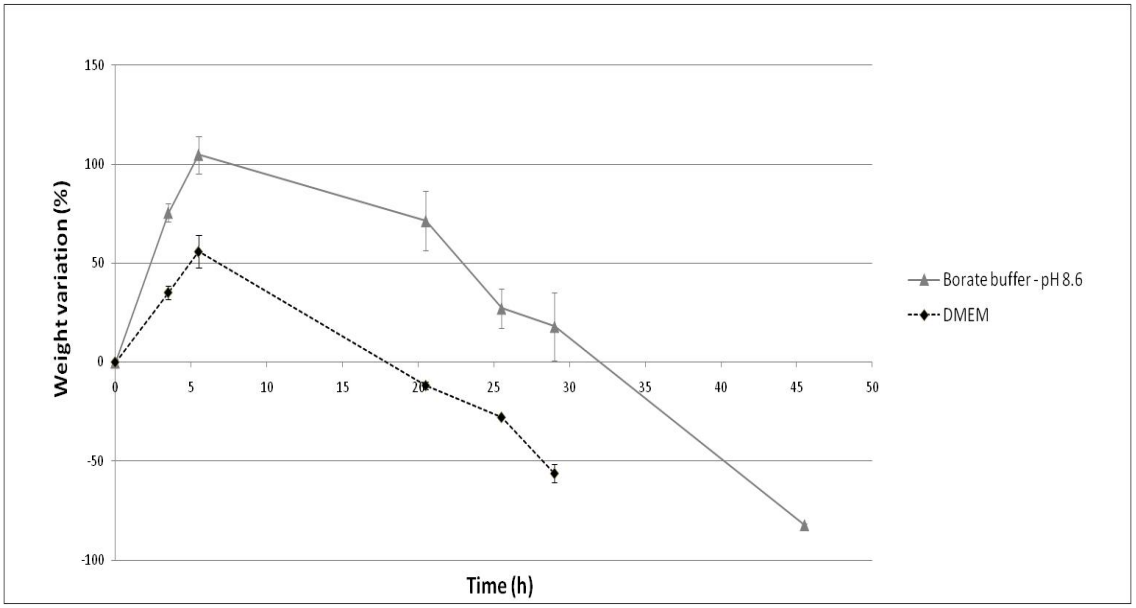

Figure 4.3 Weight variation (%) vs time for photopolymerized hydrogels in borate buffer (pH ~ 8.6) and in DMEM (pH varying from 7.5 – 8.7).

As shown in Figure 4.3, hydrogels in DMEM degrade within 20 h, with a behavior similar to the one in pH 8.6.

Such a fast degradation does not allow long *in vitro* studies of cell-hydrogel interactions. In addition, the degradation induces a fast release of the basic molecules of HA in the medium, thus altering the osmolarity of cell environment with negative effects on cells.

The pictures in Figure 4.4 show the difference in hydrogel external appearance of samples maintained for 25 h in physiological solution (no noticeable change from the hydrogel polymerization), in borate buffer and in DMEM.

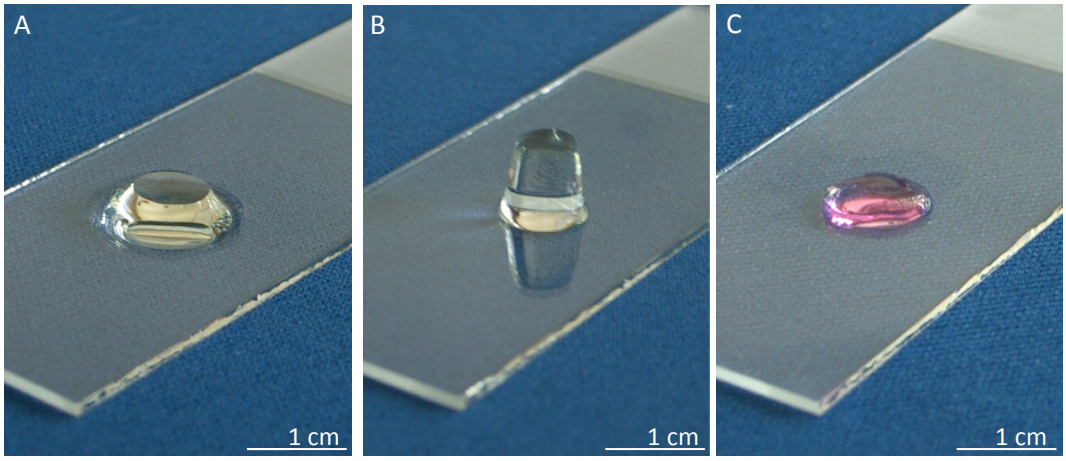

Figure 4.4 External appearance of hydrogel maintained for 25 h in borate buffer (A), in physiological solution (B), in DMEM (C).

4.3.3 In vitro analysis of cell viability (cells cultured in monolayer)

Preliminary *in vitro* tests are performed using the murine myoblast cells C2C12 to investigate the influence of UV light exposure on cell viability.

C2C12 are seeded on a gelatin coated well with culture medium. Two hours later, growth medium is removed leaving only few microliters. Cells are then exposed to UV light. Different UV lamp exposition time (40 s, 50 s, 60 s, 80 s, 100 s) and distance (10 cm, 5 cm, and 2 cm) are chosen.

4.3.3.1 Results and discussion

Figure 4.5 shows the data of cell viability analysis by Live & Dead assay (Invitrogen) performed 2 hours after exposure to UV light. For UV light distance = 10 cm, the irradiations do not affect cell viability, whereas, the UV light source distance reduction up to 2 cm induces an enhancement of dead cell number to 95% in 40s is detectable.

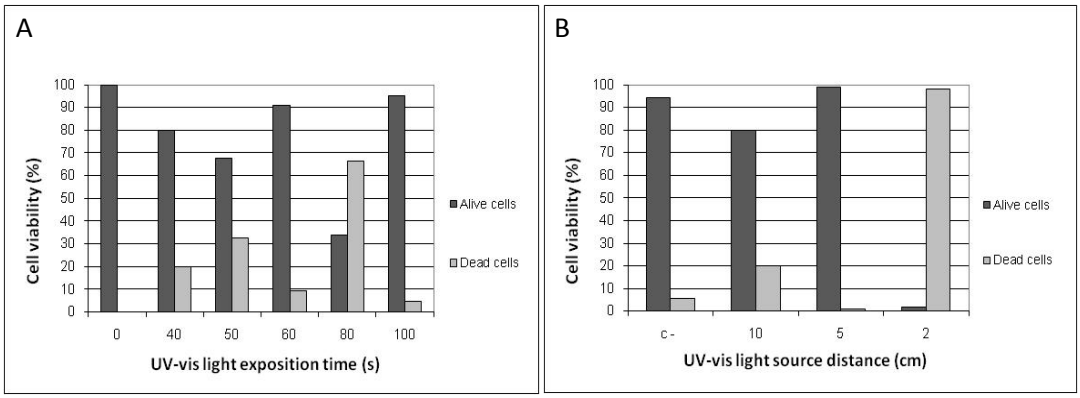

Figure 4.5 Cell viability depending on the distance of UV light and the time exposure. IN (A) the left UV source is maintained at 10 cm, IN (B) cells are irradiated for 40 s.

From these preliminary evidences it is possible to conclude that the energy emitted by the UV light source does not compromise cell viability if kept properly spaced from the cells.
It is known that cells can respond in different ways depending on their density and their adhesion conditions (2D or 3D systems). Thus, an analysis of cell viability after encapsulation in a 3D hydrogel structure would be more representative of cell survival *in vivo* during clinical applications.

4.3.4 In vitro analysis of cell viability (cells cultured in 3D)

C2C12 cells are suspended at a density of 5 x 10^6 cells/ml in a defined volume of 40 mg/ml Hyaff120 polymer. The polymer mixed to the cell suspension is photopolymerized using the 366 nm UV light in a cylinder of 5 mm diameter. Cell-hydrogel constructs are maintained in culture medium for 20 h. The cylinder is then longitudinally sectioned and stained with Live & Dead assay. The section is than analyzed by fluorescent microscopy and cell viability quantified.

4.3.4.1 Results and discussion

The results from the analysis of cell viability by Live & Dead are shown in Figure 4.6.
The intense red fluorescence which is visible in the bottom-left part of the hydrogel is due to high hydrogel autofluorescence.

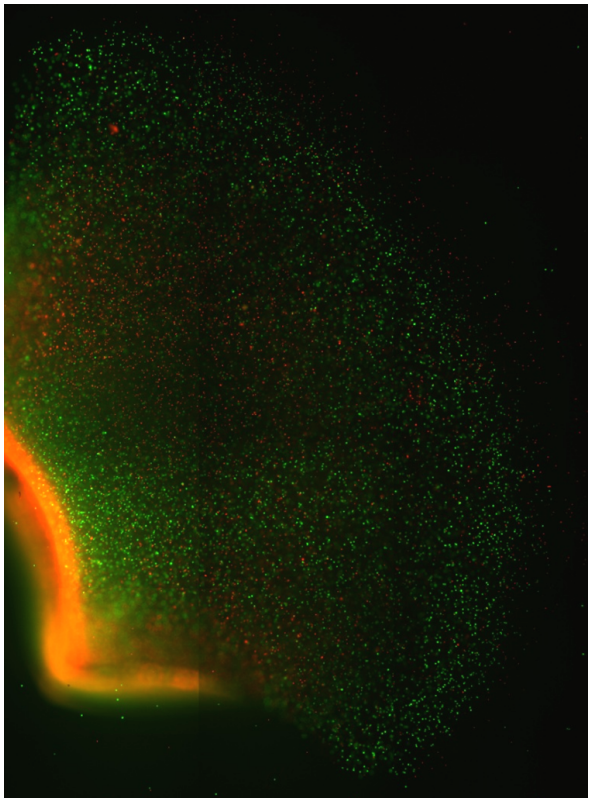

Figure 4.6 Live & Dead assay showing the viability of C2C12 after 24 h from encapsulation into the hydrogel. The picture presents the central section of the hydrogel.

Single pictures taken in different areas of the hydrogel are analyzed after deconvolution (Figure 4.7).

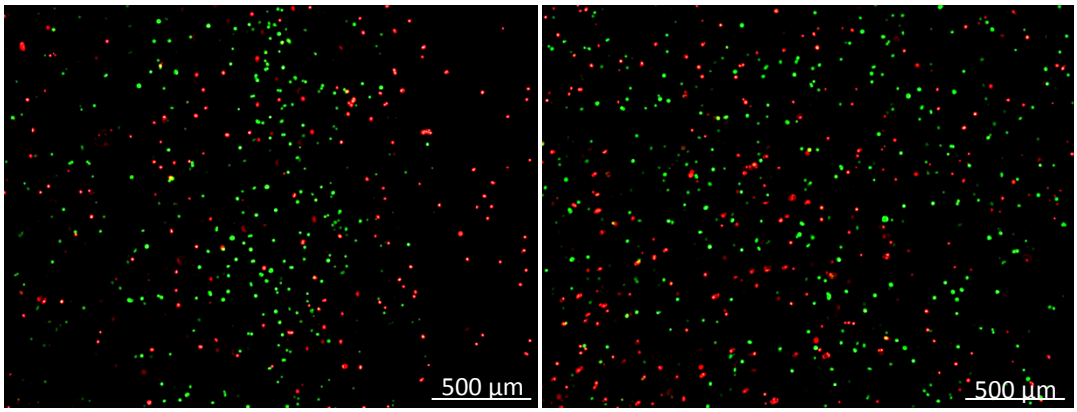

Figure 4.7 Live & Dead assay of two different areas of the hydrogel showing the viability of C2C12 after 24 h from encapsulation into the hydrogel. Green fluorescence is an indicator of live cells and red indicates the dead-cell population. The pictures present two distinct parts of the central section of the hydrogel.

Imaging analysis shows a uniform distribution of alive and dead cells with a percentage approaching 50%.

In order to quantify cell viability, 10 areas of the sectioned hydrogel have been selected and alive and dead cells counted. The results are shown in Table 4.1

Table 4.1 Cell viability percentages of cellsbelonging to different areas of the 3D hydrogel 24 h after encapsulation in 3D hydrogel.

Area	% alive cells
1	**57.4**
2	**32.3**
3	**18.9**
4	**50.7**
5	**49.1**
6	**63.4**
7	**32.6**
8	**52.3**
9	**53.8**
10	**57.6**
Average %	**46.8**
σ	**14.1**

The preliminary results regarding cell viability suggest that the hydrogel may be suitable for *in vivo* applications. On the other hand, the fast degradation rate when the hydrogel is soaked in cell culture medium does not allow long-term *in vitro* studies, as those required for studying hES cells differentiation. Because of the indications, a no-biodegradable hydrogel is required for hES cell long-term *in vitro* culture.

4.4 Biomaterial for confined cell clusters culture

Among the wide spectrum of no-biodegradable hydrogels, the polyacrilamide (PA) ones are easy to fabricate, inexpensive and can be produced as a thin film or a thick layer and covalently bonded to a functionalized glass slide showing long-term stability in culture. Moreover, PA hydrogels have a non fouling surface, which allow for performing substrate-dependent cell culture in suspension, are biocompatible, highly permeable and their geometry is easily tunable. Thanks to these properties, PA hydrogel has been considered a promising substrate for long-term hES-EBs culture. Moreover, such a biomaterial may be a tool to prevent cell aggregation allowing for culturing physically separated hES-EBs. By selective photopolymerization with appropriate photomasks, it is possible to realize a microwell array, thus obtaining a microstructured hydrogel. Such a system, can be used for further control of hES cell differentiation by exploiting the possibility to modulate the depth of the microwells and consequently the availability of endogenous factors in the cell microenvironment. The detailed rational approach together with the results of this study will be presented in Chapter 5.
In the following paragraphs, the PA microstructured hydrogel preparation is described.

4.4.1 Polyacrylamide hydrogel preparation

PA hydrogels are prepared optimizing previously developed procedures [32] as homogeneous layer with a circular shape (20 mm in diameter and an average thickness of 1mm) over a properly functionalized microscope slide.

4.4.1.1 Glass slide functionalization

Glass slides surfaces are chemically modified creating a layer of silane groups to ensure covalent binding of the hydrogel layer. Briefly, slides are washed in acetone and rinsed with distilled water, dried at 110°C. A few drops of 3 di 3-aminopropyltriethoxy silane are

deposited on the glass slides for 5 minutes (Figure 4.8). The 3-aminopropyltriethoxy silane is then removed and the glasses are rinsed with distilled water.

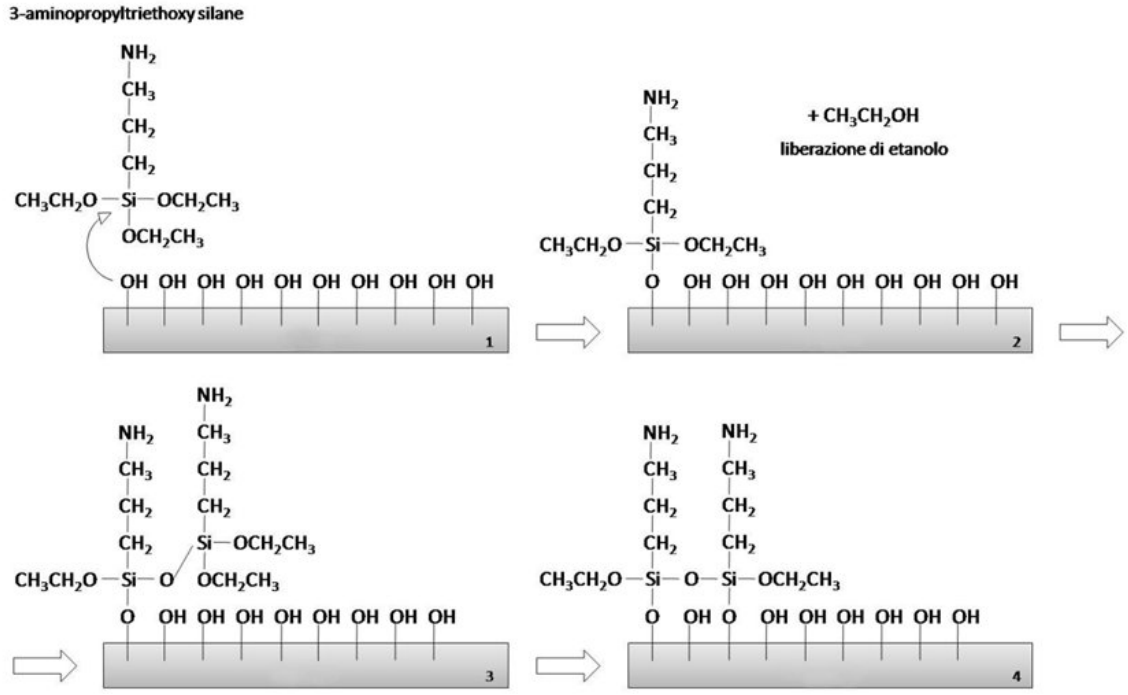

Figure 4.8 Schematics of the binding of 3 – aminopropyltriehtoxy silane to the glass slide

A drop of glutaraldehyde 0.5% in PBS 1X (-Ca^{2+}/-Mg^{2+}) is deposited on dried glasses which are stacked for 30 minutes at RT (Figure 4.9).

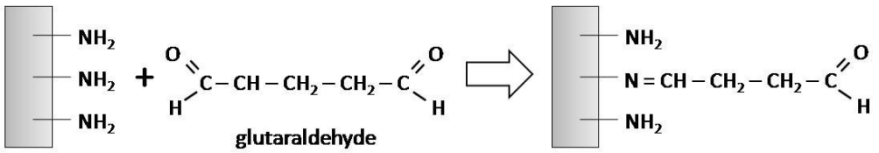

Figure 4.9 Schematics of the glutaraldehyde binding to 3 – aminopropyltriehtoxy silane groups

The solution is finally removed and the glasses rinsed with distilled water.
The complete chemical reaction for glass functionalization is reported in Figure 4.10.

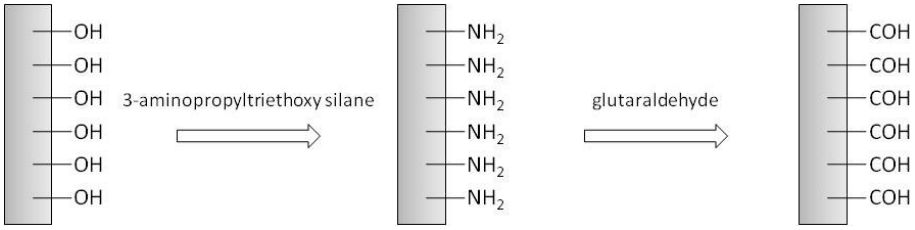

Figure 4.10 Schematics of the complete chemical reaction for glass functionalization

4.4.1.2 Microstructured hydrogel photopolymerization

The prepolymer solution acrylamide/bisacrylamide 29:1 is diluted in phosphate-buffered saline (PBS) to the final desired concentration (generally 10%). The photoinitiator (Irgacure 2959; Ciba Specialty Chemicals) is initially dissolved in methanol (200 mg/ml) and then added to the acrylamide/bis-acrylamide solution in order to obtain a final concentration of 20mg/ml, and mixed thoroughly. Hydrogel polymerization occurred first by exposing the 20 µl prepolymer solution, dropped over the functionalized glass surface and with a glass coverslip floating over it, to UV light for 3 min (high-pressure mercury vapor lamp (Philips HPR 125 W) to obtain a thin film of hydrogel. The thin film of hydrogel is then covered by a second volume of 500 µl prepolymer solution to be exposed to UV light for 1 minute for selective photopolymerization. Non-polymerized acrylamide is removed using distilled water. Selective photopolymerization of acrylamide solution on the glass surface is achieved by interposing a photomask with the desired geometry between the light source and the glass slide. Such procedures resulted in homogeneous hydrogel with an average thickness of 1 mm, covalently bounded to the functionalized glass, as schematized in Figure 4.12.

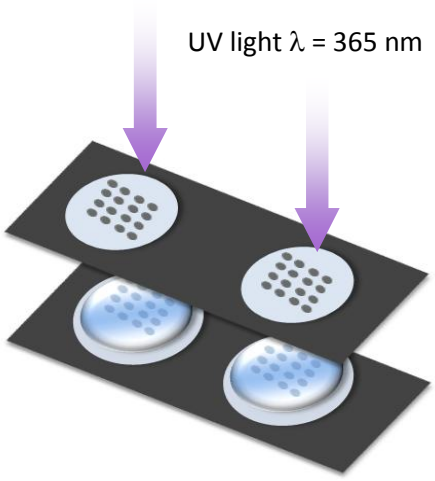

Figure 4.11 Hydrogel preparation. A volume of acrylamide/bis-acrilamyde mixture is deposed over a glass coverslip (25 mm in diameter). The hydrogel is polymerized with UV light through a photomask.

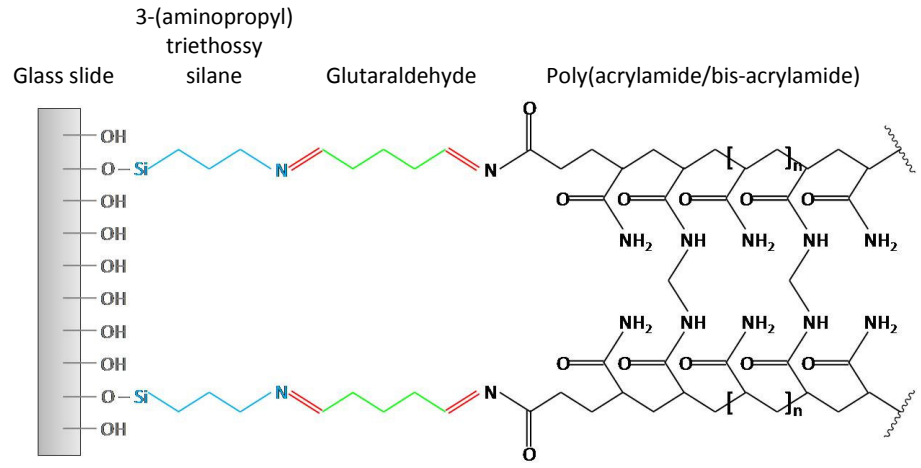

Figure 4.12 The circular polyacrylamide hydrogel covalently binds to the glass slide through 3-aminopropyltriethoxysilane and glutaraldehyde, as shown in the schematic representation of the chemistry.

4.4.1.3 Hydrogel sterilization

Glass slides with covalently bonded hydrogel films are immersed in ultra-pure distilled water for 96 hours to ensure complete removal of the un-reacted monomeric units or photoinitiator. After rinsing, hydrogel are sterilized by exposure to UV light for 30 minutes under a sterile hood.

4.4.2 Characterization of the polyacrilamide hydrogel

In order to fully characterize the permeability of the hydrogel, it is necessary to know the molecular cut-off that excludes free diffusion through the hydrogel network. Using the Fluorescent recovery after photobleaching (FRAP) technique, we could obtain local diffusion coefficient values for fluorescent probes of different molecular weight.

In Figure 4.13 a rapid drop of the diffusion coefficient is shown when a threshold in acrylamide concentration is reached. At this point, the cross-linking characteristic distance is becoming relevant in the exclusion phenomena of the tracers. At the increasing of tracer molecular weight, the threshold dicreases. In conclusion, the high permeability of the hydrogel (for molecules up to 19nm) is ensured for acrylamide concentration below 10% weight, which corresponds to the one we use.

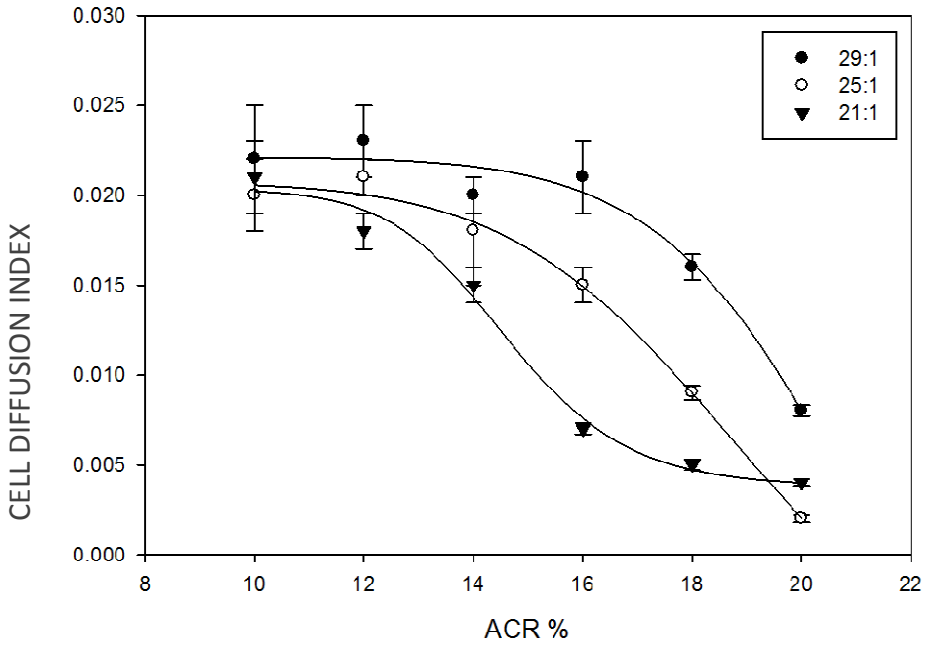

Figure 4.13 Cell diffusion index versus acrylamide concentration, with different cross-linker content. As expected, the self diffusion decreases as hydrogel network becomes more dense.

4.4.3 Optimization of the microstructured polyacrilamide hydrogel

4.4.3.1 Array design

In order to obtain the proper shape of the microwells, different array designs have been performed. The shape of the desired microwells should meet different requirements. First, the microwells shape should be designed in order to maximize the percentage of cells entrying the wells after seeding, thus, a conical shape would be ideal. Second, the microwells distance should permit the manipulation of single embryoid body with the tips of a micropipette, without interfering with other EBs. In order to satisfy both the requirements, different masks have been designed, as indicated in Figure 4.14

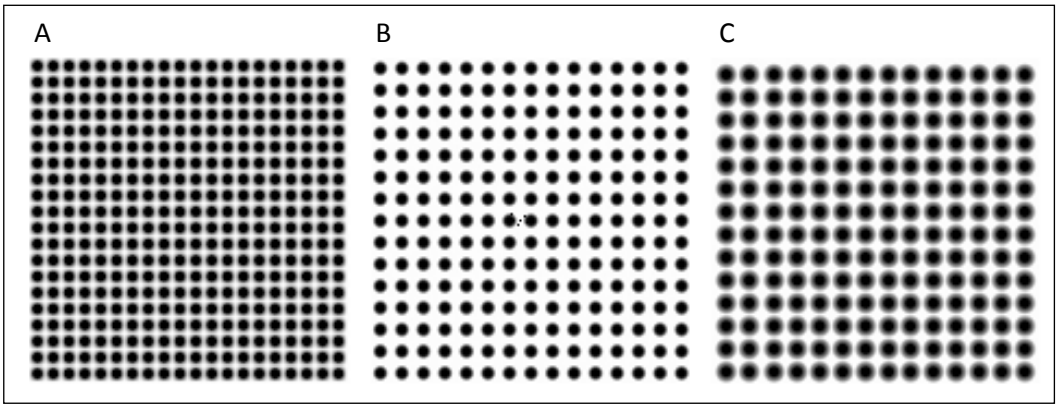

Figure 4.14 Fotomask models. The fotomasks models differ in the number and in the distribution of the spots and in the diameter of the shaded circular crowns.

All the requirements were satisfied using the photomask in Figure 4.14C.

The desired array design has been realized in digital form with AutoCAD and consisted of 196 spots of 1 mm diameter (composed by a black spot of 500 µm diameter and a shaded circular crown), as shown in the enlargement in Figure 4.15 The distance between each microwell is 1mm.

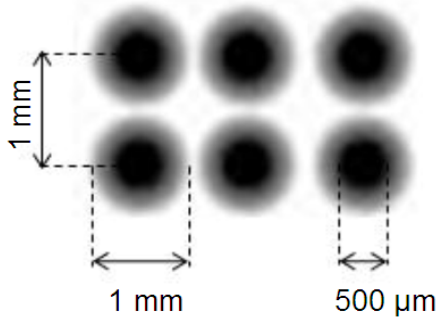

Figure 4.15 Enlargement of the photomask in Figure 4.14C Each spot is vertically and horizzontally 1mm distant from the others. The black spot is 500 µm diameter and is surrounded by a shaded circular crown.

This pattern has been printed onto an overhead transparency and used as photomask for the obtainment of the hydrogel microwells.

4.4.3.2 Modulation of microwells depth

Microwells of different depth have been realized properly varying light collimation.

Collimated light from optic fiber leads to the obtainment of microwells of 1 mm depth; whereas more diffuse light leads to microwells of 450 µm depth.

Figure 4.16 shows the distribution of microwells depth which have been obtained varying light collimation. As shown, the distribution of the measurements is very close to the targets, thus underlying the reproducibility of the techniques for the obtainment of microwells of different depths.

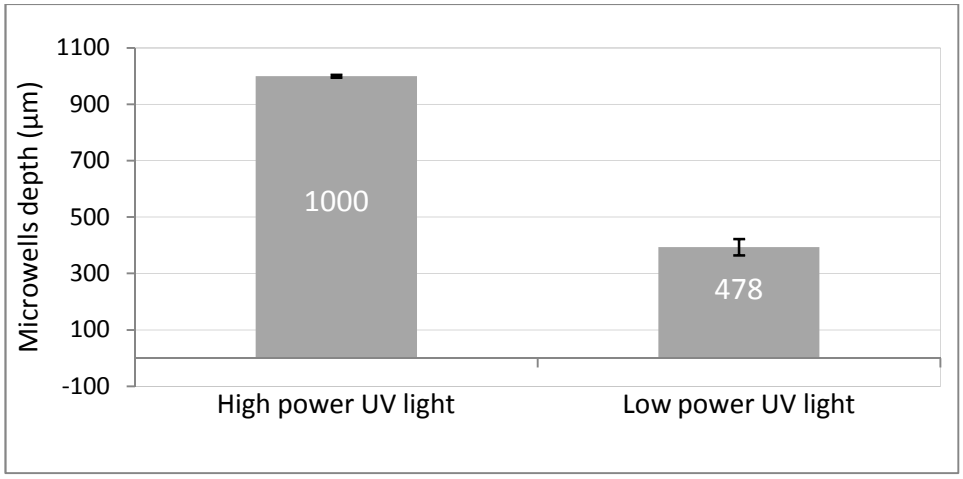

Figure 4.16 Measurements of the depth of the microwells obtained by modulating UV light collimation.

Figure 4.17 shows the external appearance of the microstructured hydrogel. The array of microwells is clearly visible. The pink color of the hydrogel is due to the presence of medium which keeps the hydrogel hydrated.

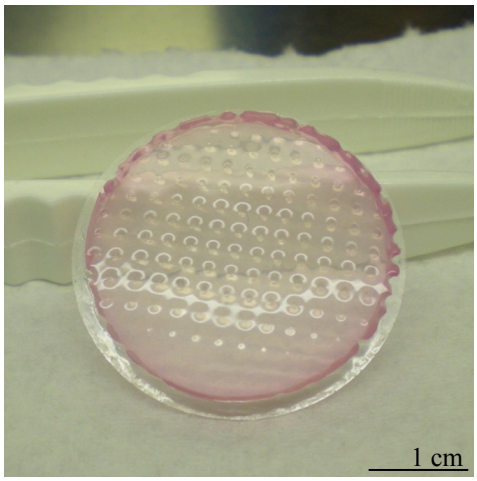

Figure 4.17 The microstructured hydrogel. The pink colour is due to the presence of cell culture medium which keep the hydrogel hydrated

4.4.4 In vitro analysis of cell viability (cells seeded onto the microwells

Preliminary *in vitro* tests are performed using C2C12 murine skeletal muscle cells to verify the biocompatibility of the hydrogel.
2 x 10^5 C2C12 are seeded onto both the microstructured hydrogels and viability is analyzed by Live & Dead assay 72h, 96h and 144h after seeding. Figure 4.18 reports brigh field images of microwells before and after cell seeding in A) and B), respectively.

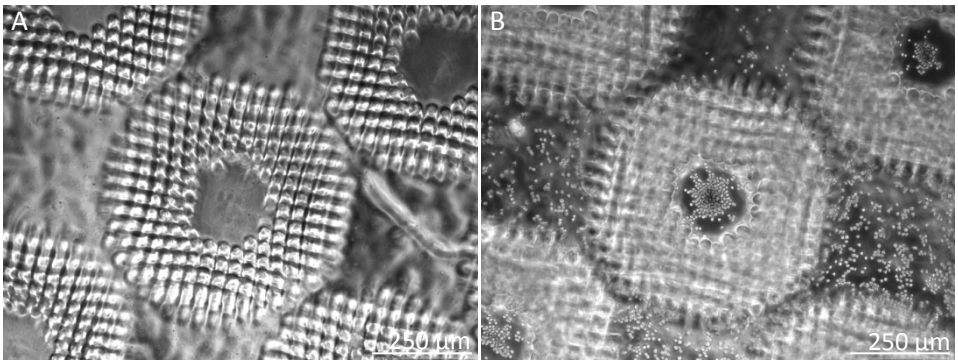

Figure 4.18 Bright field image of microwells before cell seeding (A) and immediately after C2C12 seeding (B).

At each time point cells have been retrieved from the microwells with a micropipette and Live & Dead assay has been performed on the cells suspension dropped onto a glass slide.

4.4.4.1 Results and discussion

C2C12 cells remain alive during the entire duration of the experiments, without evident differences among the hydrogel, as shown in Figure 4.19.

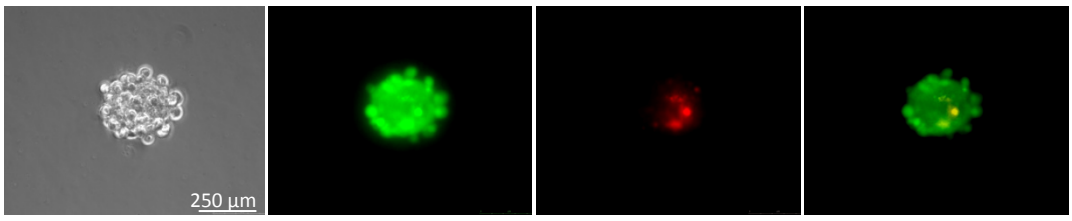

Figure 4.19 Live & Dead-assay showing the viability of C2C12 after 144 h from encapsulation into the hydrogel.

As shown in Figure 4.19, the majority of cells are still alive in the hydrogel. This indication suggest that hydrogel does not interfere with C2C12 cell viability.
Further investigations are needed to investigated whether the microwell confinement may be inadequate for ensuring human stem cells viability and culture.

4.5 Conclusions

As discussed in § 2.7, the developed stirred bioreactor for stem cell culture was suitable for HSC culture, but undesirable cell aggregation issues due to the stirring were observed with hES-EBs cultures. Cell aggregation should be avoided since it represents a source of heterogeneity within the culture. Albeit many solutions have been attempted to overcome such limitation, none of the strategies resulted in a homogeneous population of cells within the bioreactor. In that scenario, the possibility to use biomaterials to prevent cell aggregation arose as an alternative promising possibility. In particular, a biomaterial possessing the chemico-physical properties for supporting stem cell cultures was required. Among the wide range of biomaterials, hydrogels address all the requirements, thus representing the best solution for the aims of this research. Hydrogels are biocompatible biomaterials that allow for proper mass transport and are characterized by high water content, essential requisites for cell culture. In addition hydrogel are high transparent biomaterials, thus allowing for the visualization of living cells and optical image analyses without disrupting the culture; present a non fouling surface, thus permitting to perform substrate-dependent cell culture in suspension and, especially for photopolymerizable hydrogels, their geometry is also easy modulable and controllable.

In the present chapter two photopolymerizable hydrogels are tested for single stem cell dispersion or confined stem cell clusters cultures. For single cell dispersion, the attention has been directed to a promising hyaluronic acid based hydrogel which, beside presenting all the requirements for stem cell culture, was proved by Gerecht *et al.* (2007) to be suitable for hES cell culture. Albeit encouraging results from biological experiments using murine myoblast cells, the low *in vitro* stability made this substrate inadequate for supporting the long-term culture required by hES cell differentiation protocols.

Because of these evidences, we turned our attention towards no-biodegradable hydrogels. Among the wide spectrum of hydrogels addressing this requisite, the polyacrylamide ones captured our attention. PA hydrogels present all the fundamental characteristics of photopolymerizable hydrogels and, in addition, are no-biodegradable, easy to fabricate, and inexpensive. Moreover, such a biomaterial was supposed to be a feasible tool for preventing cell aggregation. Exploiting these exciting properties, a microwell array has been realized by selective photopolymerization with different photomasks, thus obtaining a microtructured hydrogel. The innovative culture system has been tested to verify the idoneity for long-term cell clusters cultures. Viability assays performed on murine myoblast cell custers used for

preliminary tests showed alive cells for all the duration of the experiments. Moreover, the clusters remained physically separated and confined within the microwells.

Such a system, can be used for further control of hES cell differentiation by exploiting the possibility to modulate the depth of the microwells, properly modifying the photomasks geometry and the UV light collimation, thus the availability of endogenous factors in the cell microenvironment. The detailed rational approach together with the results of this study will be presented in Chapter 5.

Such small-scale culture systems, which require volumes in the order of micro-liters and can be used for performing up to 196 parallel experiments, may be used for wide screening of different experimental conditions. The developed system may be a tool for optimizing cell culture protocols in the critical phase of process optimization, where product quality control and cost reduction are required. This aspect is particularly important when working with human embryonic stem cells which lack of robust and well defined protocols for *in vitro* culture and differentiation.

4.6 References

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Chapter 5

Application of microstructured hydrogel for hES differentiation pattern control

As discussed in § 4.4, the microstructure hydrogel developed for culturing physically separated hES-EBs might be a tool for the further control of hES differentiation. In this context, as introduced in § 1.4 and 1.6, the attention is focused especially towards cardiac differentiation. The chapter is organized as follows: § 5.1 describes the motivations, the aim and the rational approach of this research, whereas § 5.2 and § 5.3 introduces cardiogenesis *in vivo* and the current approaches for hES cells *in vitro* cardiac differentiation. § 5.4, reports the experimental plan together with the results of the *in vitro* studies. In § 5.5 the future perspective of performing this research using iPS cells is introduced. In § 5.6 and in § 5.7 the conclusive remarks and the cited literature are reported, respectively.

5.1 Motivations and aim of the research

The heart is one of the least regenerative organs in the body and, consequently, loss of myocardium to infarction or other diseases often leads to heart failure [1]. Moreover, due to the poor prognosis of patients with advanced heart failure and the shortage in donor organs for heart transplantation, a search for new therapeutic paradigms has become imperative [2]. Stem cells offer the possibility of repairing organs of the human body, and there is an intensive effort to develop stem cell–based strategies for cardiac repair. Both adult and embryonic stem cells are being studied in preclinical models, and at least four types of autologous cells (skeletal myoblasts, bone marrow mononuclear cells, mesenchymal stem cells and endothelial progenitor cells) are being tested in early-stage clinical trials [3][4][5]. Apparently, several of these cell types do not transform to generate significant amounts of new myocardium [6][7]. This has led to the investigation of other types of cells that may truly help cardiac repair. At present the two most promising cell sources for this task are human embryonic stem cells and resident cardiac progenitor cells. hES cells are an attractive cell

population for cardiac repair because they self-renew indefinitely while retaining their capacity to differentiate into cell lineages of all three primary germ layers including cardiomyocytes. These cells can be expanded *in vitro* in culture and differentiated into definitive cardiomyocytes [8][9][10][11][12]. The intriguing feature of hES cell-derived cardiomyocytes for myocardial regeneration is that they can integrate with host cardiomyocytes via gap-junctions [13]. However, the control of their differentiation is still a challenge. Thus, the major obstacle to clinical applications, is the inability to obtain cells in the proper quantity and quality.

In this context, the possibility to control the local concentration of endogenous factors in the cell microenvironment, would represent a tool to elucidate the mechanisms of cell differentiation and to make a powerful step forward in the reproducible differentiation of hES cells toward cardiac cells.

It is well accepted that *in vitro* systems present substantial differences from the *in vivo* environment. *In vivo* cells reside in the specific microenvironment of the tissue they belong to and interact with several factors, including the extracellular matrix (ECM), neighbor cells and various soluble factors, whereas isolated cells cultured in standard cell culture dishes are exposed to a bulk environment which is very different from the environment *in vivo*. In petri dishes, in fact, any molecule is rapidly distributed over the total medium volume, with consequences for cell signaling transduction. The mechanisms underneath these events are not well outlined in part because of a lack of spatial and temporal control of parameters in standard cell culture systems.

Thus, the control of the microenvironment would be important to better mimic native tissue by providing the appropriate conditions for cellular dynamics such as migration, spreading, proliferation and differentiation.

To address this question, we design an experimental investigation in order to evaluate the effects of local endogenous factors concentrations on hES-EB differentiation. With this aim, we cultured cells in three different conditions. EBs are cultured in parallel in 1) conventional ultra low adhesive petri dishes (for negative control) and in physically separated hydrogel microwells of 2) 500 μm diameter and 450 μm depth, 3) hydrogel microwells of 500 μm diameter and 1 mm depth. Figure 5.1 show schematic representation of the three different conditions.

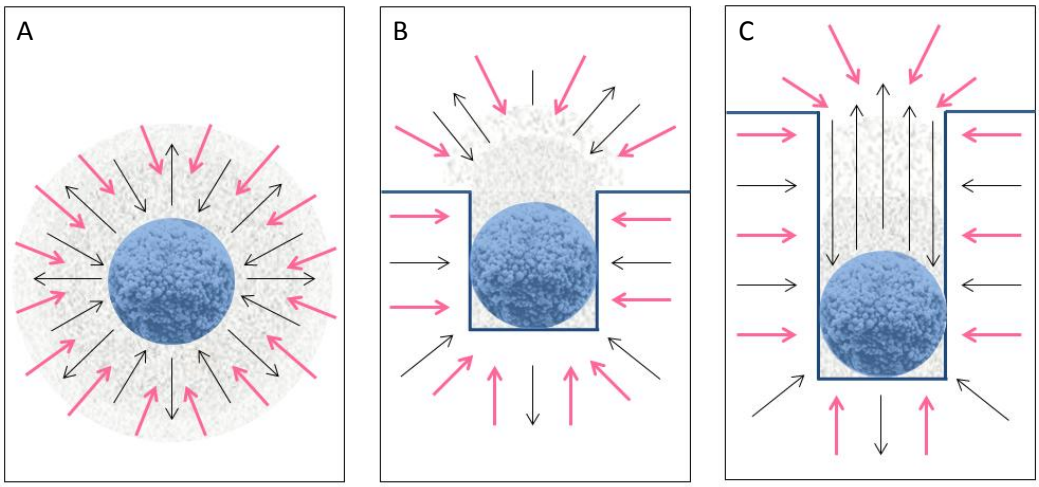

Figure 5.1 EBs in suspension (A); EBs in microwells 500µm x 450µm (B), 500µm x 1mm (C). Black arrow represent endogenous factors diffusion, pink arrows indicate exogenous factors diffusion.

The culture within the microstructure hydrogel is expected to increase cell homogeneity in the population preventing the formation of random cell aggregates. Morover, exploiting the exploiting the possibility to modulate the depth of the microwells and consequently the availability of endogenous factors in the cell microenvironment, this system can be used for further control of hES differentiation. The main difference among the three conditions is the diffusion of endogenous factors through the hydrogel, which is likely to lead to an accumulation of factors over times which are relevant for cell proliferation and differentiation.

By enabling the formation of controlled microenvironments, we aim at studying and controlling hES cardiac differentiation process. To this end, cells will be analyzed during the organization of the three germ layers, focusing the attention on mesodermal differentiation, which is the intermediate phase for cardiac lineage commitment, as it will be described in § 5.2.

5.2 Cardiogenesis and embryonic cardiac progenitors

Myocardial cells are lineage descendents of the developing mesoderm, which emerges from the primitive streak during gastrulation [14]. From the primitive streak, cardiac precursors divide into two populations, one on either side of the midline. Cells then extend toward the midline, forming the cardiac crescent. The cardiac crescent then fuses along the midline, forming the linear heart tube, which undergoes rightward looping. Finally,

with further hypertrophy of the left and right ventricles and atria, the four heart chambers undergo several phases of remodeling before assuming their mature structure.

At the primitive streak stage, cardiac precursors are not irreversibly committed to a cardiac lineage and can also contribute to the paraxial mesoderm forming the skeletal muscle in the head and neck [15].

Markers such as Mesp1 and Mesp2 have been used to identify these earliest cardiac and skeletal precursors [16][17]. When mesodermal precursors restrict their fate to cardiovascular and hematopoietic lineages, they begin to express Mesp1 and Flk1[18]. Flk1 is used to denote primitive precursors for cardiovascular cells [19], Mesp1 and Mesp2 are expressed transiently during the primitive streak stage, moreover, descendants of Mesp1+ and Mesp2+ cells colonize the entire myocardium [20], enabling Mesp1 and Mesp2 to be reliably used as cardiac progenitor markers. Mesp1 drives commitment of mesodermal precursors to the cardiac lineage by promoting the stable expression of cardiomyogenic transcription factors, including Nkx2.5, Gata4, Isl1, and myocardin [21] and at the cardiac crescent stage, cardiac precursors begin to express factors such as Nkx2.5, Gata4, and Isl1 [22][23], while undergoing rapid expansion.

The expression of Nkx2.5 is cardiac selective (Komuro and Izumo, 1993), while Isl1 is transiently expressed in cardiac mesoderm but is then turned off during cardiomyocyte maturation [24]. In addition to myocardial expansion within the heart tube, two heart fields contribute cardiac progenitor cells at the anterior and venous poles of the heart tube. Moreover, recent studies support the existence of an epicardium-derived cardiac progenitor cell population [25][26], which appear to contribute to all four chambers of the heart [25][26].

5.3 Cardiac differentiation of human embryonic stem cells *in vitro*

The initial observation that hES cells could mature into spontaneously contracting cardiomyocyte-like cells was reported as a result of experiments in which hES cells were differentiated into embryoid bodies [27]. Although it is almost 10 years ago since the initial report on the generation of cardiomyocytes from hES cells, the most common way to induce cardiomyocyte differentiation *in vitro* remains the embryoid body formation in suspension cultures. An EB consists of ectodermal, mesodermal and endoderal tissues, which recapitulate many aspects of cell differentiation during early mammalian embryogenesis. For this reason EB generation has been used widely for *in vitro* differentiation of both mouse and

human ES cells. Interestingly, the hES cell-aggregation process self-activates cell differentiation and within only a few days the embryoid bodies consist of cells representing all three germ layers, including cells expressing cardiac markers [27][28]. Typically, the embryoid bodies are plated onto a matrix coated tissue culture plate for further differentiation and within a few days post-plating, the appearance of rhythmically contracting outgrowths can be observed. Analyses of the beating areas verify that they contain cells with cardiomyocyte characteristics [29].

For completeness, another approach that has been applied broadly to induce cardiomyocyte differentiation *in vitro*, is based on co-culture with a mouse visceral endoderm-like cell line (END-2) [30]. Based on the knowledge from developmental biology and embryo development, the cardioinductive signals are thought to originate from direct cell-cell contact and/or secreted factors from the END-2 cells which simulate the presence of embryonic endoderm [31].

Several protocols have been established to promote an efficient and reproducible development of the cell type of interest. The following are three basic strategies to induce *in vitro* differentiation of ES cells: suspension culture in non adherent-dishes or bacterial-grade dishes, culture in methylcellulose semisolid media, culture in hanging drops.

In the suspension culture in bacterial grade dishes, a nontreated polystyrene dish with hydrophobicity is used for liquid suspension culture of ES cells to induce EB formation. This technique has been utilized to initiate the differentiation of ES cells into a variety of differentiated cell types. In bacterial-grade dishes, ES cells can aggregate spontaneously, thus the number of incorporated cells into each aggregate varies and cannot be controlled. Consequently, the size and the shape of the resulting EBs tend to be heterogeneous [32]. Heterogeneous EBs will rapidly lose any sincrony in differentiation.

In the methylcellulose culture, ES cells are seeded onto semisolid methylcellulose media. This strategy allows reproducible formation of EBs from single ES cells because seeded cells tend to remain single and isolated by the matrix of cellulose. Methylcellulose culture has been used for the study of hematopoietic differentiation of ES cells [33][34][35][36]. Wiles and Keller reported that hematopoietic cells are found in more than 50% of EBs generated by methylcellulose culture. This data might be due to a local accumulation of factors in the matrix of methylcellulose surrounding the EBs. In spite of these interesting results, the matrix hinder the mass transfer of factors in the medium, single isolated hES cells often fail to aggregate and form an EB, and the handling of semisolid solution is not easy. Besides

hematopoietic differentiation, this method is also used in the differentiation of endothelial cells [37] and in hematopoietic colony-forming assays (as presented in Chapter 3)[38][39].
Hanging drop culture is a method that has been widely used to differentiate ES cells into several type of specialized cells. The rounded bottom of a hanging drop promote ES cells aggregation and the number of the cells in a hanging drop can be controlled by varying the number of the initial cell suspension, thus this method allows the formation of homogeneous EBs from a defined number of cells. Beside the described advantages, the suspension volume for the formation of a drop is limited to 40 – 50 µl (due to maintaining hanging drops by surface tension), medium replacement is impracticable and microscopic observations of EBs in drop is quite difficult.

The quality of formed EBs affects further differentiation occurring in the EB afterwards, thus far the strategies for EB generation have been chosen individually by researcher to attain their objectives.

5.3.1 Stage protocol for hES-EBs cardiac differentiation

Production of hES cell-derived cardiomyocytes (hESC-CMs) *in vitro* has been demonstrated by several groups [2][28][30][40][41][42] and protocols are well established [43].
Currently, the most efficient strategy for cardiomyocytes derivation from ES cells is reported in [42]. The developmental biology approach has made it possible to recapitulate in hES cultures the key events that regulate early lineage commitment in the embryo, resulting in the efficient and reproducible generation of highly enriched differentiated cell populations [44].

The methodology developed by Keller's laboratory reproduces *in vitro* the signaling effecting cardiac differentiation during embryogenesis, and is based on the addition to EB culture medium of specific soluble factors in a defined temporal sequence. To this end, Keller *at al.* designed a staged protocol of 20 days (Figure 5.2) that involved the formation of a primitive-streak-like population (stage 1), the induction and specification of cardiac mesoderm (stage 2) and the expansion of the cardiovascular lineages (stage 3).

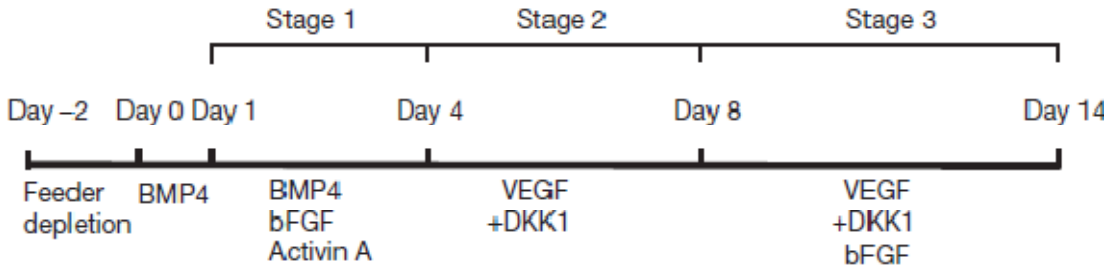

Figure 5.2 Outline of the protocol used for cardiac differentiation of hES cells (modified from [42])

After induction with combinations of activin A, BMP4, bFGF, VEGF and DKK1 in serum-free media, a KDR_{low}/C-KIT$(CD117)_{neg}$ hES-EBs population displays cardiac, endothelial and vascular smooth muscle potential *in vitro* and, after transplantation, *in vivo*. When plated in monolayer cultures, these KDR_{low}/C-KIT_{neg} cells differentiate to generate populations consisting of greater than 50% contracting cardiomyocytes [42]. Keller and colleagues, applied the same strategies for the regulation of primitive streak formation, primary germ layer induction, and tissue specification (hematopoietic, vascular, pancreatic) from differentiated mouse ES cells. A schematic representation of the methodology is reported in Figure 5.3.

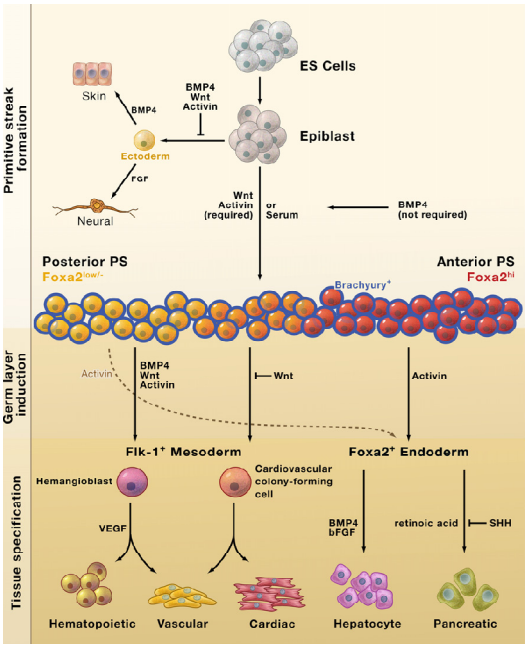

Figure 5.3 ES cells differentiation in culture towards several lineages and cell types (modified from Murry et al [44]).

Thanks to the collaboration with Keller's research group at Toronto University, cardiac differentiation protocol has been acquired and 21-day-HES2-EBs periodically received from Keller's laboratory. HES2-EBs have been kept in culture for additional 20 days and, after differentiation, embedded in OCT for immunofluorescence analysis of specific cardiac markers expression on 10 µm crio-preserved sections. Results from immunofluorescence analysis for Cardiac TroponinT (cTnT), Connexin 43 (Cnx43) and Cardiac troponin I (cTnI) are presented in Figure 5.4.

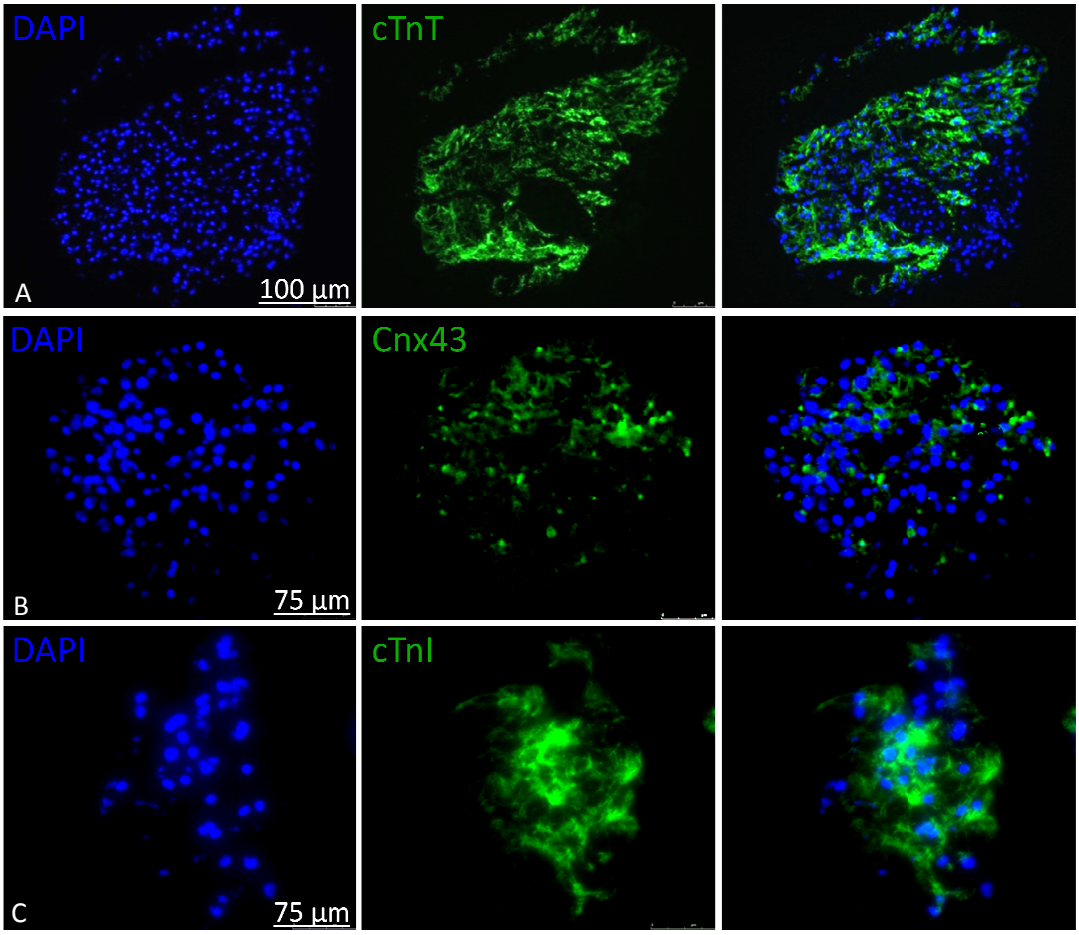

Figure 5.4 Immunofluorescence of cTnt4 (A), Cnx43 (B) and cTnI (C). Nuclei are counterstained with DAPI

Cardiac differentiation of EBs is confirmed by the presence of cells positive for cTNT, cnX43, cTnI. More than 50% of cells after cardiac differentiation, show cardiac characteristics. The remaining fraction of cells has not been characterized but it is presumable that those cells belong to endoderm, ectoderm or mesoderm (but cardiac cells) derivatives.

Albeit the high yield in cardiac cells after cardiac differentiation, the heterogeneity of the total cell population represent the major concern for future clinical applications.
Differentiation of cells within the EB is directed by morphogenic cues comprising the intercellular and surrounding extracellular microenvironment, including exogenously administered molecules and endogenous factors produced by the ES. Individual aspects of the microenvironement can be studied rather simply in planar culture formats, but similarly to a developing embryo, the 3D organization of an EB is comprised of a complex milieu of integrated signals that synergistically affect cell behavior. Although the 3D assembly of cells to form EBs present unique challenges for regulating the homogeneity of stem cell differentiation, attempts to control ES fate is still challenging. The limitations of traditional culture techniques have led to the development of various microscale technologies that possess the potential to regulate some of the parameters which have been identified as critical for stem cells fate, as EB size, extracellular matrix interactions, cell-cell adhesions within the EB and soluble factors.

5.3.2 EB size control

Recent developments in EB formation techniques have enabled more controlled systems capable of modulate EB formation. Non-adhesive polyethylene glycol (PEG) microwell arrays have been used to control the homogeneity of EB size and shape [45][46]. Recent studies on PEG microwell-mediated control of EB size have also investigated the effects on ES cell fate determination, specifically addressing cardiogenesis and vasculogenesis via WNT signaling pathways [47]. These studies showed that WNT11 was highly expressed in larger microwells (450 μm diameter) and ES cells cultured therein exhibited cardiogenesis. In contrast, higher expression of WNT5a in smaller microwells (150 μm in diameter) was associated with endothelial cell differentiation. In addition to PEG, polyurethane microwells containing self-assembled monolayers have been used to culture human ES cells and have been shown to maintain ES cells in their undifferentiated state [48]. In those microwells, a triethylene glycol-terminated alkanethiol selfassembled monolayer prevents cell and protein attachment. Moreover, a polydimethylsiloxane (PDMS)-based hollow sphere strategy has been realized for culturing EBs [49]. The resulting hollow sphere structure contains 500 mL medium, allowing for long-term (10–15 days) culture of EBs *in vitro* without medium loss. The microcontact printing has also been developed to regulate EB size-dependent ES cell differentiation and to investigate the underneath stem

cell biology [50][51][52][53]. In addition, microfabricated adhesive stencils have been used for murine ES cell aggregation within micropatterned substrates [51]. These studies demonstrated that mesoderm and endoderm differentiation were strongly induced in larger cell aggregates, while ectoderm differentiation was promoted in smaller aggregates. Concave [54][55], convex-based [55] thin PDMS membrane arrays (10 μm thickness) and stimuli-responsive microwells [56] for culturing the cells and better mimic the countour of EBs have also been developed. Three-dimensional cuboidal microwell systems have also been developed to culture hES cell colonies of defined dimension for the further study of cardiac differentiation.

EB size can be controlled also in other ways. For example, as discussed before, the hanging drop technique allows for the obtainment of uniform size-controlled EBs and it has been observed that the number of cells used to form hanging drops can influence the chondrogenic differentiation potential of EBs [57]. Force centrifugation studies examining hematopoietic differentiation of hES cells of varying sizes indicated that a minimum starting cell number (500 cells/EB) promoted myeloid differentiation, whereas 1000 cells/EB promoted erythroid one [58].

5.3.3 Extracellular matrix interactions control

The extracellular matrix (ECM) can have an important role in stem cell differentiation by providing a complex milieu of morphogenic cues to cells. The ECM can influence cell behavior through integrin-mediated signaling events and through release of growth factors during matrix remodeling [59][60]. The effects of ECM on EB differentiation have been studied in particular seeding ES cells within natural hydrogel [61][62][63][64], varying the elasticity of ECM [65] and encapsulating EBs within ECM matrices [66].

5.3.4 Cell-cell interactions control

EBs are initially formed through cell adhesive interactions mediated by cadherins. These interactions trigger intracellular pathways that will result in regulation of cell differentiation. Modulated presentation of cadherins, for example through the exposition of cadherins on biomaterial substrate, is a promising and not deeply investigated research field.

5.3.5 Soluble factors control

Controlling the compositions of culture medium to direct ES differentiation is one of the most attempted approach (as discussed in § 5.5.1), therefore barriers to diffusion due to EB structure make difficult the obtainment of homogeneous concentration of factors throughout the EBs. Beside controlling medium compositions, delivery of morphogenic factors within the EB using polymer microspheres is an innovative approach attempted by Carpenedo *et al.* (2009).

An attempt to study the spatial and temporal organization of germ layers during the early stages of differentiation has been also realized. In this work, eight hES cell lines have been compared through the analysis of expression of mesoderm, endoderm and ectoderm markers in order to evaluate whether the differences in EB characteristics would correlate with cardiac differentiation Pekkanen-Mattila *et al.* (2010).

5.4 Experimental plan

For the aim of the research, hES cells have been first cultured, expanded and characterized to verify the maintenance of their undifferentiated state. In order to evaluate the effects of modulated local endogenous factors availability in the immediate hES cell microenvironment, the microstructured hydrogel presented in § 4.4 has been used as the experimental platform for culturing physically separated hES-EBs and modulate the local availability of endogenous factors.

To this end, HES2 have been seeded in 1) conventional ultra low adhesive dishes (for negative control), 2) hydrogel microwells of 500 μm diameter and 450 μm depth, 3) hydrogel microwells of 500 μm diameter and 1 mm depth.

Preliminary analyses are performed to verify cell viability in the developed culture systems by Live & Dead assay.

At 4, 8 and 12 days after seeding, EB diameters will be measured in order to evaluate the distribution of EB sizes in conventional and microwells culture systems.

After reaching mesodermal differentiation (8 days), EBs will be collected for gene expression analysis of specific mesodermal markers by Real Time PCR.. Oct4 has been chosen as representative of pluripotency, Brachyury T [69] and GATA4 [9] of mesodermal differentiation lineages. Whole genome analysis by microarray will be performed in order to

analyze the complete profile of hES cell differentiation. EBs will be also embedded in OCT for further immunofluorescence analysis of expression and distribution of mesodermal, endodermal and ectodermal markers on crio-preserved sections.

5.4.1 Results and discussion

5.4.1.1 Human embryonic stem cells in vitro culture

Human ES cells proliferate in long-term culture in an undifferentiated state but spontaneous differentiation is frequently observed during routine expansion [70].

These cells are particularly sensitive to chemical agents. This aspect implicates that a) antibiotics should not be added to hES culture medium, b) particular care should be addressed to the maintenance of culture sterility, c) cell passing by mechanical disaggregation should be preferred to enzymatic treatment. In addition, hES cells show high metabolism so they do fast deplete the nutrients which are present in the culture medium, requiring daily medium change.

HES2 culture is performed on six-well plates, which are pre-coated with 0.1% gelatin for promoting the adhesion and the homogeneous distribution of the feeder layer cells: inactive mouse embryonic fibroblasts. The feeder layer provides certain currently unknown factors, which support undifferentiated growth of hES cell [71]. MEF need to be inactivated to be used as feeders (Figure 5.6) to prevent their continued proliferation. These cells can be inactivated chemically by treatment with Mitomycin C or through γ-irradiation. After inactivation MEF are cultured in DMEM with 10% (v/v) Heat Inactivate Fetal Bovine Serum and 1% (v/v) Non-Essential Amino Acids. Once plated, MEF can be used for hES cells culture within 14 days maximum until they lose their properties.

The medium for HES2 cell lines culture is composed of 80% DMEM F12 supplemented with 20% (v/v) KnockOut Serum Replacement (Knock-Out SR), 10% MEF conditioned medium, 20 ng/ml Basic Fibroblast Growth Factor (bFGF), 1mM L-Glutamine, 0.1mM β-Mercaptoethanol, 1% stock solution of Non-Essential Amino Acids, 1% penicillin/streptomycin. hES cells grow in uniform colonies, with defined and regular edges, as shown in Figure 5.5.

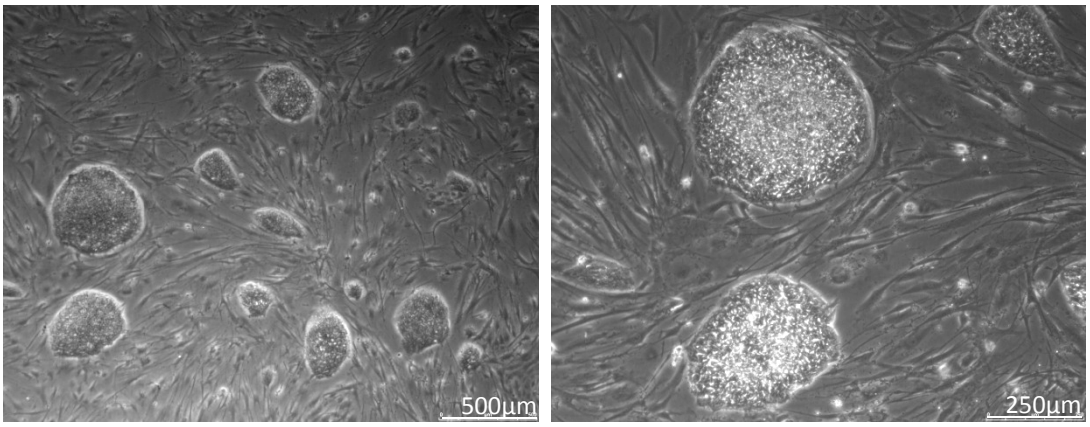

Figure 5.5 Bright field images of HES2 hES cell line colonies on a monolayer of inactive MEF. Undifferentiated colonies appear as uniform cell aggregates. Edges are defined and regular.

Cell passing is performed when MEF feeder layer are 14 days old or when colonies are reaching confluence. The passing can be done both mechanically and enzimatically; the first should be preferred, because of hES cells sensitivity to chemical agents. The mechanical removal of colonies is performed observing the cells with a stereomicroscope and dissecting the undifferentiated colonies into several pieces using a cutting pipette. These selected pieces then are replated onto new well containing fresh MEF feeders. The enzymatic treatment consist of 3 minutes of incubation with 1 mg/mL Collagenase IV (in Knock Out DMEM) at 37°C, followed by scraping the cells off the surface of the plate, using a glass pipet.

MEF has a key role in maintaining hES cells undifferentiated. Timing and density are essential: MEF should not be used after 14 days post inactivation and the optimal seeding density is $1.6\text{-}1.8\times10^4$ cells/cm^2.

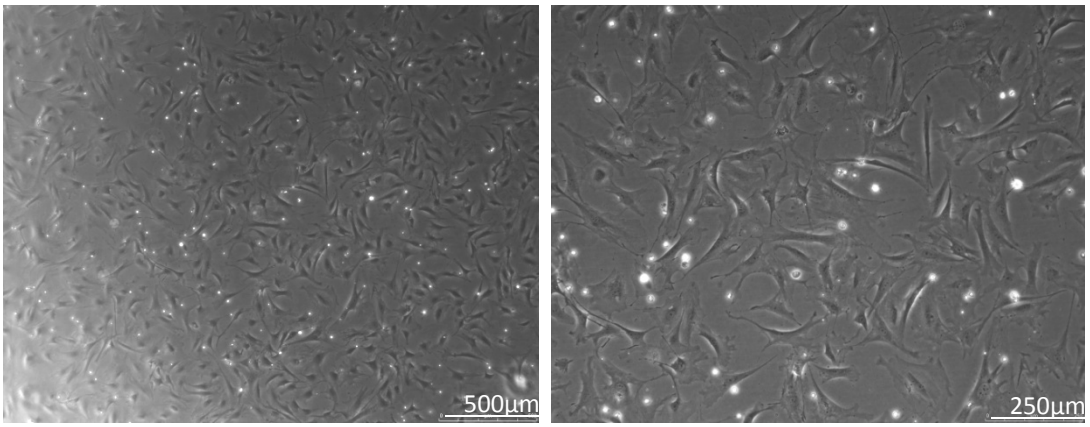

Figure 5.6 Inactive MEF feeder layer (cell density: 1.75 x 10^4 cells/cm^2) for the support of hES cells culture.

The differentiation of hES cells occurs mainly through three mechanisms. When center differentiation occurs, a darker region forms in the middle of colony (Figure 5.7A). When the colony has poorly defined borders and flat cells are distinguishable, it presents peripheral differentiation (Figure 5.7B).

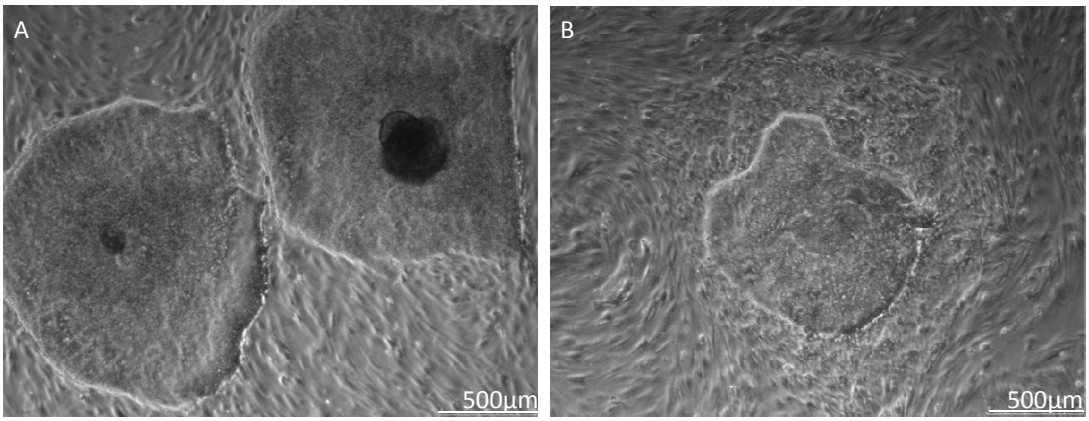

Figure 5.7 hES cells differentiation. Centre differentiation (A) Peripheric differentiation (B). The area selected with dotted line show visibly differentiated cells.

In order to keep hES cells culture undifferentiated, differentiated cells should be removed as soon as they form. Depending on the relative amount of differentiated colonies in the culture, the methodologies for cell expansion are: "picking to remove" or "picking to keep". If there are mostly undifferentiated colonies on the plate with only a few differentiated single cells and colonies, the picking to remove is preferred: the differentiated cells are picked, removed, and discarded, whereas undifferentiated colonies are kept on the plate until they are ready to passage. If there are only a few undifferentiated colonies on the plate with a large number of differentiated cells and colonies, the "picking to keep" methodology is used: the undifferentiated colonies are picked, plated and propagated. The differentiated cells are left on the plate and discarded.

The undifferentiated state of hES has been verified by analysis of karyotype stability (Figure 5.8) and immunofluorescence of the pluripotency markers Oct4, C-Myc, Klf4 (Figure 5.9), Tra1-81, Tra1-60, Tra2-49 (Figure 5.10).

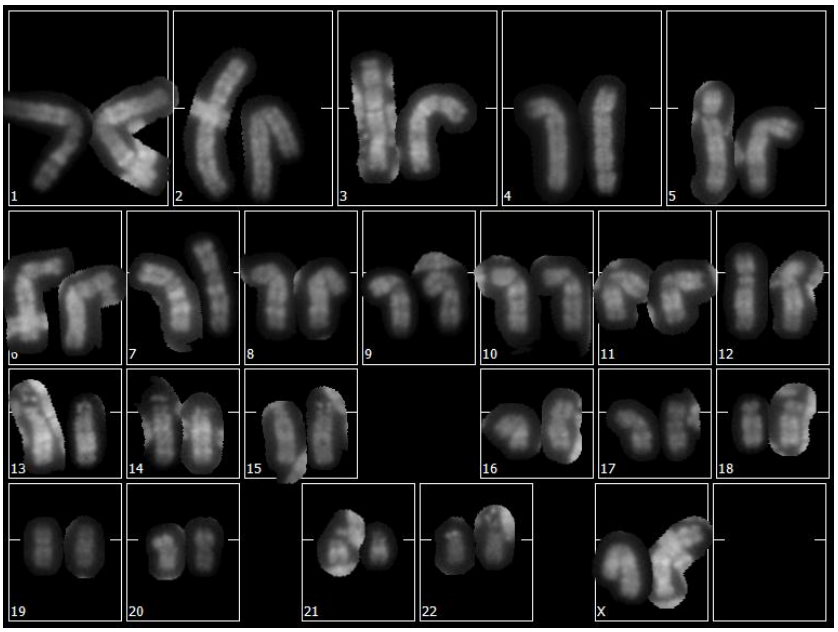

Figure 5.8 Karyotype analysis of HES2 cells. HES2 cells show normal karyotype.

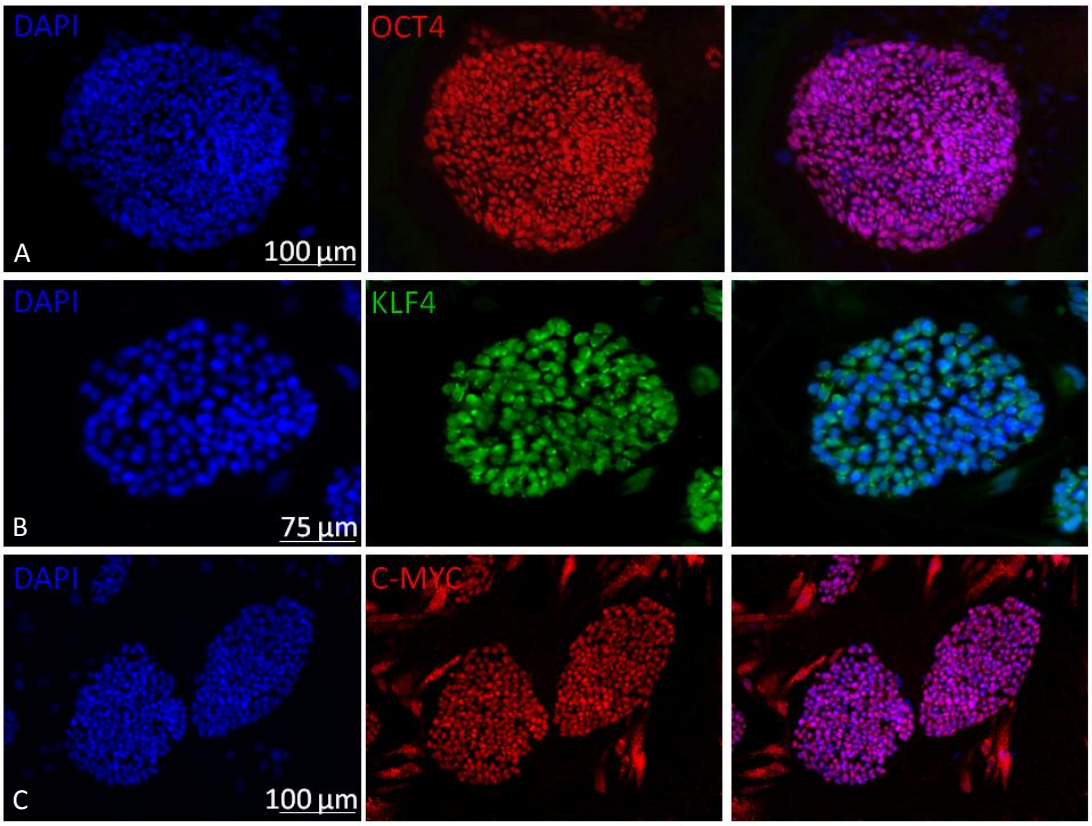

Figure 5.9 Immunofluorescence of Oct4 (A), Klf4 (B) and C-myc (C). Nuclei are counterstained with DAPI

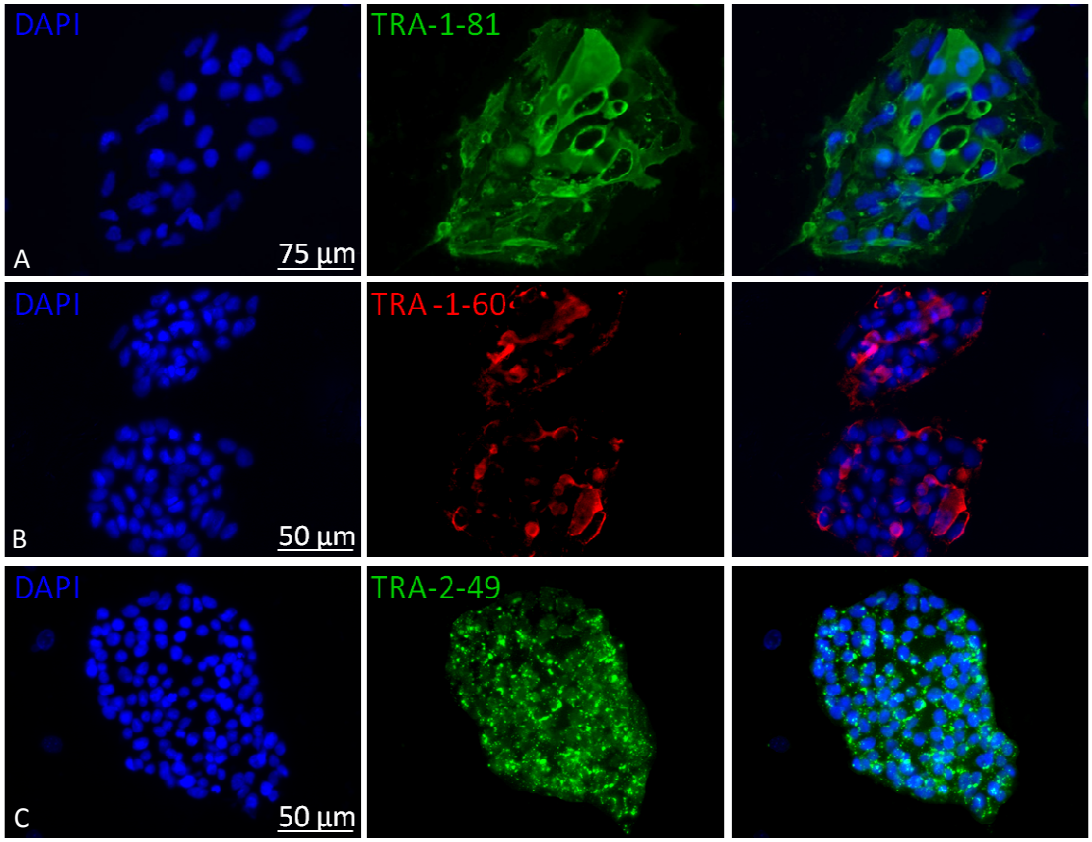

Figure 5.10 Immunofluorescence of Tra1-81 (A), Tra1-60 (B) and Tra2-49 (C). Nuclei are counterstained with DAPI

5.4.1.2 Generation of HES2-EBs

To induce the formation of human EBs, undifferentiated HES2 cells are treated with 1ml trypsin 0.25% and then transferred to matrigel coated dishes for MEF depleting. 24-48h later, HES2 cells are treated with1 mg/ml collagenase for 20 minutes followed by 1ml trypsin 0.25% in PBS for 30 seconds and then transferred to low attachment plates containing EB medium: 80% knockout Dulbecco's modified Eagle medium supplemented with 20% defined fetal bovine serum (Hyclone), 1 mM L-glutamine, 1% non-essential amino acid stock and 1% penicillin/streptomycin. Human EBs are cultured at 37 °C and 5% CO_2 in a humidified incubator, with changes of media every 3 days. Figure 5.11 presents brigh field images of 8-day-old EBs.

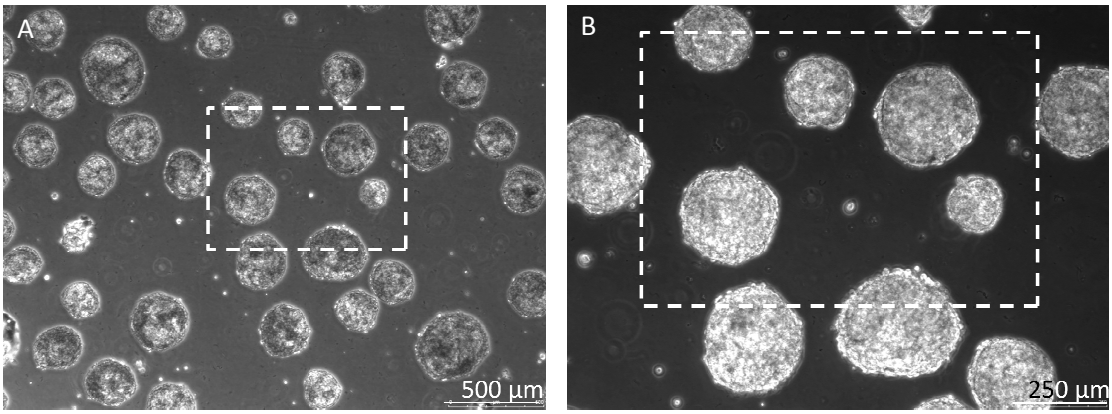

Figure 5.11 Bright field images of 8-day-old HES2-EBs in suspension. EBs of different sizes are visible (A). Magnification in (B)

5.4.1.3 Microwells cell seeding

Prior to seeding cells in microwells, each microstructured hydrogel was placed in a well of a 6-well plate, immersed in cell culture medium and incubated at 37°C for 24 hours for equilibration. hES cells were passaged from two wells of a 6-well matrigel coated plates to one microstructured hydrogel in 700 µl of cell culture medium. In order to maximize cell seeding efficiency, the cell suspension was aliquoted to the top of the microstructured hydrogel, taking care to maintain the entire volume of cell suspension on the hydrogel. Hydrogel were kept at room temperature for at least 15 minutes to allow cells to settle into wells and then placed into the incubator for 24 hours. Finally 4ml/well of cell medium were added carefully to each well of the 6-well plate to prevent cells being washed out of microwells.

After seeding, HES2 cells spontaneously form EBs within the array of microwells as presented in Figure 5.12 and Figure 5.13.

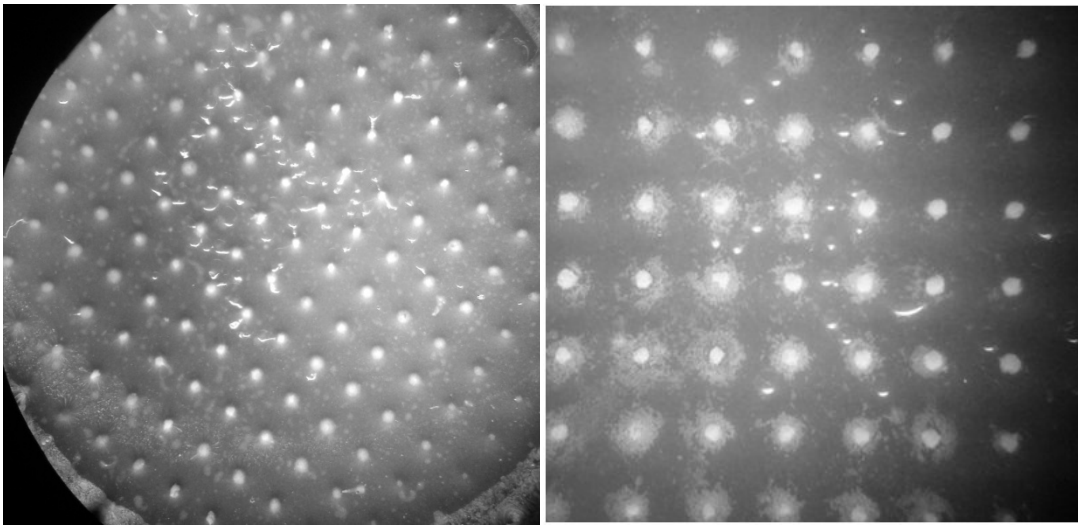

Figure 5.12 Stereoscope images of HES cells 48h after seeding onto the microstructured hydrogel.

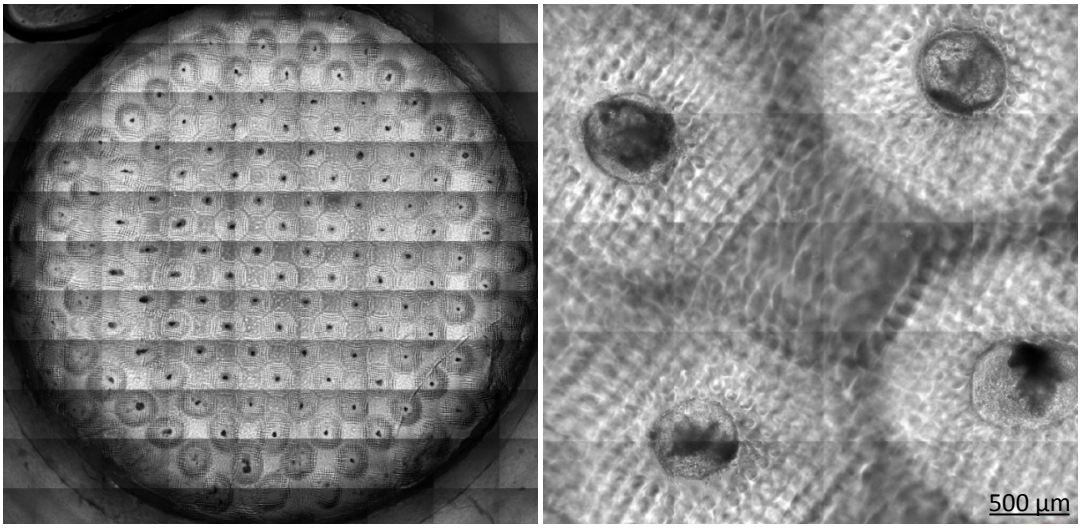

Figure 5.13 Bright field images of HES cells 48h after seeding onto the microstructured hydrogel.

5.4.1.4 Cell viability assay

2 x 10^5 HES2 cells are seeded onto both microstructured hydrogel (microwells of 450 µm and microwells of 1 mm depth) and into ultra low adhesive dishes for further analysis of cell viability. 10 days after seeding, cell viability is analyzed by Live & Dead assay. The results of the assay are presented in Figure 5.14.

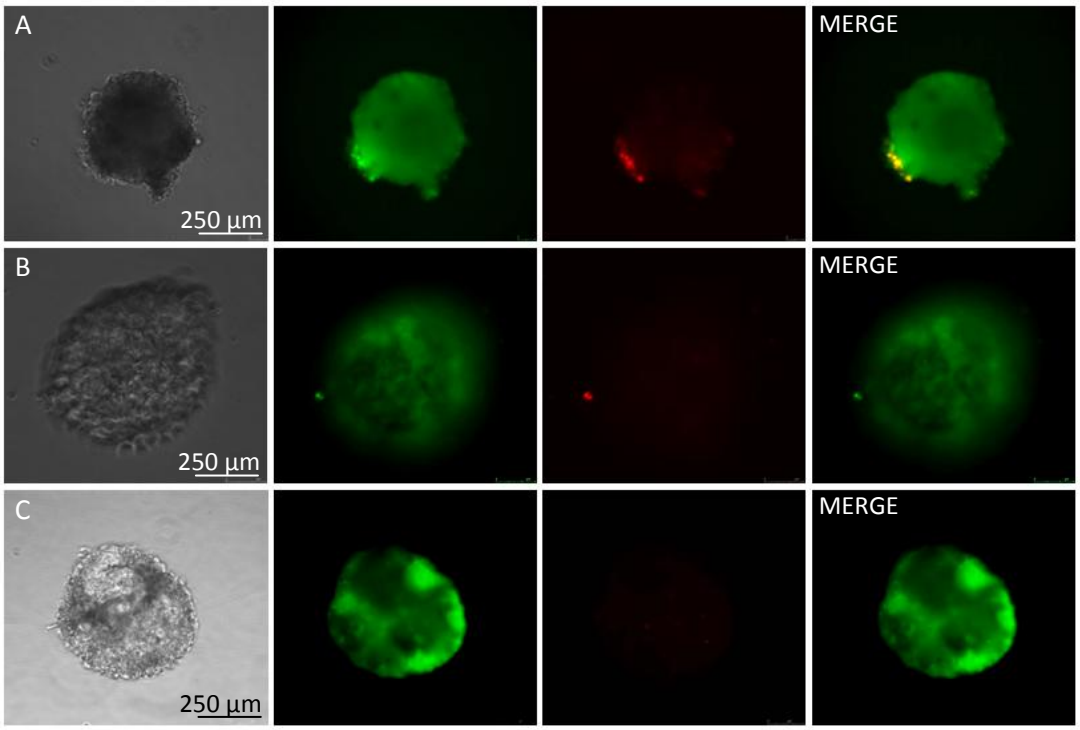

Figure 5.14 Live & Dead assay on hES2-EBs 10 days after cell seeding in (A) ultra low adhesive dishes (B) 450µm deep microwells (C) 1mm deep microwells.

Live & Dead assay results shows alive cells in all the different cell culture conditions for the duration of the entire experiments. No evident differences are detectable among EBs cultured in ultra low adhesive dishes (suspension EBs) versus EBs culture in microwells (450 µm and 1 mm EBs), thus indicating that the hydrogel do not negatively affect cell viability.

5.4.1.5 EBs size distribution

4, 8 and 12 days after seeding EB diameters have been measured at the microscope.

Quantitative analysis of diameter distribution of suspension and microwells EBs has been performed at optical microscope. Results are presented in Figure 5.15 and Figure 5.16 respectively.

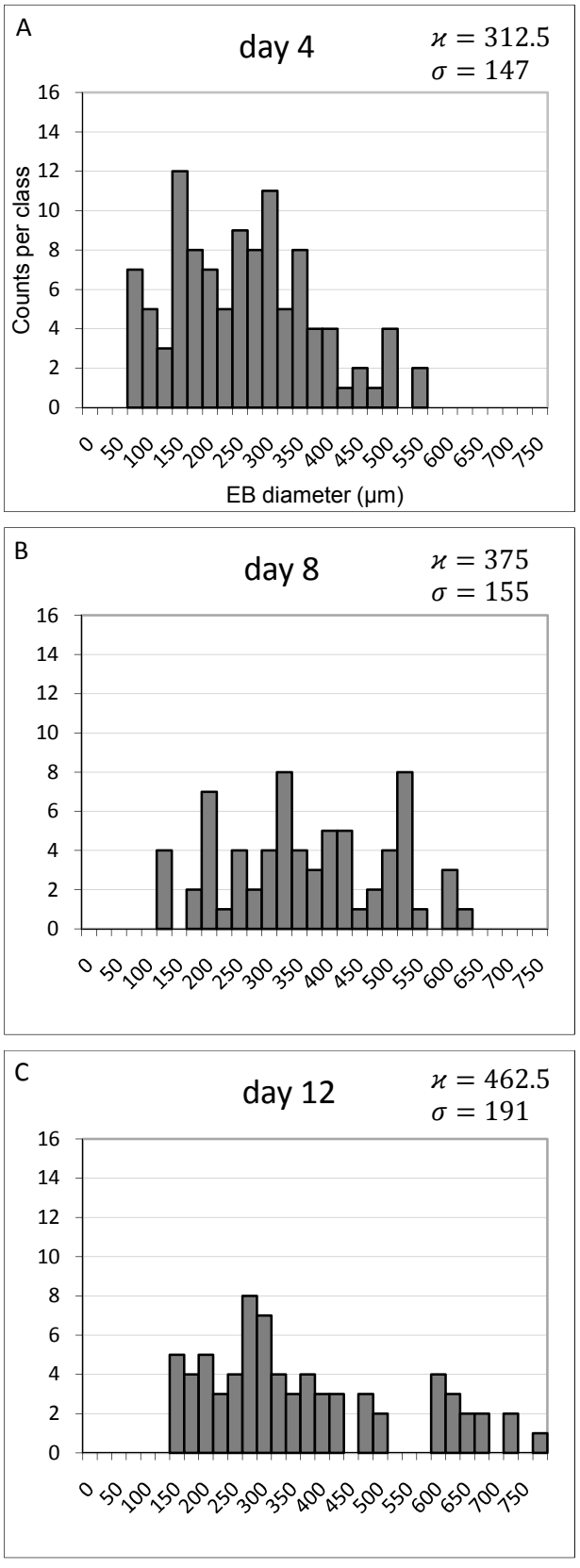

Figure 5.15 Quantitative distribution of suspension EBs diameter. Measurements of EBs diameters after 4 days (A), 8 days (B), 12 days (C) of culture in suspension.

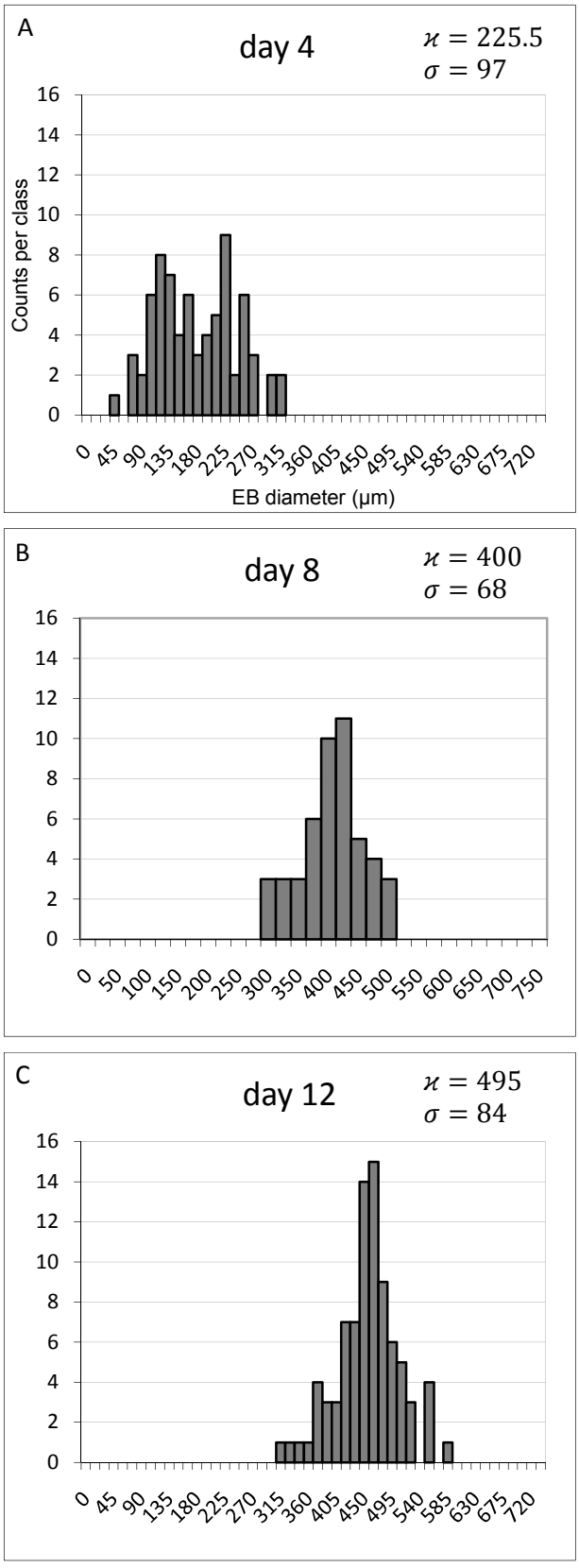

Figure 5.16 Quantitative distribution of microwells EBs. Measurements of EBs diameters after 4 days (A), 8 days (B), 12 days (C) of culture in microwells.

As shown, the width of hEBs is significantly regulated by microwell size after few days of culture. After 12 days, the mean growth of the hEBs reaches the diameters of the microwells, whereas the distribution of suspension EBs is heterogeneous. In addition, the presence of EBs with diameter >> 500 μm suggested the formation of undesiderable EBs aggregates.

5.4.1.6 QPCR analysis

Real Time PCR analysis is carried out on the EBs cultured in the three different culture conditions. Total RNA from 8-day-old EBs is isolated using TRIZOL reagent (Invitrogen) coupled to RNeasy Kit (Qiagen) according to the manufacturers' instruction. The optimized protocol for RNA extraction from hES-EBs is in Appendix D. 500ng of total RNA is used per RT reaction using High-Capacity cDNA Archive Kit (Applied Biosystems). Each PCR as performed in triplicate with TaqMan® Universal PCR Kit (Applied Biosystems) and TaqMan® gene expression assays. TaqMan Primers for:

- GAPDH (Hs99999905_m1)
- OCT4 (Hs01895061_ul)
- Brachyury T (Hs00610080_m1)
- GATA4 (Hs01034628_m1)

are used. Expression levels of genes of interest are normalized to GAPDH expression.
Real Time results are shown in Figure 5.17.

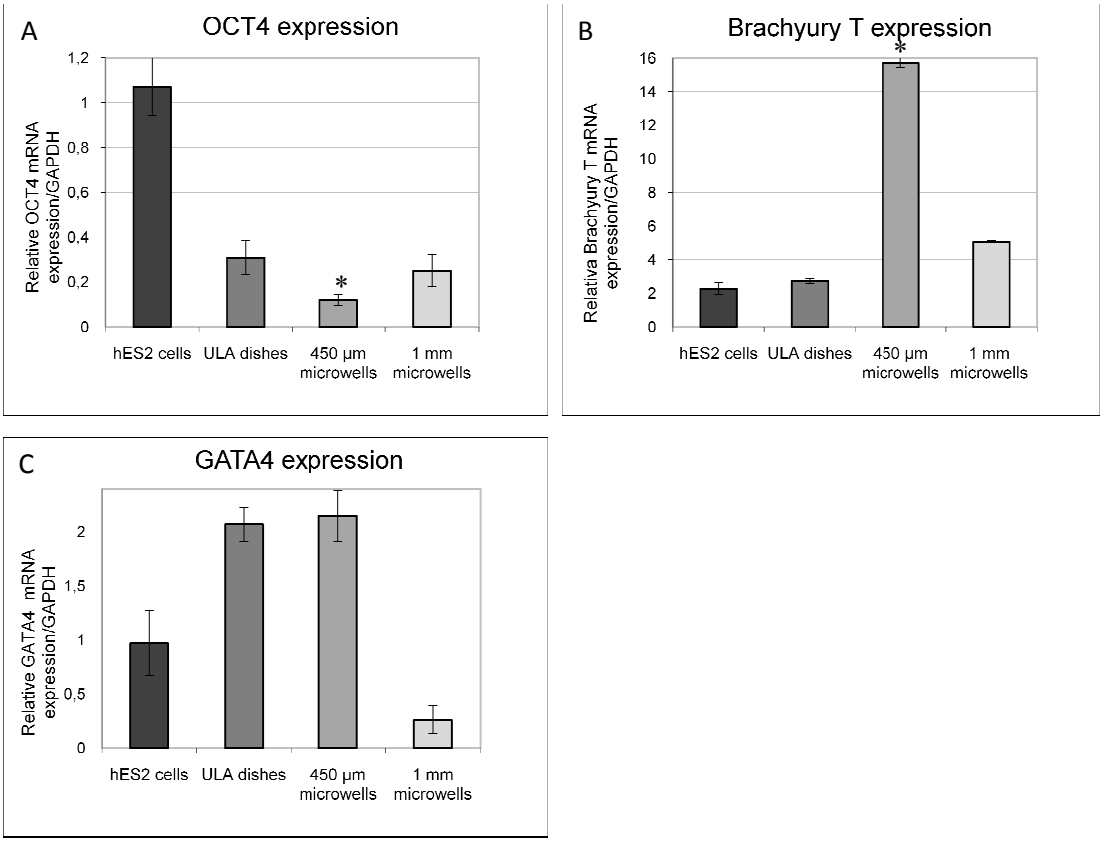

Figure 5.17 Real Time analysis of OCT4 (A), Brachyury T (B) and GATA4 (C) expression in the EBs after 8 days of culture either in ultra low adhesivet dishes (ULA dishes) or in microwells. Gene expression of hES2 cells growing as colonies on MEF feeder layer is also included in the analysis.

In Figure 5.17A, relative OCT4/GAPDH expression is presented. As expected, the expression of the pluripotency markers sensitively decreases from HES2 colonies to EBs. The considerable OCT4 expression in differentiating EBs is expected in early differentiation stages.

In Figure 5.17B, relative Brachyury T/GAPDH expression is presented. Interestingly the highest expression of Brachyury T is detected in 450 µm EBs, whereas Brachyury T expression in suspension and 1 mm EBs is comparable.

OCT4 and Brachyury T expression suggest that the microenvironmental conditions in 450 µm deep microwells may promote differentiation of cultured EBs, towards mesodermal lineage especially.

Figure 5.17C shows relative GATA4/GAPDH expression. The expression of the meso-endodermal marker GATA4 increases from hES2 colonies to suspension EBs and 450 µm EBs, whereas it is similar to the one detected in 1mm EBs.

The results from preliminary gene expression analysis suggests that hES cells are differentiating, however the indications are not sufficient to characterize the expected different differentiation profiles of hES-EBs due to modulated local availability of endogenous factors in the different cell culture conditions. In order to address this question, whole genome analysis by microarray has been performed.

5.4.1.7 Microarray analysis

DNA microarray analysis is carried out on the EBs cultured in the three different culture conditions. Total RNA is isolated using TRIZOL reagent (Invitrogen) coupled to RNeasy Kit (Qiagen) according to the manufacturers' instruction. Labelled cDNAs are synthesized by reverse transcription from the total RNA using the Quick Amp Labeling Kit, One Color (Agilent). Whole Human Genome Microarray Kit, 4x44K (Agilent) is used according to the manufacturer's protocol. Microarray analysis has been performed by Prof. Barzon's research lab from Department of Histology, Microbiology and Medical Biotechnologies, University of Padua. Statistical analysis has been performed by Dr. Di Camillo from Department of Information Engineering at University of Padua.

Microarray data pre-processing

Expression data with log_base_2 value lower than 6 shows high variability.
Loess pair-wise normalization has been applied to data.

Data selection (univariate analysis)

Significant Analysis of Microarrays (SAM) algorithm [72] has been used to identify genes with statistically significant changes in expression between different classes. All data have permutated over 1000 cycles by using the multiclass response format, not considering equal variances. Significant genes were selected based on a false discovery rate (FDR) threshold of 0.05. Significant genes have been tested using the two-classes response format (suspension EBs vs 450 µm EBs, suspension EBs vs 1mm EBs, 450 µm EBs vs 1mm EBs), not considering equal variances, and selected based on a significance level of 0.05/3 (Bonferroni correction).

Functional analysis

The probe sets have been grouped according to their biological function using Gene Ontology (GO) Biological Process descriptions [73]. The functional enrichment analysis to

identify the most relevant biological mechanisms, has performed using [74]. GO_Fat have been used instead of standard GO to filter the broadest terms so that they do not overshadow the more specific terms. p-values are corrected for multiple testing using FDR.

Selection

418 probes, corresponding to 380 unique gene IDs and 8 ESTs, have selected in the multiclass analysis. Table 5.1 shows the number of genes significantly differentially expressed in the two-classes comparisons and the sign of the differential expression.

Table 5.1 Number of genes significantly differentially expressed in the two-classes comparisons and the sign of the differential expression.

Suspension vs 450µm	Suspension vs 1mm	450 µm vs 1 mm	Selected probes
↑	↑	↑	4
↓	↓	↓	10
↑	↑	↓	7
↓	↓	↑	2
↑	↑	-	**189**
↓	↓	-	**203**
↑	-	↓	1
↓	-	↑	2

Data from microarray analysis has identified a group of 392 genes whose expression is significantly differentially detectable among suspension EBs and microwells EBs (without distinction from 450 µm and 1 mm EBs).

Functional analysis

The functional analysis on groups of genes which are respectively significantly up-regulated in microwells EBs compared to suspension EBs shows that 2 GO terms resulted significantly enriched:

- pattern specification process with genes: CER1, ARC, WNT3, EGR2, SFRP2, NKX6-2, LEFTY2, TDGF1, DLL3, BCOR, GLI2, LFNG
- regionalization with genes: CER1, ARC, WNT3, EGR2, SFRP2, NKX6-2, TDGF1, DLL3, GLI2, LFNG

One GO term in resulted significantly enriched in suspension EBs compared to microwells EBs:

- heart development: TNNT2, NRP1, ERBB4, KCNJ8, COL3A1, OBSL1, MYH6, COL5A1, CASQ2

These results suggest that EBs confinement, thus accumulation of endogenous factors in the immediate cell microenvironment, does influence cell differentiation. Robust differences among suspension EBs and microwells EBs are evident from microarray analysis. Functional analysis of the statistically differentially expressed genes shows that confinement of EBs promotes the expression of genes involved in the pattern specification process, while conventional cell culture conditions promotes the expression of genes involved in the heart development.

5.5 Future development: extension to iPS cells

The present research may be extended to iPS cells in the future. To this aim, iPS cells are currently cultured in my laboratory. After successful expansion, embryoid bodies have been generated from hiP cells and differentiation in mesodermal precursor cells is in progress. Figure 5.18 shows a contrast phase image of an iPS colony on feeder layer. However, because of the high variability and the uncomplete characterization of these cells [75], hES cells has been chosen for this first studies.

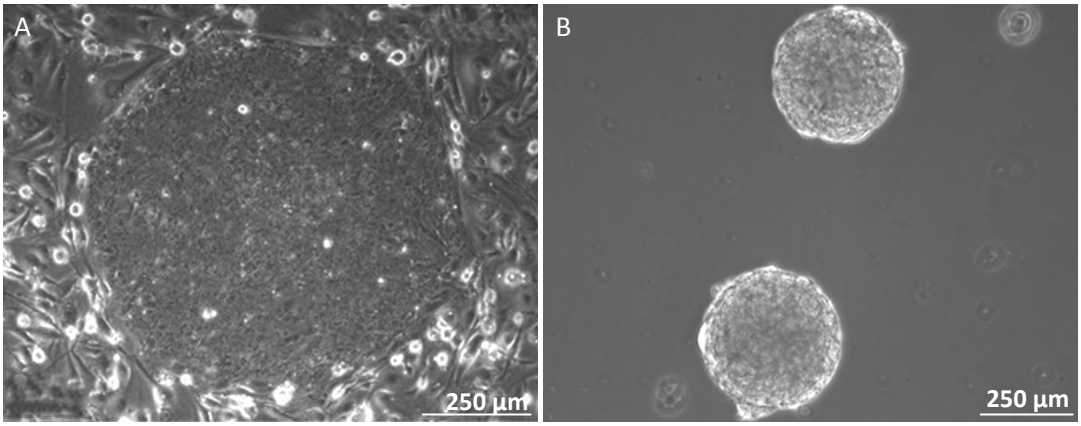

Figure 5.18 Phase contrast images of an PS colony on MEF feeder layer (A), iPS-EBs in suspension culture onto ultra low adhesive dishes (B).

5.6 Conclusions

As reported in § 2.7, the culture of hES cells in a stirred system is not straightforward, because of cell aggregation issues. That limitation has been overcome, as described in Chapter 4, by the development of a no-biodegradable microstructured hydrogel. Preliminary results from biological experiments reported in § 4.4 demonstrated that the microstructured hydrogel was suitable for the culture of physically separated cell clusters. Exploiting the possibility to easily modulate the depth and shape of the microwells, thus the availability of endogenous factors to cell microenvironment, it was supposed to proceed with further experiments aimed at controlling hES cell fate. In order to address this challenge, hES cells were cultured until reaching of mesoderm differentiation (8 days) in microwells of different depths and in conventional ultra low adhesive dishes as negative control. Measurements of EBs diameters at 4, 8 and 12 days of cultures during preliminary experiments, showed that the width of hEBs is significantly regulated by microwell size. After 12 days, the mean growth of the hEBs reached the diameters of the microwells, whereas the distribution of suspension EBs was highly heterogeneous. In addition, the presence of EBs with diameter >> 500 μm suggested the formation of undesiderable EBs aggregates. These results confirm that the microwell technology can be used to obtain many physically separated size-controlled hEBs, thus increasing homogeneity within the cell population. The cultured EBs can easily be collected by using a micropipette for further investigations without disrupting the culture, thus this technology may be applicable for high-throughput testing. Regarding the control of hES cell differentiation, after 8 days, hES-EBs were retrieved from the microwells and whole genome profile analyzed by microarray.
From the microarray analysis, performed on 8-day EBs, it emerges that the confinement of EBs in the microwells lead to the formation of spatial patterns in developing embryo creating directional signals necessary for the proper placement of the components of the organism. This indication is particularly interesting considering that the patterning of mammalian embryos *in vivo* is regulated by gradients of mophogens, including hedgehog (Hh), bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), wingless Int (Wnts), and fibroblasts growth factor (FGFs) [76]. The culture in conventional dishes, on the other hand, promotes the expression of genes involved in heart development. These findings demonstrate that the locally controlled availability of endogenous factors elicits significant differences in hES differentiation and may provide new insights into the mechanisms involved in cell differentiation. Further experiments are required in order to study the

regionalization process in the EBs. With regards to the study of regionalization processes in the EBs, it would be challenging to analyze and compare the distribution of specific markers of hES differentiation. To this end, immunofluorescence analyses on sections may be informative.

As a general remark, this technology may be a tool suitable to modulate the presentation of morphogenic cues and elucidate the mechanisms of stem cell differentiation. To this end, the cultured EBs can easily be collected using a micropipette for further investigations without disrupting the culture, thus this technology may be applicable for high-throughput testing.

The recreation of cellular microenvironments may contribute to answer fundamental questions regarding cell behavior and to guide differentiation. The small amounts of medium which are required for the obtainment of hundreds of separated EBs makes this approach a tool to consider when developing *in vitro* differentiation protocols.

5.7 References

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Chapter 6
Conclusions

The potential of stem cell-derived differentiated cells, tissues and organs promises to revolutionize the field of regenerative medicine and is a topic of intense research. For the hematopoietic system and the cardiac muscle, which suffer from a variety of diseased conditions, the perspective of cell therapy approaches is particularly attractive. However, fundamental prerequisite for transforming the promise of stem cells into therapeutically relevant treatments, is the obtainment of a sufficient number of cells that maintain the appropriate phenotype and perform the specific biological function.

In this context, this PhD thesis aimed at the development of multiscale technologies for culturing and directing differentiation of human stem cells under defined cell culture conditions, in order to control the resulting cell phenotype. A particular focus has been addressed to the development of strategies to improve the final product quality, *i.e.* the stem cell clinical therapeutic potential.

The rational approach underneath this work is based on the use of human stem cells coupled to technologies for cell environment control, as shown in the schematics in Figure 6.1.

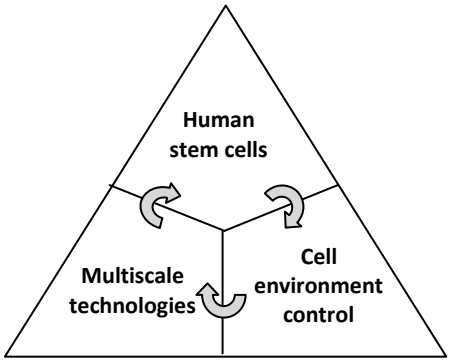

Figure 6.1 Schematic representation of the rational approach underneath the present PhD thesis work: the stem cell niche inspired multiscale technologies development, in order to study the biological and chemical-physical cues that guide human cell behavior in vivo.

A general aim is to yield the small volumes involved in the proposed technology to span simultaneously a wide range of cell culture conditions which would have prohibitive costs with volumes of hundreds of milliliters.

In the first part of the research, the attention was focused at rationally understanding biological phenomena in hematopoietic process of cell expansion *in vitro*.
In order to investigate the effects of oxygen tension on the expression of c-kit on human UCB- derived CD34+ cells have, a six-well stirred bioreactor has been designed end developed. The stirred system in fact improves homogeneity within the culture volume thus allowing for the control of the effective O_2 concentration in the cell immediate microenvironment. This control would not be possible in static cultures which are subjected to concentration gradients. Mixing is the only difference respect to conventional culture systems, where medium is stagnant. Thus, this bioreactor allows the use of already developed protocols for hematopoietic stem cell culture, looking exclusively at the effect of mixing. Considering the interdisciplinary of stem cell field, a simple and robust device that can be handled even by researchers without a strong technical background, for a wide-spread use and repeatability of the results, was developed. The innovative culture system was first tested under normoxic conditions, to exclude any negative effects respect to cell viability, morphology, proliferation and differentiation potential. Once verified these fundamental aspects, the bioreactor was used for culturing cells under hypoxic conditions, using those in normoxic as negative control. After 6 days of culture, lower proliferation was detected in hypoxic conditions compared to normoxic ones. These data are confirmed by other studies [1][2] and the low cell-cycle activity in hypoxia conditions has been associated with an increase in the proportion of long-term reconstituting hematopoietic stem cells [3]. Moreover, by flow cytometric analyses, different profiles of c-kit expression between cells cultured in hypoxic conditions and to those cultured in normoxic atmosphere were obtained.
Specifically, in hypoxia an over-expression of c-kit respect to the 21% oxygen condition was detected. These exciting findings underline the importance of oxygen tension as a metabolic regulator of stem cell biology and represent an added dimension of stem cell control that allows cells to maintain self-renewal and multilineage differentiation potential. The data obtained in the work provide the basis for further investigations in order to understand the molecular mechanisms underneath oxygen concentration and c-kit expression.

Aim of the second application of my research was the development of technology for the control of hES cardiac differentiation *in vitro*. hES cells represent the best cell source for the obtainment of an elevated number of cardiomyocytes. Despite their high sensitivity to environmental conditions and their time- and labor demanding procedures, three human embryonic stem cell lines have been successfully cultured for more than 25 passages during this study. The procedures for EB derivation, differentiation and dissociation to single cell

have been also established. Cell differentiation towards cardiac lineage has been achieved by spontaneous differentiation of hES-EBS cultured in suspension and by a stage protocol based on hES cell stimulation with a defined temporal sequence of soluble factors [4], which gave an extremely high yield in cadiomyocytes (more than 50% of cells were positive to cardiac Troponin T). In order to direct and control the differentiation of hES, two different approaches have been attempted. In the first, I did contribute to the studies for an innovative protocol based on the application of electrical stimulation, (results are reported in Appendix E) and in the second I designed and developed the microstructured hydrogel platform for controlling the effects of endogenous factors on hES cell differentiation, which has been presented in Chpater 4. The polyacrylamide hydrogel has been chosen for the aims of the work because it is highly stable *in vitro*, it is capable of absorbing large volumes of aqueous solution, thus creating 3D microenvironment suitable for cell cultures, it is biocompatible, geometrically easily modulable and characterized by a non fouling surface which is suitable for EB culture, and above all, is selectively permeant to molecules in the cell culture medium, thus adapt for the aims of this study. hES cells were cultured until reaching of mesoderm differentiation (8 days) in microwells of different depths and in conventional ultra low adhesive dishes as negative control. Measurements of EBs diameters showed that the width of hEBs is significantly regulated by microwell size, whereas the distribution of suspension EBs was highly heterogeneous. From the microarray analysis, performed on 8-day EBs, it emerges that the confinement of EBs in the microwells lead to the formation of spatial patterns in developing embryo creating directional signals necessary for the proper placement of the components of the organism. The culture in conventional dishes, on the other hand, promotes the expression of genes involved in heart development. These findings demonstrate that the locally controlled availability of endogenous factors elicits significant differences in hES differentiation and may provide new insights into the mechanisms involved in cell differentiation. Further experiments are required in order to study the regionalization process in the EBs. With regards to the study of regionalization processes in the EBs, it would be challenging to analyze and compare the distribution of specific markers of hES differentiation by immunofluoresce. As a general remark, this technology may be a tool suitable to modulate the presentation of morphogenic cues and elucidate the mechanisms of stem cell differentiation and may contribute to answer fundamental questions regarding cell behavior and to guide differentiation. This type of studies, aiming at control the differentiation pathway of hES cells, are nowadays one of the most important challenge in hES cell research, since this is the major prerequisite for clinical application of hES cells.

The results obtained in small scale system may be translated when planning large-scale expansion. The advent of human embryonic stem cells opened new and exciting perspective in the field of tissue engineering and regenerative medicine. The possibility of deriving all cell types composing human body holds the promise and the potential of treating and healing those diseases based on cellular disfunction and of generating artificial tissues and organs. These possibilities belong to a long-term perspective, but they rise from a comprehensible enthusiasm for the latest results obtained in the field. Important steps forward in the control over hES cell *in vitro* specific differentiation for clinical applications have been accomplished. The coupling of biological science with engineering offer new possibilities, as demonstrated by the promising results obtained in this thesis. Novel bioengineering technologies should be integrated into cell biology as they evolve to further enhance control of cell product identity, reproducibility, and efficacy. Cellular reprogramming for obtaining induced Pluripotent Stem cells (iPS) [5] was named Breakthrough of the Year for 2008 by Science magazine. iPS cells are adult somatic cells reprogrammed to an embryonic state, which can then be differentiate to any cell type. The approach overcomes the ethical issues involving the use of embryos and opens the possibility of creating patient-specific stem cell lines to study different disease mechanisms *in vitro*, to provide new platforms for toxicology studies and drug discovery. The use of iPS coupled to the multiscale technologies presented in this thesis is straightforward. iPS cell culture are fundamentally based on knowledge and technical methodologies established for hES cells. The medicine in the future will be founded on patient-specific data obtained from patient-derived cells for customized therapy development.

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APPENDIX A

"Norme in materia di procreazione medicalmente assistita"

pubblicata nella *Gazzetta Ufficiale* n. 45 del 24 febbraio 2004

CAPO I
PRINCÌPI GENERALI

ART. 1.
(Finalità).

1. Al fine di favorire la soluzione dei problemi riproduttivi derivanti dalla sterilità o dalla infertilità umana è consentito il ricorso alla procreazione medicalmente assistita, alle condizioni e secondo le modalità previste dalla presente legge, che assicura i diritti di tutti i soggetti coinvolti, compreso il concepito.

2. Il ricorso alla procreazione medicalmente assistita è consentito qualora non vi siano altri metodi terapeutici efficaci per rimuovere le cause di sterilità o infertilità.

ART. 2.
(Interventi contro la sterilità e la infertilità).

1. Il Ministro della salute, sentito il Ministro dell'istruzione, dell'università e della ricerca, può promuovere ricerche sulle cause patologiche, psicologiche, ambientali e sociali dei fenomeni della sterilità e della infertilità e favorire gli interventi necessari per rimuoverle nonché per ridurne l'incidenza, può incentivare gli studi e le ricerche sulle tecniche di crioconservazione dei gameti e può altresí promuovere campagne di informazione e di prevenzione dei fenomeni della sterilità e della infertilità.

2. Per le finalità di cui al comma 1 è autorizzata la spesa massima di 2 milioni di euro a decorrere dal 2004.

3. All'onere derivante dall'attuazione del comma 2 si provvede mediante corrispondente riduzione dello stanziamento iscritto, ai fini del bilancio triennale 2004-2006, nell'ambito dell'unità previsionale di base di parte corrente "Fondo speciale" dello stato di previsione del Ministero dell'economia e delle finanze per l'anno 2004, allo scopo parzialmente utilizzando l'accantonamento relativo al Ministero della salute. Il Ministro dell'economia e delle finanze è autorizzato ad apportare, con propri decreti, le occorrenti variazioni di bilancio.

ART. 3.
(Modifica alla legge 29 luglio 1975, n. 405).

1. Al primo comma dell'articolo 1 della legge 29 luglio 1975, n. 405, sono aggiunte, in fine, le seguenti lettere:

"d-bis) l'informazione e l'assistenza riguardo ai problemi della sterilità e della infertilità umana, nonché alle tecniche di procreazione medicalmente assistita;

d-ter) l'informazione sulle procedure per l'adozione e l'affidamento familiare".

2. Dall'attuazione del presente articolo non devono derivare nuovi o maggiori oneri a carico della finanza pubblica.

CAPO II
ACCESSO ALLE TECNICHE

ART. 4.
(Accesso alle tecniche).

1. Il ricorso alle tecniche di procreazione medicalmente assistita è consentito solo quando sia accertata l'impossibilità di rimuovere altrimenti le cause impeditive della procreazione ed è comunque circoscritto ai casi di sterilità o di infertilità inspiegate documentate da atto medico nonché ai casi di sterilità o di infertilità da causa accertata e certificata da atto medico.

2. Le tecniche di procreazione medicalmente assistita sono applicate in base ai seguenti princípi:

a) gradualità, al fine di evitare il ricorso ad interventi aventi un grado di invasività tecnico e psicologico più gravoso per i destinatari, ispirandosi al principio della minore invasività;

b) consenso informato, da realizzare ai sensi dell'articolo 6.

3. È vietato il ricorso a tecniche di procreazione medicalmente assistita di tipo eterologo.

ART. 5.
(Requisiti soggettivi).

1. Fermo restando quanto stabilito dall'articolo 4, comma 1, possono accedere alle tecniche di procreazione medicalmente assistita coppie di maggiorenni di sesso diverso, coniugate o conviventi, in età potenzialmente fertile, entrambi viventi.

ART. 6.
(Consenso informato).

1. Per le finalità indicate dal comma 3, prima del ricorso ed in ogni fase di applicazione delle tecniche di procreazione medicalmente assistita il medico informa in maniera dettagliata i soggetti di cui all'articolo 5 sui metodi, sui problemi bioetici e sui possibili effetti collaterali sanitari e psicologici conseguenti all'applicazione delle tecniche stesse, sulle probabilità di successo e sui rischi dalle stesse derivanti, nonché sulle relative conseguenze giuridiche per la donna, per l'uomo e per il nascituro. Alla coppia deve essere prospettata la possibilità di ricorrere a procedure di adozione o di affidamento ai sensi della legge 4 maggio 1983, n. 184, e successive modificazioni, come alternativa alla procreazione medicalmente assistita. Le informazioni di cui al presente comma e quelle concernenti il grado di invasività delle tecniche nei confronti della donna e dell'uomo devono essere fornite per ciascuna delle tecniche applicate e in modo tale da garantire il formarsi di una volontà consapevole e consapevolmente espressa.

2. Alla coppia devono essere prospettati con chiarezza i costi economici dell'intera procedura qualora si tratti di strutture private autorizzate.

3. La volontà di entrambi i soggetti di accedere alle tecniche di procreazione medicalmente assistita è espressa per iscritto congiuntamente al medico responsabile della struttura, secondo modalità definite con decreto dei Ministri della giustizia e della salute, adottato ai sensi dell'articolo 17, comma 3, della legge 23 agosto 1988, n. 400, entro tre mesi dalla data di entrata in vigore della presente legge. Tra la manifestazione della volontà e l'applicazione della tecnica deve intercorrere un termine non inferiore a sette giorni. La volontà può essere revocata da ciascuno dei soggetti indicati dal presente comma fino al momento della fecondazione dell'ovulo.

4. Fatti salvi i requisiti previsti dalla presente legge, il medico responsabile della struttura può decidere di non procedere alla procreazione medicalmente assistita, esclusivamente per motivi di ordine medico-sanitario. In tale caso deve fornire alla coppia motivazione scritta di tale decisione.

5. Ai richiedenti, al momento di accedere alle tecniche di procreazione medicalmente assistita, devono essere esplicitate con chiarezza e mediante sottoscrizione le conseguenze giuridiche di cui all'articolo 8 e all'articolo 9 della presente legge.

ART. 7.
(Linee guida).

1. Il Ministro della salute, avvalendosi dell'Istituto superiore di sanità, e previo parere del Consiglio superiore di sanità, definisce, con proprio decreto, da emanare entro tre mesi dalla data di entrata in vigore della presente legge, linee guida contenenti l'indicazione delle procedure e delle tecniche di procreazione medicalmente assistita.

2. Le linee guida di cui al comma 1 sono vincolanti per tutte le strutture autorizzate.

3. Le linee guida sono aggiornate periodicamente, almeno ogni tre anni, in rapporto all'evoluzione tecnico-scientifica, con le medesime procedure di cui al comma 1.

CAPO III
DISPOSIZIONI CONCERNENTI LA TUTELA DEL NASCITURO

ART. 8.
(Stato giuridico del nato).

1. I nati a seguito dell'applicazione delle tecniche di procreazione medicalmente assistita hanno lo stato di figli legittimi o di figli riconosciuti della coppia che ha espresso la volontà di ricorrere alle tecniche medesime ai sensi dell'articolo 6.

ART. 9.
(Divieto del disconoscimento della paternità e dell'anonimato della madre).

1. Qualora si ricorra a tecniche di procreazione medicalmente assistita di tipo eterologo in violazione del divieto di cui all'articolo 4, comma 3, il coniuge o il convivente il cui consenso è ricavabile da atti concludenti non può esercitare l'azione di disconoscimento della paternità nei casi previsti dall'articolo 235, primo comma, numeri 1) e 2), del codice civile, né l'impugnazione di cui all'articolo 263 dello stesso codice.

2. La madre del nato a seguito dell'applicazione di tecniche di procreazione medicalmente assistita non può dichiarare la volontà di non essere nominata, ai sensi dell'articolo 30, comma 1, del regolamento di cui al decreto del Presidente della Repubblica 3 novembre 2000, n. 396.

3. In caso di applicazione di tecniche di tipo eterologo in violazione del divieto di cui all'articolo 4, comma 3, il donatore di gameti non acquisisce alcuna relazione giuridica parentale con il nato e non può far valere nei suoi confronti alcun diritto né essere titolare di obblighi.

CAPO IV
REGOLAMENTAZIONE DELLE STRUTTURE AUTORIZZATE ALL'APPLICAZIONE DELLE TECNICHE DI PROCREAZIONE MEDICALMENTE ASSISTITA

ART. 10.
(Strutture autorizzate).

1. Gli interventi di procreazione medicalmente assistita sono realizzati nelle strutture pubbliche e private autorizzate dalle regioni e iscritte al registro di cui all'articolo 11.

2. Le regioni e le province autonome di Trento e di Bolzano definiscono con proprio atto, entro tre mesi dalla data di entrata in vigore della presente legge:

a) i requisiti tecnico-scientifici e organizzativi delle strutture;

b) le caratteristiche del personale delle strutture;

c) i criteri per la determinazione della durata delle autorizzazioni e dei casi di revoca delle stesse;

d) i criteri per lo svolgimento dei controlli sul rispetto delle disposizioni della presente legge e sul permanere dei requisiti tecnico-scientifici e organizzativi delle strutture.

ART. 11.
(Registro).

1. È istituito, con decreto del Ministro della salute, presso l'Istituto superiore di sanità, il registro nazionale delle strutture autorizzate all'applicazione delle tecniche di procreazione medicalmente assistita, degli embrioni formati e dei nati a seguito dell'applicazione delle tecniche medesime.

2. L'iscrizione al registro di cui al comma 1 è obbligatoria.

3. L'Istituto superiore di sanità raccoglie e diffonde, in collaborazione con gli osservatori epidemiologici regionali, le informazioni necessarie al fine di consentire la trasparenza e la pubblicità delle tecniche di procreazione medicalmente assistita adottate e dei risultati conseguiti.

4. L'Istituto superiore di sanità raccoglie le istanze, le informazioni, i suggerimenti, le proposte delle società scientifiche e degli utenti riguardanti la procreazione medicalmente assistita.

5. Le strutture di cui al presente articolo sono tenute a fornire agli osservatori epidemiologici regionali e all'Istituto superiore di sanità i dati necessari per le finalità indicate dall'articolo 15 nonché ogni altra informazione necessaria allo svolgimento delle funzioni di controllo e di ispezione da parte delle autorità competenti.

6. All'onere derivante dall'attuazione del presente articolo, determinato nella misura massima di 154.937 euro a decorrere dall'anno 2004, si provvede mediante corrispondente riduzione dello stanziamento iscritto, ai fini del bilancio triennale 2004-2006, nell'ambito dell'unità previsionale di base di parte corrente "Fondo speciale" dello stato di previsione del Ministero dell'economia e delle finanze per l'anno 2004, allo scopo parzialmente utilizzando l'accantonamento relativo al Ministero della salute. Il Ministro dell'economia e delle finanze è autorizzato ad apportare, con propri decreti, le occorrenti variazioni di bilancio.

CAPO V
DIVIETI E SANZIONI

ART. 12.
(Divieti generali e sanzioni).

1. Chiunque a qualsiasi titolo utilizza a fini procreativi gameti di soggetti estranei alla coppia richiedente, in violazione di quanto previsto dall'articolo 4, comma 3, è punito con la sanzione amministrativa pecuniaria da 300.000 a 600.000 euro.

2. Chiunque a qualsiasi titolo, in violazione dell'articolo 5, applica tecniche di procreazione medicalmente assistita a coppie i cui componenti non siano entrambi viventi o uno dei cui componenti sia minorenne ovvero che siano composte da soggetti dello stesso sesso o non coniugati o non conviventi è punito con la sanzione amministrativa pecuniaria da 200.000 a 400.000 euro.

3. Per l'accertamento dei requisiti di cui al comma 2 il medico si avvale di una dichiarazione sottoscritta dai soggetti richiedenti. In caso di dichiarazioni mendaci si applica l'articolo 76, commi 1 e 2, del testo unico delle disposizioni legislative e regolamentari in materia di documentazione amministrativa, di cui al decreto del Presidente della Repubblica 28 dicembre 2000, n. 445.

4. Chiunque applica tecniche di procreazione medicalmente assistita senza avere raccolto il consenso secondo le modalità di cui all'articolo 6 è punito con la sanzione amministrativa pecuniaria da 5.000 a 50.000 euro.

5. Chiunque a qualsiasi titolo applica tecniche di procreazione medicalmente assistita in strutture diverse da quelle di cui all'articolo 10 è punito con la sanzione amministrativa pecuniaria da 100.000 a 300.000 euro.

6. Chiunque, in qualsiasi forma, realizza, organizza o pubblicizza la commercializzazione di gameti o di embrioni o la surrogazione di maternità è punito con la reclusione da tre mesi a due anni e con la multa da 600.000 a un milione di euro.

7. Chiunque realizza un processo volto ad ottenere un essere umano discendente da un'unica cellula di partenza, eventualmente identico, quanto al patrimonio genetico nucleare, ad un altro essere umano in vita o morto, è punito con la reclusione da dieci a venti anni e con la multa da 600.000 a un milione di euro. Il medico è punito, altresí, con l'interdizione perpetua dall'esercizio della professione.

8. Non sono punibili l'uomo o la donna ai quali sono applicate le tecniche nei casi di cui ai commi 1, 2, 4 e 5.

9. È disposta la sospensione da uno a tre anni dall'esercizio professionale nei confronti dell'esercente una professione sanitaria condannato per uno degli illeciti di cui al presente articolo, salvo quanto previsto dal comma 7.

10. L'autorizzazione concessa ai sensi dell'articolo 10 alla struttura al cui interno è eseguita una delle pratiche vietate ai sensi del presente articolo è sospesa per un

anno. Nell'ipotesi di più violazioni dei divieti di cui al presente articolo o di recidiva l'autorizzazione può essere revocata.

CAPO VI
MISURE DI TUTELA DELL'EMBRIONE

ART. 13.
(Sperimentazione sugli embrioni umani).

1. È vietata qualsiasi sperimentazione su ciascun embrione umano.

2. La ricerca clinica e sperimentale su ciascun embrione umano è consentita a condizione che si perseguano finalità esclusivamente terapeutiche e diagnostiche ad essa collegate volte alla tutela della salute e allo sviluppo dell'embrione stesso, e qualora non siano disponibili metodologie alternative.

3. Sono, comunque, vietati:

a) la produzione di embrioni umani a fini di ricerca o di sperimentazione o comunque a fini diversi da quello previsto dalla presente legge;

b) ogni forma di selezione a scopo eugenetico degli embrioni e dei gameti ovvero interventi che, attraverso tecniche di selezione, di manipolazione o comunque tramite procedimenti artificiali, siano diretti ad alterare il patrimonio genetico dell'embrione o del gamete ovvero a predeterminarne caratteristiche genetiche, ad eccezione degli interventi aventi finalità diagnostiche e terapeutiche, di cui al comma 2 del presente articolo;

c) interventi di clonazione mediante trasferimento di nucleo o di scissione precoce dell'embrione o di ectogenesi sia a fini procreativi sia di ricerca;

d) la fecondazione di un gamete umano con un gamete di specie diversa e la produzione di ibridi o di chimere.

4. La violazione dei divieti di cui al comma 1 è punita con la reclusione da due a sei anni e con la multa da 50.000 a 150.000 euro. In caso di violazione di uno dei divieti di cui al comma 3 la pena è aumentata. Le circostanze attenuanti concorrenti con le circostanze aggravanti previste dal comma 3 non possono essere ritenute equivalenti o prevalenti rispetto a queste.

5. È disposta la sospensione da uno a tre anni dall'esercizio professionale nei confronti dell'esercente una professione sanitaria condannato per uno degli illeciti di cui al presente articolo.

ART. 14.
(Limiti all'applicazione delle tecniche sugli embrioni).

1. È vietata la crioconservazione e la soppressione di embrioni, fermo restando quanto previsto dalla legge 22 maggio 1978, n. 194.

2. Le tecniche di produzione degli embrioni, tenuto conto dell'evoluzione tecnico-scientifica e di quanto previsto dall'articolo 7, comma 3, non devono creare un numero di embrioni superiore a quello strettamente necessario ad un unico e contemporaneo impianto, comunque non superiore a tre.

3. Qualora il trasferimento nell'utero degli embrioni non risulti possibile per grave e documentata causa di forza maggiore relativa allo stato di salute della donna non prevedibile al momento della fecondazione è consentita la crioconservazione degli embrioni stessi fino alla data del trasferimento, da realizzare non appena possibile.

4. Ai fini della presente legge sulla procreazione medicalmente assistita è vietata la riduzione embrionaria di gravidanze plurime, salvo nei casi previsti dalla legge 22 maggio 1978, n. 194.

5. I soggetti di cui all'articolo 5 sono informati sul numero e, su loro richiesta, sullo stato di salute degli embrioni prodotti e da trasferire nell'utero.

6. La violazione di uno dei divieti e degli obblighi di cui ai commi precedenti è punita con la reclusione fino a tre anni e con la multa da 50.000 a 150.000 euro.

7. È disposta la sospensione fino ad un anno dall'esercizio professionale nei confronti dell'esercente una professione sanitaria condannato per uno dei reati di cui al presente articolo.

8. È consentita la crioconservazione dei gameti maschile e femminile, previo consenso informato e scritto.

9. La violazione delle disposizioni di cui al comma 8 è punita con la sanzione amministrativa pecuniaria da 5.000 a 50.000 euro.

CAPO VII
DISPOSIZIONI FINALI E TRANSITORIE

ART. 15.
(Relazione al Parlamento).

1. L'Istituto superiore di sanità predispone, entro il 28 febbraio di ciascun anno, una relazione annuale per il Ministro della salute in base ai dati raccolti ai sensi dell'articolo 11, comma 5, sull'attività delle strutture autorizzate, con particolare

riferimento alla valutazione epidemiologica delle tecniche e degli interventi effettuati.

2. Il Ministro della salute, sulla base dei dati indicati al comma 1, presenta entro il 30 giugno di ogni anno una relazione al Parlamento sull'attuazione della presente legge.

ART. 16.
(Obiezione di coscienza).

1. Il personale sanitario ed esercente le attività sanitarie ausiliarie non è tenuto a prendere parte alle procedure per l'applicazione delle tecniche di procreazione medicalmente assistita disciplinate dalla presente legge quando sollevi obiezione di coscienza con preventiva dichiarazione. La dichiarazione dell'obiettore deve essere comunicata entro tre mesi dalla data di entrata in vigore della presente legge al direttore dell'azienda unità sanitaria locale o dell'azienda ospedaliera, nel caso di personale dipendente, al direttore sanitario, nel caso di personale dipendente da strutture private autorizzate o accreditate.

2. L'obiezione può essere sempre revocata o venire proposta anche al di fuori dei termini di cui al comma 1, ma in tale caso la dichiarazione produce effetto dopo un mese dalla sua presentazione agli organismi di cui al comma 1.

3. L'obiezione di coscienza esonera il personale sanitario ed esercente le attività sanitarie ausiliarie dal compimento delle procedure e delle attività specificatamente e necessariamente dirette a determinare l'intervento di procreazione medicalmente assistita e non dall'assistenza antecedente e conseguente l'intervento.

ART. 17.
(Disposizioni transitorie).

1. Le strutture e i centri iscritti nell'elenco predisposto presso l'Istituto superiore di sanità ai sensi dell'ordinanza del Ministro della sanità del 5 marzo 1997, pubblicata nella *Gazzetta Ufficiale* n. 55 del 7 marzo 1997, sono autorizzati ad applicare le tecniche di procreazione medicalmente assistita, nel rispetto delle disposizioni della presente legge, fino al nono mese successivo alla data di entrata in vigore della presente legge.

2. Entro trenta giorni dalla data di entrata in vigore della presente legge, le strutture e i centri di cui al comma 1 trasmettono al Ministero della salute un elenco contenente l'indicazione numerica degli embrioni prodotti a seguito dell'applicazione di tecniche di procreazione medicalmente assistita nel periodo precedente la data di entrata in vigore della presente legge, nonché, nel rispetto delle vigenti disposizioni sulla tutela della riservatezza dei dati personali, l'indicazione nominativa di coloro che hanno fatto ricorso alle tecniche medesime a seguito delle quali sono stati formati gli embrioni. La violazione della disposizione del presente comma è punita con la sanzione amministrativa pecuniaria da 25.000 a 50.000 euro.

3. Entro tre mesi dalla data di entrata in vigore della presente legge il Ministro della salute, avvalendosi dell'Istituto superiore di sanità, definisce, con proprio decreto, le modalità e i termini di conservazione degli embrioni di cui al comma 2.

ART. 18.
(Fondo per le tecniche di procreazione medicalmente assistita).

1. Al fine di favorire l'accesso alle tecniche di procreazione medicalmente assistita da parte dei soggetti di cui all'articolo 5, presso il Ministero della salute è istituito il Fondo per le tecniche di procreazione medicalmente assistita. Il Fondo è ripartito tra le regioni e le province autonome di Trento e di Bolzano sulla base di criteri determinati con decreto del Ministro della salute, da emanare entro sessanta giorni dalla data di entrata in vigore della presente legge, sentita la Conferenza permanente per i rapporti tra lo Stato, le regioni e le province autonome di Trento e di Bolzano.

2. Per la dotazione del Fondo di cui al comma 1 è autorizzata la spesa di 6,8 milioni di euro a decorrere dall'anno 2004.

3. All'onere derivante dall'attuazione del presente articolo si provvede mediante corrispondente riduzione dello stanziamento iscritto, ai fini del bilancio triennale 2004-2006, nell'ambito dell'unità previsionale di base di parte corrente "Fondo speciale" dello stato di previsione del Ministero dell'economia e delle finanze per l'anno 2004, allo scopo parzialmente utilizzando l'accantonamento relativo al Ministero medesimo. Il Ministro dell'economia e delle finanze è autorizzato ad apportare, con propri decreti, le occorrenti variazioni di bilancio.

APPENDIX B

Parere del Comitato Nazionale per la Bioetica su ricerche utilizzanti embrioni umani e cellule staminali

1. In relazione all'avvio del VI Programma Quadro di Ricerca dell'U.E. il Ministro Moratti ha richiesto al CNB se sia eticamente lecito:
a) svolgere sul territorio nazionale ricerche utilizzanti embrioni umani anche soprannumerari che ne determinino la distruzione;
b) svolgere ricerche utilizzanti cellule staminali derivate da embrioni umani prodotte in data successiva all'avvio del VI Programma Quadro di Ricerca dell'Unione Europea;
c) produrre cellule staminali derivate da embrioni umani anche soprannumerari.

2. Considerando:
a) che gli embrioni umani sono vite umane a pieno titolo;
b) che esiste quindi il dovere morale di sempre rispettarli e sempre proteggerli nel loro diritto alla vita, indipendentemente dalle modalità con cui siano stati procreati e indipendentemente dal fatto che alcuni di essi possano essere qualificati - con una espressione discutibile, perché priva di valenza ontologica - soprannumerari;
c) che (secondo il dettato della c.d. Convenzione di Oviedo) la sperimentazione a loro carico è giustificata unicamente se praticata nel loro specifico interesse e non possa essere giustificata dal pur rilevante interesse generale della società e della scienza e che quindi non possa in alcun modo sostanziarsi nella loro distruzione;
d) che la Carta dei diritti fondamentali dell'Unione europea, proclamata a Nizza, riconosce la dignità di tutti gli esseri umani e l'esistenza di diritti fondamentali quali il diritto all'integrità fisica e psichica di ogni individuo nei confronti delle applicazioni della medicina e della biologia;
e) che l'eventuale finanziamento pubblico alla ricerca sugli embrioni non può che rafforzare e avallare ingiustificatamente l'erronea opinione che gli embrioni siano un mero insieme di cellule, prive di valore intrinseco, e quindi conseguentemente l'idea dell'irrilevanza bioetica della vita umana nella fase embrionale;
f) che la limitazione della sperimentazione agli embrioni soprannumerari, oltre a non avere motivazione logica, ma solo occasionale e pragmatica, favorirebbe surrettiziamente la pratica di produzione di embrioni in vitro a soli scopi di ricerca, indipendentemente cioè da specifiche finalità inerenti alla fecondazione assistita e in violazione quindi di consolidati principi bioetici;
g) che il prelievo di cellule staminali umane da embrioni, comportando la distruzione di questi ultimi, deve essere a pari titolo stigmatizzato, anche per l'ulteriore effetto eticamente inaccettabile di non orientare la ricerca verso la sempre più promettente ed eticamente impeccabile utilizzazione di cellule staminali prelevate da cordone ombelicale o da feti spontaneamente abortiti o di cellule staminali "adulte";
h) che le precedenti considerazioni trovano conferma nelle valutazioni espresse da alcuni membri del CNB, segnatamente nel documento Identità e statuto dell'embrione umano (del 22.6.1996) e nel Parere del CNB sull'impiego terapeutico delle cellule staminali (del 27.10.2000).

3. Di conseguenza, su ciascuno dei tre quesiti, il CNB, nella seduta plenaria dell'11.04.2003, dopo votazione per appello nominale, ha espresso parere negativo.

4. Hanno espresso voto favorevole a questo testo i seguenti membri del C.N.B.: Mario Fiori, Adriana Loreti Beghè, Salvatore Amato, Luciana Rita Angeletti, Sergio Belardinelli, Paola Binetti, Adriano Bompiani, Carlo Casini, Mario Condorelli, Bruno Dallapiccola, Maria Luisa Di Pietro, Renzo Dionigi, Luciano Eusebi, Giovanni Federspil, Silvio Ferrari, Enrico Garaci, Gianfranco Iadecola, Aldo Isidori, Corrado Manni, Luca Marini, Benedetto Marino, Vittorio Mathieu, Laura Palazzani, Elio Sgreccia, Bruno Silvestrini, Giancarlo Umani Ronchi.

Allegato A
Mozione presentata dal Prof. Demetrio Neri nella seduta plenaria dell'11 aprile 2003

1. In relazione all'avvio del VI Programma Quadro di Ricerca dell'U.E. il Ministro Moratti ha richiesto al CNB se sia eticamente lecito:
d) svolgere sul territorio nazionale ricerche utilizzanti embrioni umani anche soprannumerari che ne determinino la distruzione;
e) svolgere ricerche utilizzanti cellule staminali derivate da embrioni umani prodotte in data successiva all'avvio del VI Programma Quadro di Ricerca dell'Unione Europea;
f) produrre cellule staminali derivate da embrioni umani anche soprannumerari;

Risposte alle domande

1) La prima domanda evoca la più generale e, come è noto, controversa questione dello status morale dell'embrione umano, questione che - è appena il caso di rammentarlo - è indipendente dalla localizzazione territoriale come anche dalla condizione in cui si trova l'embrione. Prescindendo qui da questa più generale questione, i sottoscritti membri del CNB si riconoscono e fanno propria la posizione espressa nel documento del CNB del 27 ottobre 2000 (Parere sull'impiego terapeutico delle cellule staminali), ai punti 22 (primo e secondo capoverso) e al punto 31 (primo capoverso), che qui sinteticamente si riassume. Il ragionamento sviluppato da una parte del Comitato si è confrontato col fatto dell'esistenza, anche in Italia, di un notevole numero di embrioni formati nel corso di procedure di fecondazione in vitro, ma, per varie ragioni, non utilizzati per il trasferimento in utero e crioconservati presso vari centri. Sulla base di un attento bilanciamento dei valori in gioco - che tiene anche conto del fatto che gran parte di tali embrioni sarebbero comunque destinati ad essere distrutti - , questa parte del Comitato ha ritenuto che "la rimozione e la coltura in laboratorio di cellule staminali da un embrione che non può essere impiantato non significhino una mancanza di rispetto nei suoi confronti, ma possano considerarsi se mai un contributo, da parte della coppia donatrice, alla ricerca di terapie per malattie difficilmente curabili e spesso inguaribili, che deriva da un atto di solidarietà"; e ha quindi concluso di ritenere "eticamente lecita la derivazione di cellule staminali a fini terapeutici dagli embrioni non più in grado di essere impiantati".
2) Circa la seconda domanda (la liceità etica delle ricerche utilizzanti cellule staminali derivate da embrioni umani, prodotte in data successiva all'avvio del VI Programma quadro), i sottoscritti membri del CNB non sono in grado di formulare un argomento morale (né di individuare un principio o valore morale) capace di sostenere la non liceità (o la liceità) morale di una condotta unicamente in base alla data in cui viene messa in atto: non può essere moralmente illecito il 1° gennaio 2002 quel che era lecito il 31 dicembre 2001 (anche se, ovviamente, può diventare giuridicamente illecito).La plausibilità di segnare il limite del 31 dicembre 2001 fa riferimento, con tutta evidenza, a ragioni di natura politica e il limite non può che essere il risultato di una decisione scaturente da procedure di "diplomazia bioetica". In questa luce - e affinché tali procedure non risultino in compromessi di basso profilo - possono tornare utili le seguenti considerazioni.

a) La scelta - già operata dal nostro Governo, stando alla lettera del Ministro Moratti - di ritenere ammissibili le ricerche utilizzanti cellule staminali prodotte in data antecedente all'avvio del VI Programma Quadro evidenzia una posizione non pregiudizialmente contraria alla ricerca sulle cellule staminali embrionali. Tuttavia, affinché questa posizione possa produrre effetti pratici, è necessario soddisfare le seguenti condizioni: 1) le linee cellulari esistenti prima della data prescelta siano quantitativamente sufficienti alle esigenze della ricerca; 2) tali linee cellulari siano ben caratterizzate e accessibili ai ricercatori che ne facciano richiesta, con le procedure più idonee ad evitare discriminazioni nell'accesso. Il dibattito successivo alla decisione (analoga a quella che si vorrebbe perseguire a livello comunitario) assunta da George Bush il 9 agosto 2001 ha mostrato quanto sia difficile assicurare fattualmente il soddisfacimento di quelle due condizioni. Delle più di 60 (sembra circa 70-72) linee cellulari scrutinate dall'NIH e depositate presso compagnie private o università, solo poche sono così ben caratterizzate da poter essere utilizzabili per successive ricerche e su queste poche (non più di 10-12) gravano problemi di natura legale che le rendono difficilmente accessibili. Ad esempio, in Europa l'NIH ha registrato 6 linee cellulari possedute dal Karolinska Institute di Stoccolma (nessuna delle quali attualmente disponibile) e ben 19 possedute dall'Università di Goteborg, delle quali solo 3 sono disponibili. In tale situazione, segnare una data-limite significa introdurre un vincolo che renderà praticamente impossibile il prosieguo delle ricerche.
b) Qualora le sopra esposte difficoltà tecniche potessero essere superate, si prospetta un ulteriore problema. Segnare una data-limite significa di fatto conferire alle istituzioni pubbliche o private in possesso di linee cellulari embrionali un monopolio nel settore, con tutte le conseguenze (sia in relazione alla protezione della proprietà intellettuale, sia in termini di equità nell'accesso, in futuro, ai trattamenti ottenuti con queste ricerche) che possono essere facilmente immaginate. Ad esempio, la WICells, una società creata dall'Università del Wisconsin per la distribuzione delle linee cellulari prodotte da J. Thomson, sottopone l'accesso gratuito alle linee cellulari alla condizione che vengano usate solo ed esclusivamente per scopi di ricerca e riservandosi ogni diritto sullo sfruttamento commerciale dei risultati. Sulla base di queste considerazioni fattuali, si suggerisce dunque che non venga posto il limite del 31 dicembre 2001, consentendo quindi ai ricercatori di poter utilizzare i fondi europei per ricerche su linee cellulari prodotte anche successivamente all'avvio del VI programma quadro.
3) Quanto alla terza domanda (la produzione di cellule staminali da embrioni soprannumerari), si suggerisce di consentire ai ricercatori europei, che lo richiedano, di operare tale produzione, sia per le ragioni indicate in precedenza circa l'opportunità di evitare posizioni di monopolio, sia per le ragioni scientifiche qui di seguito sintetizzate. La separazione tra ricerca tendente a derivare le cellule staminali embrionali e ricerca sulle cellule staminali già derivate è artificiosa e insostenibile da un punto di vista scientifico. Le proprietà delle linee cellulari dipendono anche dalle condizioni e dai metodi usati per derivarle e le conoscenze che si possono acquisire dal processo di derivazione e mantenimento delle linee cellulari in stato di indifferenziazione sono essenziali per lo sviluppo di ulteriori ricerche. Ciò significa che impedire ai ricercatori europei di utilizzare i fondi del VI programma quadro per operare direttamente la derivazione delle linee cellulari da embrioni soprannumerari equivale a privarli della possibilità di acquisire le conoscenze di base che possono rivelarsi essenziali allo sviluppo della ricerca in direzione terapeutica e, quindi, implica un impiego non ottimale dei fondi europei.
4. Hanno espresso voto favorevole a questo testo i seguenti membri del C.N.B.: Demetrio Neri, Mauro Barni, Luisella Battaglia, Cinzia Caporale, Lorenzo d'Avack, Carlo Flamigni, Renata Gaddini, Alberto Piazza, Michele Schiavone, Annalisa Silvestro.

Allegato B

Mozione presentata dal Prof. Francesco Donato Busnelli nella seduta plenaria dell'11 aprile 2003

1. In relazione all'avvio del VI Programma Quadro di Ricerca dell'U.E. il Ministro Moratti ha richiesto al CNB se sia eticamente lecito:
g) svolgere sul territorio nazionale ricerche utilizzanti embrioni umani anche soprannumerari che ne determinino la distruzione;
h) svolgere ricerche utilizzanti cellule staminali derivate da embrioni umani prodotte in data successiva all'avvio del VI Programma Quadro di Ricerca dell'Unione Europea;i) produrre cellule staminali derivate da embrioni umani anche soprannumerari;
2. Ricordando che in sede di redazione del documento su Identità e statuto dell'embrione umano (del 27 giugno 1996) questo Comitato "è pervenuto unanimemente a riconoscere il dovere morale di trattare l'embrione umano, sin dalla fecondazione, secondo i criteri di rispetto e tutela che si devono adottare nei confronti degli individui umani a cui si attribuisce comunemente la caratteristica di persone, e ciò a prescindere dal fatto che all'embrione venga attribuita sin dall'inizio con certezza la caratteristica di persona nel senso tecnicamente filosofico";
3. Ricordando altresì che in sede di redazione del Parere su La clonazione come problema bioetico (del 21 marzo 1997) questo Comitato ha avuto modo di ribadire, nell'esprimere una "condanna etica" della clonazione di individui umani, "il diritto di ciascun essere umano alla propria dignità";
4. Considerando che la "Carta di Nizza" ("Progetto di Carta dei diritti fondamentali dell'Unione europea" del 28 settembre 2000) esordisce affermando nei termini più ampi e indifferenziati che "la dignità umana è inviolabile" (art. 1), specificando poi, sempre in termini generali e categorici, che "ciascuno (everyone) ha diritto alla vita" (art. 2, comma 1);
5. Prendendo atto del divieto di "costituzione di embrioni umani a fini di ricerca" stabilito dalla "Convenzione sui diritti dell'uomo e della biomedicina" (c.d. Convenzione di Oviedo del 4 aprile 1997), ferma restando comunque l'imprescindibile esigenza di assicurare "una protezione adeguata all'embrione" qualora una legge nazionale consenta "la ricerca su embrioni in vitro formati con fini riproduttivi" (art. 18);
6. Considerando, conclusivamente, che i documenti di natura bioetica e di natura normativa (o, comunque, giuridica) fin qui menzionati offrono un quadro d'insieme relativamente coerente e non possono essere ragionevolmente disattesi in sede di risposta ai quesiti de quibus,

il Comitato Nazionale per la Bioetica,
con l'intento di superare per quanto possibile le divergenze radicali espresse al suo interno al fine di corrispondere alle aspettative del Ministro richiedente, formula le seguenti risposte:
quanto al quesito sub a): si propende, allo stato, per la negazione di un principio generale di liceità etica della ricerca utilizzante embrioni umani anche soprannumerari che ne determini la distruzione. La possibilità di riscontrare un'area eccezionale di liceità etica limitata a ricerche su embrioni soprannumerari dipenderà dalla verifica di una serie di fattori non ancora compiutamente definiti - l'accertata inadeguatezza dell'"obiettivo ottimale di 'riprogrammare' cellule mature": una documentata concretezza della prospettiva di applicazioni terapeutiche innovative di straordinaria importanza da valutarsi caso per caso; l'individuazione di criteri di accertamento della ragionevole impossibilità di impianto dell'embrione - e dalla conseguente introduzione di una legge che stabilisca preventivamente presupposti e condizioni tali da giustificarne la legittimità costituzionale alla stregue dell'imprescindibile bilanciamento tra il valore individuale dell'embrione, anche soprannumerario, e il valore sociale della tutela della salute come "interesse della collettività" (art. 32 Cost.);

quanto al quesito sub b): si propende per una risposta negativa, in quanto la delimitazione temporale prospettata appare allo stesso tempo ingiustificatamente restrittiva e arbitrariamente generalizzante. Ingiustificata risulta la discriminazione tra condotte omogenee sotto il profilo di un giudizio etico; eticamente illecita, per le ragioni esposte in risposta al precedente quesito, sarebbe una "liberalizzazione" delle ricerche afferenti a un periodo temporale definito, secondo una logica "politica" arieggiante alla tecnica del condono;

quanto al quesito sub c): il verbo "produrre" getta una luce di ambiguità sul quesito. Se, verosimilmente, si vuol fare riferimento a una "produzione" che si distingue dalla "ricerca" - alla quale si riferiscono in modo generale i precedenti quesiti - la risposta sarebbe negativa, specie se la produzione, non ulteriormente definita, dovesse estendersi a iniziative onerose, andando così a urtare contro il divieto di profitto sancito dall'art. 21 della Convenzione di Oviedo, secondo cui, com'è noto, "il corpo umano e le sue parti non possono essere, in quanto tali, fonte di profitto". Se, invece, la "produzione" evocata dal quesito dovesse, meno verosimilmente, essere intesa come mera modalità della ricerca (in senso generale), allora il quesito diverrebbe superfluo, dovendosi considerare ricompreso nei quesiti precedenti.

7. Hanno espresso voto favorevole a questo testo i seguenti membri del C.N.B.: Francesco Donato Busnelli, Isabella Maria Coghi, Luigi De Carli, Simonetta Matone, Stefano Racheli.

APPENDIX C

"Financing for research on embryonic stem cells: The situation in Italy and its origins"

3rd Italian National Congress of the Group of Italian Researchers on Embryonic Stem Cells (IES Group)

Rome, 1st July 2008

Report by E. Serena, E. Cimetta and M. Zagallo (University of Padua)

Published on: "Notizie di Politeia. Rivista di Etica e Scelte Pubbliche"
Vol. 91/Anno XXIV, 2008, pp. 110-113

"*Opened up to this vast and most excellent science, of which my work is mere the beginning, ways and means by which other minds more acute than mine will explore in remotest corners*"

These are the words of Italian astronomer and physicist Galileo Galilei, who was sentenced to indefinite prison in San Macuto Palace, on which site the Group of Italian Researchers on Embryonic Stem Cells (IES Group) organized its 3rd National Congress on "Research on Embryonic Stem Cells" (1 July 2008). These scientists strongly believe in the potential of human embryonic stem cells (hESCs), which are already the main focus of their research. A parallel thus emerges, as innovative and revolutionary scientific issues are discussed once again in this place.

In the magnificent structure of the Refectory Room of the Chamber of Deputies, IES scientists presented their research and discussed ethical aspects of its implications.
The elegant Refectory Room of the Deputy Chamber at San Macuto Palace. In this site Galileo Galilei (1564-1642) was tried by the Inquisition for his advocacy of
Copernican theory, which held that the Earth revolves around the sun. Since 1974 the Palace is an official site of the Italian Chamber of Deputies.

The main aim of the Congress was to highlight the need for an immediate and tangible opening to research on hESC in Italy. In fact, even while it is legally allowed to work on established hESC lines, it is prohibited to derive new lines from embryos; and even

more limiting is the lack of funding from the Government. Consequently, a first and most crucial step is to improve the dialogue between science and both society and politics, in order to make known the potential, in terms of therapeutic outcomes, of hESC research. Opening speakers of the congress were Elena Cattaneo, pioneer of hESC research in Italy, and Andrew Smith, project manager from ESTOOLS. Cattaneo's speech highlighted the main aims of the meeting, to identify the role of the IES group and to inform the audience on the research carried by its members. Since 2002, the interest of the international scientific community in hESCs has strongly increased, shown by the augmented financial investment and by the number of participants at specific conferences. In this scenario, IES scientists must create a solid network in order to facilitate financial and practical aspects of their research (training of personnel, development of protocols, reporting of results, etc) and to sensitize the public towards the relevant ethical aspects of their research. In underlining the tremendous potential of hESCs as tools for studying human physiology, disease and new therapies development, she stated that IES researchers do not exclusively work on embryonic but also believe in the potential of adult stem cells. Prof. Cattaneo focused the attention on the nr.1 scientific discovery of 2007 (according to The Times): induced pluripotent stem cells[1] now receiving much interest by some Italian laboratories. This discovery, overcoming the ethical issues involving the use of embryos, would have never been possible without preceding research on hESCs.

Last but not least she expounded the chronic problem of fund-raising in Italy and Europe, and of the long and laborious iter leading to a project approval by the EU.

Taking a look outside academia, Andrew Smith described how the ESTOOLS consortium endorses lab research but at the same time tries to make it accessible to the public. Rescaled on an European level, with their 21 labs in 10 countries, the main targets of ESTOOLS resemble those listed by Cattaneo with regard to IES. In particular, ESTOOLS focuses on four different areas of activity: research and technology development (integration of funding and projects), dissemination of results (website, international public symposia), training (fellowships, lab staff exchanges, videos of lab techniques, and media training) and outreach (newsletters, trans-Europe telescope, ethic workshops). Most importantly, ESTOOLS promotes the development of integrated projects involving both academics and industrial partners: successful scientists should thus possess the skills of a good researcher and a good manager, be able to communicate with the public, be open minded and flexible.

After the welcome note of Carlo Flamigni, chairman of the session, Marisa Jaconi and Tiziano Barberi, top Italian scientists working on hESC outside Italy presented the

results of their research on cardiac and skeletal muscle, respectively. Jaconi (University of Geneve, Switzerland), in particular, is working to develop animal

free protocols and GMP-grade cell lines and to understand and control hES differentiation process for possible clinical applications. She also pointed put the pros and cons of iPS: no embryos are involved and no immune response is elicited but, on the contrary, a strong teratogenic effect would be possible due to their derivation through viral infection. Barberi (Beckman Research Institute of the City of Hope, Duarte, California) works on mesodermal progenitors isolation from pluripotent stem cells; he reported his latest achievements that follow his previous results on skeletal muscle3. The major efforts of his lab are directed on the development of more solid and reproducible protocols in order to take a step further towards clinical applications in general.

E.1 Research activities inside the IES

Research on hESC is applied in three different directions: studying embryonic development, controlling the differentiation in specific lineages, and clinical applications (e.g. drug screening and therapy development). All these research themes are equally important and tightly related, each of them needing the knowledge derived from the others to proceed; the IES group has competencies to over them all.

IES new members

During the last year, four more research labs (Elvassore, Bianco, Gambari, Mantovani) joined the IES ranks and thus had the chance to present their projects to the audience and to the other members. Nicola Elvassore, Chemical Engineer from Padova University, supported the idea of coupling engineering and biotechnology skills to develop technologies serving biology. His laboratory already established hESC culture, and is currently developing devices (e.g. substrates, micro and macro-scaled bioreactors, ..) for the control of the culture microenvironment and thus of the cellular differentiation process. Bianco and Gambari's labs are studying two specific genetic diseases: McCune-Albright syndrome and β-Thalassemia respectively. In particular, Riminucci (Bianco's lab) explained that such syndrome is caused by a mutation occurring in embryonic cells and Gambari underlined the importance of studying the activation of fetal γ-globin in thalassemic patients. For these reasons, they now both aim at using hESC for developing therapeutic strategies as they represent the closest in vitro human model. Mantovani, studying the transcription

factors regulating stemness, recognizes how this research would greatly benefit from the use of hESC.

IES senior members

Cardiac differentiation is the leading theme for research by Condorelli and Cerbai. The first aims at reproducing the protocols for cardiomyocytes derivation from hESCs developed by Keller[2] and showed interest in adopting iPS cells cultures. The second is studying cardiac functionality and physiology in diseased heart in vitro, in vivo and in silico, using hESC as a cellular model. The teams led by Brevini and Oliviero focus on deriving new cell lines not originating from human embryos and thus overcoming the possible ethical issues. Brevini's group works on human parthenogenetic stem cell, which possess all of the main features of hESC but their potential for clinical application has yet to be tested. Riding the clamor of iPS cells, Oliviero succeeded in inducing iPS and obtaining a 100-fold increased yield by adding myc transcription factor to the original Yamanaka recipe (Takahashi et al., Cell, 2007). Another important application of hESC is gene therapy; the Sangiuolo group works on this application with regards to spinal muscular atrophy and cystic fibrosis. Elena Cattaneo and her prominent team working on neural differentiation have derived a population of neural crest precursors from hESC and are currently testing its differentiation potential, in vitro and in vivo, with a view to possible clinical application. Italian scientists here gathered, unequivocally demonstrated how their research hold an enormous social relevance and all expressed hope in the future possibilities deriving from their integration within the IES and in the common efforts in remodeling the Italian scenario.

E.2 Round table discussion: financing research on hESC

Why use embryos and why finance hESC-based research? These two major issues emerged during the meeting and the final discussion. In the near future there may no longer be the need for to discuss the rightness of using embryos for progressing science and research, said Neri, followed by Corbellini who underlined that it would be desirable that prejudices should not hinder successful research. In addition, besides the lack of funding for research in general and on hESCs in particular, it is clear that Italy needs deeply to reform its financing mechanisms. Even Senator Ignazio Marino, Deputy at the Italian Parliament and invited participant to the meeting, expressed his opinion firmly in these terms. It is unfortunately clear that government does not recognize science as crucial for the future of the country's economy. Mindful of his past experience as a scientist in the US, Senator

Marino affirmed how Italy should not pose any ideological barrier to scientific research and, most important, should adopt absolutely new project-evaluation criteria. To date, only 10% of the research investments are allocated through peer review; the remaining 90% through unclear top-down methods. A reform of the entire system must thus be done, basing the entire distribution of funding on the mechanism of peer review in order to enhance meritocracy and transparency in the selection of projects. Barberi, active participant in the discussion, finally stated that there's no need for further speculation on the validity of peer review as it must be the sole and only method for a meritocratic evaluation of research projects. With a favourable legislative and financial environment supporting all areas of human embryonic stem cell research, the future promises to be very exciting for scientists interested in this field, and crucial for progress in clinical applications.

APPENDIX D

Extraction of RNA from hES – derived embryoid bodies

Purpose

This protocol details the extraction of RNA from hES – derived embryoid bodies.
The RNA is used for the gene expression analysis.

Precautions for Preventing RNase Contamination:

RNases can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper technique. Because RNase activity is difficult to inhibit, it is essential to prevent its introduction. The following guidelines should be observed when working with RNA.
Always wear disposable gloves. Skin often contains bacteria and molds that can contaminate an RNA preparation and be a source of RNases. Practice good microbiological technique to prevent microbial contamination.
Use sterile, disposable plasticware and automatic pipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipment.
In the presence of TRIZOL Reagent, RNA is protected from RNase contamination. Downstream sample handling requires that nondisposable glassware or plasticware be RNase-free. Glass items can be baked at 150°C for 4 hours, and plastic items can be soaked for 10 minutes in 0.5 M NaOH, rinsed horoughly with water, and autoclaved.

Equipment

- Sterile biosafety cabinet
- Fume hood
- Microcentrifuge – perform all centrifugation at 20-25$_{o}$C.
- Pipet-Aid
- p1000 Pipetman
- Liquid waste disposal systems for aspiration, consisting of vacuum source and side-arm flask trap
- NanoDrop

Materials

- 5ml sterile serological pipets
- 1.5ml microcentrifuge tubes
- P1000 pipet tips
- Baked Pasteur pipets
- RNeasy Mini Kit (Qiagen, 74104/74106)
- 20-guage needle and syringe
- PBS (pH 7.2)
- Ethanol
-

Isolation of RNA

Start material for isolation:

- Cells Grown in Suspension
- 1 well of a 6-well ultra low adhesive plate (between 3-4x10^6 cells)

Pellet cells by centrifugation (200g, 5 minutes). Lyse cells in Trizol by repetitive pipetting. Use 1 ml of the reagent per 5-10 x 10^6 cells.

Phase separation

Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of Trizol. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12,000g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol used for homogenization.

RNA purification

Add 700µl ethanol 70% and transfer the solution into a RNeasy Mini Kit column.
Centrifuge for 1 minute at 2°C at > 8000 g (during this phase, total RNA will bind to the column).

Discard the flow through and reuse the collection tube.
Add 700µl of Buffer RW1 Qiagen (mix of ethanol and Guanidinium thiocyanate) to the spin column and centrifuge for 1 minute at 8000 g at 2°C. Discard the flow through and reuse the collection tube.
Add 500µl of Buffer RPE Qiagen to the spin column and centrifuge for 1 minute at 8000 g to wash the column. Discard the flow through and reuse the collection tube.
Add 500µl of Buffer RPE to the spin column and centrifuge for 2 minutes at 8000 g to wash and dry the column.
Place the spin column into a clean 1.5ml collection tube RNasi/DNasi-free LoBind and spin for 1 minute at full speed to completely dry the column.
Add 30µl RNase free water to the column. Wait 5 minutes and centrifuge for 1 minute at 8500 g to elute the RNA.
Add an additional 15µl of RNase free water to the column. Wait 5 minutes and centrifuge for 1 minute at 8500 g to elute the RNA.

Quality control check of extracted RNA

Measure the concentration of the sample using the NanoDrop.
Store the isolated RNA at -80°C until sample is used for gene expression analysis.
The 260/280 ratio (measure of protein presence) is >1.9
The 230/260 ration (measure of organic contamination) is >1.85

APPENDIX E

Microscale *in vitro* model for screening pathological conditions on human embryonic stem cells-derived cardiomyocytes

to be submitted to Stem Cells

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Introduction

The heart is one of the least regenerative organs in the body[1] and any major insult, due to ischemia, viral infection or other pathologies, causing significant heart cell loss can result in the progression to irreversible heart failure. For instance, congestive heart failure is already the most common cause of hospitalization in US citizens over 65[2] and is a growing epidemic. At the moment, the two major and effective therapies are the administration of β-blockers and cardiac transplantation. Due to the relevant side effects of both these strategies, the search for new therapeutic paradigms has become imperative[3] and several lines of research have been investigated[4-6]. Recent advances in stem cell biology (e.g. cardiac stem cells and induced pluripotent stem cells) and the use of stem cells in cardiac tissue replacement had raised considerable enthusiasm in the field of cardiac cell therapy[7]. Numerous cell types have already been tested, such as skeletal myoblasts, bone marrow stem cells, embryonic stem cells[8], but no effective solution has been proposed. Nevertheless, most pre-clinical and clinical studies have yielded modest results, while opening several questions on the causes of the limited survival and poor functional integration of the transplanted cells[9, 10]. In this perspective, new strategies for selecting and developing the cell therapy approaches that have the highest possibility of becoming clinical applicable in the shorter time possible avoiding the risk of chronic pathological or side effects have to be developed and integrated in the conventional therapy development process[11].

So far, cardiac cell therapy has been mainly investigated *in vivo* in animal models, with a lot of efforts in both time and money and bringing up all limitations and constrains of *in vivo* studies. In addition, the animal models cannot be fully representative of human biology and physiology. Such a huge amount of experimental effort being put into the improvement of cardiac cell therapy strategies could greatly benefit from the development of an *in vitro* based test. However, the next generation of *in vitro* assay must overcome some important limitations of actual screening system which are mainly based on cytotoxicity measurements of cardiomyocytes randomly plated on protein coated plastic surface. In particular, new assays should provide information directly related to human cardiac biology and physiology, possibly, integrating technology able to reproduce *in vitro* the time evolution of acute and chronic physiopathological condition landscape.

In this perspective, the development of *in vitro* model based on artificially engineered cardiac tissue have been recently proposed[12-16] for mimicking cardiac cell therapy *in vitro*[17, 18]. For instance, Habeler and colleagues used a slice of rat or human heart for testing hESC cardiac differentiation, whereas, Song and colleagues investigated the utility of an engineered heart tissue as a predictive test-bed for functional cell integration. They examined

the electrophysiological properties of a rat engineered cardiac tissue and the improvement or worsening due to the injection of rat cardiomyocytes, cardiac fibroblasts and mouse embryonic stem cell (ESC)-derived cardiac progenitors. The methodology used for electrophysiological measurements is based on morphometric measurements previously published and widely accepted among researchers [16, 19-21].

Despite the originality of the former papers, they investigate cardiac cell therapy on animal derived systems mainly analyzing the benefic action of transplanted cells to the functionality of the organs. However, the success of cell therapy is related to a high number of parameters that need to investigate simultaneously; for example, it would be extremely relevant to test the effects of physio-pathological conditions not only on the survival of transplanted cell but also on their functional properties when exposes to pathological environment.

In this work, we aimed at developing a multiparametric functional assay through micro-scale technology for testing pathological conditions on human cell model under well defined experimental conditions. In particular, hESC-derived cardiomyocytes (hCMs) will be used a human cell model because they are potentially obtained in large number following cardiac differentiation protocols that are continuously improving, both in terms of yield and physiological cardiac characteristics[22].

As mentioned above, the effects of pathological conditions on cardiac tissue are investigated *in vitro* on cardiomyocytes monolayer cultured onto Petri dishes or glass slides[23, 24]. Despite being the most easy and fast way to culture cardiomyocytes *in vitro*, this configuration carries several disadvantages such as: multiple samples should be prepared for multiparametric analysis; cell detachment often occurs after cardiomyocytes begin to contract; the tight cell-cell interactions makes it difficult to identify the spontaneously contracting cells from the ones that are merely passively stretched; samples of human origin show rare and instable spontaneous contractions.

In this scenario, the multiparametric functional assay was designed coupling of micro-technology and stem cell engineering to achieve the following features: i) hundreds of parallel independent experimental replicates through hCMs micropatterning in array of circular dots (300 μm in diameter) with a consistent and repeatable number of cells; ii) elastic substrate with optimal stiffness supporting hCMs contractions; iii) electrophysiological stimulation assisting on line morphometric analysis of hCMs contractions. We aimed to fully characterize and standardize the described system proving that it is show functional proper properties responsive to cardiac drugs and physiological stimuli.

Moreover, as a proof of concept, we were interested in studying the effects of reactive oxygen species (ROS) not only based on hCMs citotoxicity but also onto their functional properties. ROS are one of the typical stresses encountered by transplanted cells after injection and/or transplantation and are one of the main cause of death of cardiomyocytes (e.g.: during ischemia reperfusion) and poor engraftment. In general, an *in vitro* model mimicking their effects could be a valuable tool to screen new strategies or new drugs to enhance hCMs survival after *in vivo* injection, in particular, to provide a predictably on functional integration of transplanted cells which is one of the main problems in cardiac cell therapy.

Material and methods

Human cardiomyocytes derivation and culture

Human cardiomyocytes (hCMs) were derived from HES2 cell line as previously described by Yang et al[25]. Briefly, HES2 colonies were detached from matrigel coated dish with collagenase IV (Invitrogen) and trypsin (Invitrogen) and transferred to low adhesive dishes for the EB formation in aggregation medium: basal medium (Stempro 34 (invitrogen), 1% Penicillin/streptomicyn (Gibco), 1% L-Glutamine (Gibco), 150 µg/mL Transferrin (Roche), 50 ng/mL ascorbic acid (sigma), 0.45 mM mono-Thioglycerol (MTG, Sigma Aldrich)) additioned with 10 ng/mL BMP4 (R&D system). From day 1 to day 4 EB were cultured in stage I medium: basal medium with 10 ng/mL BMP4, 5 ng/mL βFGF (R&D system) and 6 ng/mL Activin A (R&D system). EB were then cultured in stage II medium, day 4 to day 8, consisting of basal medium and 10 ng/mL VEGF (R&D system) and 150 ng/mL DKK (R&D system). Finally, from day 8 to day 14, the culture medium consisted of basal medium and 10 ng/mL VEGF and 5 ng/mL βFGF. Cultures were maintained in a 5% CO_2/5% O_2/90% N_2 environment for the first 10–12 days and were then transferred into a 5% CO_2/air environment.

Hydrogel production and protein micropatterning

Polyacrilammide hydrogel were prepared as previously described[26, 27]. Briefly, clean and dry glass slides surfaces were chemically modified by subsequent deposition of 20 µl of a solution of 3-amminopropyltrimethoxysilane (Sigma-Aldrich) and glutaraldeide 0.5% in PBS 1X; this resulted in the formation of a hydrophobic layer ensuring covalent binding of the hydrogel films. Acrylamide/bisacrylamide 29:1 40% solution (Sigma-Aldrich) was diluted in phosphate-buffered saline (PBS, Sigma-Aldrich) to the final concentrations of 10% and 20%. The photoinitiator (Irgacure 2959; Ciba Specialty Chemicals), was initially dissolved in methanol at 200 mg/ml and then added to the acrylamide/bis-acrylamide

solution in order to obtain a final concentration of 20 mg/ml, and mixed thoroughly. 20 µl of the prepolymer solution were dropped over the functionalized glass surface. Hydrogel polymerization occurred by UV light exposure for 3 min (high pressure mercury vapor lamp, Philips HPR 125 W) emitting at 365 nm with an incident light intensity of 20 mW/cm2). Selective photo-polymerization of acrylamide solutions on the glass surface was achieved by interposing a photomask with circular spot of 16 mm in diameter between the light source and the glass slide. Non-polymerized acrylamide was removed using distilled water. Such procedures resulted in homogeneous hydrogel films with 16 mm diameter and an average thickness of 40 µm. Glass slides with covalently bonded hydrogel films were immersed in ultra-pure distilled water for 48 hours to ensure complete removal of the unreacted monomeric units or photoinitiator and then soaked in a 70% ethanol solution. After rinsing with ultra-pure distilled water, hydrogels were allowed to completely dry overnight; final sterilization occurred after 20 min exposure to UV light under a sterile hood.

The micropatterning of adhesion proteins was performed onto the hydrogel surface as previously described[26, 27]. Laminin (Sigma Aldrich) as used at a concentration of 100 µg/ml in PBS. Briefly, the PDMS stamp, microstructured with a geometry of an array of circular spots (300 µm in diameter and 700 µm spaced), was inked in the laminin solution for a few seconds, and the excess removed. Conformal contact between the dry hydrogel surface and the stamp was then achieved by applying a gentle pressure, thus transferring the desired protein micropattern on the hydrogel surface.

hCMs microstructured culture

Fully differentiated EBs, ranging from 27 to 39 days old, were dissociated to single cells in order to obtain the microstructured hCMs array. EBs were treated with 0.2% Collagenase Type I (Invitrogen) for 45 minutes at 37 °C and with trypsin for 5 minutes at 37°C. Trypsin was quenched with Stop solution (50% FCS, 50% IMDM (Invitrogen)). Gentle resuspension of the loosened EBs ensures the obtainment of a single cell suspension. 300 µL of cell suspension (2.7×10^5 cells/mL) was dropped over the hydrogel, previously micropatterned (Fig. 1A), and the hCMs were allowed to adhere for 5-8 hours. Cell cultures were kept at 37°C, 5% CO_2.

Immunohystochemistry

Primary antibodies were against cardiac troponin T (cTnT, mouse monoclonal, clone 13-11, NeoMarkers) , α-actinin (mouse monoclonal, clone 1A4, Sigma Aldrich), connexin 43 (Cx43, mouse monoclonal, clone 4E6.2, Chemicon), Nkx2.5 (goat polyclonal, clone A-16, Santa Cruz Biotechnology), adult isoforms of cardiac troponin I[28] (Ti1, kindly gifted by

Professor Schiaffino, Padova University) and adult/fetal isoform of cardiac Troponin T[29] (RVC2, kindly gifted by Professor Schiaffino, Padova University).

Cells were fixed with 2% PFA (Sigma Aldrich) for 7 min at room temperature, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) and blocked in PBS-2% horse serum (HS) for 45 min at room temperature. For Cx43 the fixation and permeabilization steps were performed in acetone for 10 minutes at -20°C. Primary antibodies were individually applied for 1 hour at 37°C. Cells were washed and incubated with Alexa488 and Alexa495 fluorescence-conjugated secondary antibody (Invitrogen) against mouse or goat for 45 min at 37°C. Nuclei were counterstained with hoechst (invitrogen), samples were mounted with Elvanol® and viewed under a fluorescence microscope (Leica, CTR6500).

Live and Dead assay

Cell viability was evaluated with the LIVE/DEAD assay (Invitrogen). Briefly, hCMs were incubated with 150 µl of 3 µM calcein and 3 µM ethidium bromide in D-PBS (Gibco) for 45 minutes at room temperature. Following incubation, the cells were washed with PBS and labeled cells were observed under a fluorescence microscope.

TUNEL assay

Cell apoptosis was evaluated with the Click-iT TUNEL assay (Molecular Probes, Invitrogen). Briefly the cells were fixed with 4% PFA for 15 minutes at room temperature, permeabilized with 0.25% Tryton X-100 in PBS for 20 minutes at room temperature and washed twice with deionized water. hCMs were then incubated with TdT reaction buffer for 10 minutes at room temperature, for 1 hour at 37°C with the TdT reaction cocktail and washed twice with 3% BSA in PBS. Finally, Click-iT reaction cocktail was added to the cells for 30 minutes at room temperature and washed with 3% BSA in PBS. Nuclei counterstain with Hoechst followed and hCMs were analyzed with a fluorescence microscope.

Gap-FRAP analysis

In order to evaluate the functional interconnection between hCM, gap junction functionality was quantitatively determined in living cells by gap-FRAP assay. hMCs were loaded with calcein AM (3 µM, 45 min, Invitrogen). Due to its low molecular weights (622 Da), calcein AM has been shown to permeate gap junction channels[30]. The cell culture was washed several times with PBS to remove the fluorochrome-ester and prevent further dye loading during subsequent measurements. FRAP was performed using a confocal laser scanning microscope (Leica), constituted by a reversed microscope equipped with an argon laser source at 496 nm. The intensity of the fluorescence signal was measured between 500 and 560 nm (emission band-pass filter) with a 20X objective and 5X optical zoom. The laser

output power was adjusted using a powermeter to to 55 μW for 30 s to achieve sufficient photobleaching for fluorescence recovery observations, without causing visible damage. Fluorescence recovery analysis was performed for 450 s with a measurement every 10 s, with the laser output power being adjusted to 10 μW.

The fluorescence recovery kinetics was divided into several phases. First, the intensity of fluorescence measured in the target cell before photobleaching was recorded (F0). The fluorescence intensity value was measured at each time point during the recovery (F). For each experiment, this value was normalized by the intensity value of the reference cell (Fr) chosen at the edge of the microscopic field to account for possible photodegradation caused by the successive acquisitions and the leakage of the fluorescent dye. The ratio (F/Fr) allowed us to correct for changes in fluorescence intensity due primarily to photobleaching caused by exposure of the whole field to the excitation light source. The exponential fluorescence recovery as function of time was correlated by the following perturbation–relaxation equation:

$$\frac{F(t)}{F_r(t)} = A\left(1 - e^{-t/\tau}\right) \quad (1)$$

where t is time [s] after photobleaching, A is an adimensional parameter that sets the asymptotic level of recovery and τ the inverse value of transfer constant [s]. Values of fitting parameter were obtained from 20 replicates.

Morphometric analysis

The morphometric analysis of hCMs contractions were performed coupling the acquisition of contraction displacement by a fast acquisition camera (blue fox, Matrix Vision Gmbh) with a system for electrical stimulation of the culture[19, 31, 32].

The contraction frames were acquired for 5 or 10 seconds every 50 ms. hCMs displacement was analyzed by fixing circular regions of interest (ROIs, 2 μm diameter) and analyzing their intensity with the Image. The intensity values were then plotted in a graph showing the intensity, in arbitrary unit, versus time, seconds (see figure 4). 6 to 10 ROIs of 6-8 independent hCMs spots were analyzed for each acquired series of frames.

The electrical stimulation was applied to the hCMs array using two carbon electrodes (Ladd Research, 3 mm in diameter and 20 mm in length) placed at 10 mm distance and held by a PDMS holder designed to fit a 35 mm Petri dish and to keep electrodes immersed in the culture medium during the analysis. The electrodes were connected via platinum wires to a function generator (Amel, model 568) programmed to produce a square wave with a 0 V baseline and impulses ranging from 1 V/cm to 6.8 V/cm for 5 ms with frequency ranging

from 1 to 4Hz. A third platinum electrode was inserted to monitor the electrical stimulation by an oscilloscope (LeCroy, LT322).

The chronotropic agents noreinephrine and propanolol (all Sigma Aldrich) were added to the medium to the final concentrations of 100 and 200 μM.

Results

Micropatterned hCMs array characterization

hCMs were obtained from the human embryonic stem cell line HES2, following the protocol for cardiac differentiation described by Yang and colleagues[25]. The hCMs characterization has been previously reported by the same group[25 14].

After EB disgregation and cell seeding onto the microstructured substrate, the culture resulted in a 10×10 mm array of cellularized circular spots (Fig 1, C) defined with micrometric precision (Fig. 1, B-D). The percentage of cardiomyocytes per spot was high, around 90% (as semi-quantitatively evaluated based on Troponin T expression), with a good distribution on the entire array (Fig. 1, E-F). Indeed the expression of cardiac Troponin T (cTnT) (Fig. 1, E-F) was maintained for several days (up to 7 days). We will thus refer to this culture as hCMs.

The maintenance of cardiac markers on micropatterned cells was verified after 5-7 days of culture onto the hydrogel (Fig. 2). Cx43, α-actinin and Nkx2.5 were analyzed. At this time point, spontaneous and electrically induced contractions were observed (see supplementary video).

In sight of developing an *in vitro* model of human cardiac tissue, the differentiation stage of cardiomyocytes is a crucial point. It is worth to underline that, in a case of long term culture (hCMs derived from 39 days old EBs and cultured onto micropatterned hydrogel for 6 days), we observed hCMs expressing adult isoforms of cardiac Troponin I and T (Fig. 3, A-D).

Although cardiac markers expression was verified and monitored in the developed array, we investigated the cell-cell coupling in sight of obtaining a high-hierarchic functional tissue, rather than clusters of single scarcely communicating cardiac cells. Thus, gap-FRAP experiments have been performed to test the functionality of hCMs gap junctions[30] (Fig. 3, E-I). Confocal microscopy allowed to record target cell's fluorescence recovery on a single plan. A labeled target cell was photobleached by scanning argon laser beam (Fig. 3F), after recording the initial level of fluorescence (Fig. 3E). Fluorescence recovery (Fig. 3G) is related to dye transport across gap junction; the normalized fluorescence was plotted as a function of time (Fig. 3H). The fitting of these curves gave the fitting parameters k and A values (Fig. 3I). To exclude the eventuality of a recovery due to transplanar diffusion in the

same cell, it has been necessary to evaluate fluorescence recovery in isolated calcein loaded cells. Results of the fitting showed the presence of functional gap junctions in hCMs. The percentage of recovery for control cells was about 5 times smaller than the percentage calculated for hCMs, indicating that cultured hCMs have gap functionality and consequently proving their functional interconnection within a single spot.

Evaluation of hCMs functional property: method validation

Once verified the cardiac phenotype of the hCMs, we moved our investigations to the biophysical properties of these cells. We observed that the spontaneous contractile activity of arrayed hCMs was synchronous intra single spot, additional proof of functional cell-cell interactions, while the contractions become non-synchronous inter-spots. We thus decided to couple exogenous electrical stimulation for verifying hCMs capability to respond to external stimuli (E-C coupling) and for synchronizing the contractions of the spots (evaluating the maximum contraction frequency). The applied voltage ranged from 1 to 6.8 V/cm with a frequency ranging from 1 to 4Hz and a duration of 5 ms.

With this aim, we developed a "morphometric analyses" methodology based on the following steps: a) recording of hCMs contractions, b) analyses of the hCMs displacements from the obtained movies, c) representation of the displacements as a graph showing the variation of light intensity against time, d) calculation of the contraction frequency (see Materials and Methods section).

Micropatterned hCMs showed spontaneous beating (Fig. 4A), but in some cases they were induced to contract with exogenous electrical stimulation (Fig. 4B). In both cases, we were able to monitor hCMs contractions and calculate their frequency (Fig. 4).

The robustness of the developed methodology is shown in figures 4B and C. Figure 4B shows the contraction traces obtained with an off-on-off sequence of electrical stimulation: 0V/cm; 6 V/cm at 2Hz for 4.5 s; 0 V/cm (red line). No spontaneous contractions are present in absence of electrical filed, while during electrical stimulation hCMs are induced to contract and paced to a frequency of 2 Hz. Similarly, figure 4C shows a contraction-graph of spontaneously contracting hCMs. The applied stimulation has amplitude of 6 V/cm, frequency of 1 Hz and it stops after 5 s. The obtained graph clearly shows that hCMs are paced during the application of an exogenous electrical stimulation (from 1 to 5 s their contraction frequency is 1 Hz), while after the stimulation they return to a spontaneous contraction frequency of 0.6 Hz.

To further support the robustness of our methodology, we investigated the capability of detecting the chronotropic effects of the β-adrenergic agonist norepinephrine (Fig. 5A) and the β-AR antagonist propranolol (Fig. 5B). hCMs stimulation with norepinephrine led to an

increase in the frequency of hCMs capturing with electrical stimulation. The maximum capturing frequency for hCMs with control medium was 2 Hz, while 100 µM norepinephrine increased the capturing frequency to 3 Hz (Fig. 5A). On the contrary, treatment with 200 µM propranolol inhibited the norepinephrine action and lowered the contraction frequency to 1 Hz, even during electrical stimulation at 2 Hz (Fig. 5B).

These results, besides being an experimental validation of the methodology used, indicated that the cultured hCMs expressed β-AR. Norepinephrine acts specifically on β1 receptors, and propanolol is a non-specific blocker of β-receptors.

In order to further optimize the developed system and analyses methodology, we investigated the optimal substrate stiffness for hCMs culture. In particular, it has been reported that chicken cardiomyocytes contraction is inhibited when cultured onto a substrate whose stiffness ranges from 35 to70 kPa. Such a range of substrate elasticity is representative of the non-contractile fibrotic tissue formed after a myocardial infarction, while a normal myocardium has an elastic modulus of E ≈ 10 kPa[33]. The elastic modulus of the PA hydrogel was easily tuned varying the composition of the pre-polymer solution, as previously reported[26]. In order to mimic normal cardiac tissue, hCMs microstructured cultures were performed on a 10% PA hydrogel with E ≈ 15 kPa, while the fibrous tissue was mimicked with a 20% PA hydrogel with E ≈ 35 kPa.

After 4 days of culture we evaluated hCMs viability (Live&Dead assay), apoptosis (TUNEL) and contractility (morphometric analyses). We didn't observe any difference between hCMs cultured on 15 kPa and 35 kPa hydrogel, in terms of viability and apoptosis (in both groups, less than 1% of hCMs were TUNEL assay positive, data not shown), nor in their functional properties (Fig. 6, A and C) nor in their sarcomeric organization of cTnT (Fig. 6, B and D).

In sight of giving a proof of concept that our system could be used as an *in vitro* based test, we investigated the effects of a pathological condition on hCMs. We exposed micropatterned hCMs to increasing levels of H_2O_2 (0.01 and 0.1 mM) for 1 and 16 hours[34]. At these time point, hCMs viability and functional properties were verified. We observed that hCMs viability after 1 and 16 hours was not affected by any of the H_2O_2 concentration tested (Fig. 7B). Positive control of hydrogen peroxide citotoxicity was performed using 0.5 mM H_2O_2 solution. Interestingly the contractility and E-C coupling capability were maintained for all conditions, except for the highest concentration of H_2O_2 after 16 hours (Fig. 7A). hCMs were still alive after exposure to 0.1 mM H_2O_2 for 16 hours, but their functional properties (cell contractions) were suppressed, suggesting that a level of oxidative stress ineffective for hCMs viability could instead compromise their functionality.

Taken together, these results clearly indicate that an *in vitro* test merely based on cell toxicity could give misleading outcomes, especially in the case of cardiac drug development or screening. Physiological/functional tests are also required in order to have a complete and reliable set of data.

Discussion

This study demonstrated the feasibility of developing an *in vitro* based test for hCMs, able to give insight on both viability and functionality of hCMs. Secondly, we demonstrated that collecting information about the functional properties of hCMs could give a clearer scenario about possible secondary effects of a particular environment, in our case increasing level of H_2O_2.

The cardiomyocytes derived from hESC used for *in vitro* studies are mainly obtained and cultured as cell cluster derived by dissection of EB contracting area or as monolayer by co-culture[35, 36]. In this study we realized a microstructured array of hCMs, arranged in 20×20 spots with a consistent and high number of hCMs per spot (> 90% of cTnT-positive cells). hCMs cultured as monolayer onto standard substrates are tightly connected, having each cell influenced by the neighboring one. Conversely, microstructured and arrayed hCMs offer the possibility of analyzing different spots at the same time, exposed to identical conditions. Each spot could represent an independent sample, with a consistent/repeatable number of hCMs in each experiment and the advantage of deriving from the same batch of cells. In this way, being the system composed of 400 spots, each of them could be potentially analyzed in parallel. Consequently the system could give an high number of output information per experiment.

In addition, the microstructured culture, in contrast to standard hCMs cluster and monolayer, allowed us to analyze the spontaneous and electrically induced contractions through the development of a morphometric methodology. This technique, for testing the biophysical properties of hCMs, has been developed based on a methodology widely accepted from researchers in this area: imaging analyses of contracting areas. Studying contracting cells or tissues by video recording and digitalizing of the captured images has been applied for contractions of both three-dimensional tissue constructs[16, 19, 21, 32, 37] and two-dimensional cell culture[20, 38, 39]. Through micropatterning technique, each hCMs spot has clear and defined edge, contracting cardiomyocytes are thus easier to detect and analyze.

In summary, the developed array of micropatterned hCMs lead us to gain repeatability and robustness, and allowed multiple analysis per batch of cells.

Increasing the acquired data for reducing experimental variability is a key point when a human cell source is used. In particular, hESC are known for their variability and heterogeneity[40]. Recently, it has been shown that the hESC colony size itself could affect the differentiation trajectory[41]. We overcame this source of variability by hCMs micropatterning in spot of regular size and cell number. Moreover, we used HES2 as source for cardiomyocytes because it is a human cell line, and the obtained hCMs could better resemble the human physiology and response to environmental stimuli. In addition, being an hESC line, HES2 are an highly proliferative population, potentially giving an unlimited number of hCMs. We do not underestimate the fact that the obtained hCMs are not fully representative of adult cardiomyocytes, they retain some fetal characteristics. In this sight, it is worth to underline that we observed adult isoforms of cardiac troponin I and T in hCMs derived from 39 days old EB, indicating that longer time point of differentiation coupled with hCMs culture as monolayer onto the hydrogel, could increase the percentage of hCMs with adult phenotype.

Recently, the mechanical environment of cell culture has captured increasing interesting among researchers, especially in the field of stem cell expansion and differentiation[42]. In particular, the development of sarcomeric structures of human striated muscles[26] and the contractile functionality of chicken cardiomyocytes[33] are influenced by substrate stiffness. For these reasons, we did not cultured hCMs onto standard rigid Petri dishes or glass coverslip, but a soft polyacrilamyde hydrogel has been choose. In order to optimize our system and hCMs culture, we investigated two levels of hydrogel stiffness ($E \approx 15$ and 35 kPa) and their effects on hCMs viability or contractility, the lower being representative of an healthy myocardium and the higher of a fibrous non contractile myocardium[33]. Our data did not show any differences in either hCMs viability, as expected, or contractility. We could thus conclude that substrate stiffness ranging from 15 to 35 kPa support hCMs culture and functionality. We also retain that this part of the study needs to be further explored because the results could have been influenced by two factors: a) 35 kPa is the lowest value of elastic modulus for a fibrotic tissue, further studies should thus be conducted with hydrogel of higher elastic moduli; b) since hCMs have the tendency to aggregate in clusters of 10-20 cells, also after EB dissociation to single cells, and to adhere in multilayer, it is possible that hCMs did not sense the actual stiffness of the hydrogel itself, but the stiffness of the underneath cells/hCMs. Anyway, the use of the hydrogel as substrate sustained and favored hCMs contractions and avoided the problem of cell detection, which is quite frequent when working with contracting and force generating cells.

The obtained hCMs spots and the morphometric analysis developed showed that these cells give rise *in vitro* to a good cardiac tissue: proper expression of cardiac markers, functional and conductive gap junctions, responsive to external stimuli such as pharmaceutical, chronotropic agents and electrical stimulation.

These hCMs should thus be a good cell source for cardiac cell therapy, and *in vivo* they should contribute to the restoration of heart function. However, it is well know that the *in vivo* environment encountered after injection is an hostile niche that compromise cell viability. What is still unclear is why the surviving injected cells do not integrate or have a poor functional integration with the host tissue. Several hypothesis have been formulated such as anoikis, mitochondrial death, induction of apoptotic pathways[43].

In this scenario, the *in vitro* based model developed in this study could be used to simulate the environment of an infracted heart and could give new insights on the causes of failed integration . We gave a proof of concept of this application with the exposure of hCMs to H_2O_2. In fact, we examined the effects of increasing oxidative stress onto hCMs, trying to mimic the inflammatory environment to which the cells injected in an ischemic heart are subjected. Reactive oxygen species are generated during both ischemia and reperfusion phases[44] and the inflammatory environment of an healing infarct could present high levels of oxygen-free radicals[10]. We showed that investigating hCMs contractility besides their viability had lead to additional information about the effects of an oxidative environment.

Conclusions

In conclusion, we developed *in vitro* an array of functional hCMs, able to respond to pharmaceutical agents and external electrical stimulation. We developed a morphometric analysis methodology to monitor hCMs contraction capability and frequency. Importantly, this study emphasize the importance of multiple readouts from an *in vitro* model, both in terms of the number of data acquired in parallel (array of 20×20 spot) and in terms of the type of analysis (citotoxicity and functional contractions).

We further hypothesized that the developed system could represent a powerful *in vitro* model of human cardiac tissue for conducting preliminary studies on cardiac cell therapy.

Acknowledgments

We thank Riello Massimo (Padova University), Giulitti Stefano (Padova University), Martewicz Sebastian (Padova University) for help with experimental work.

Figures

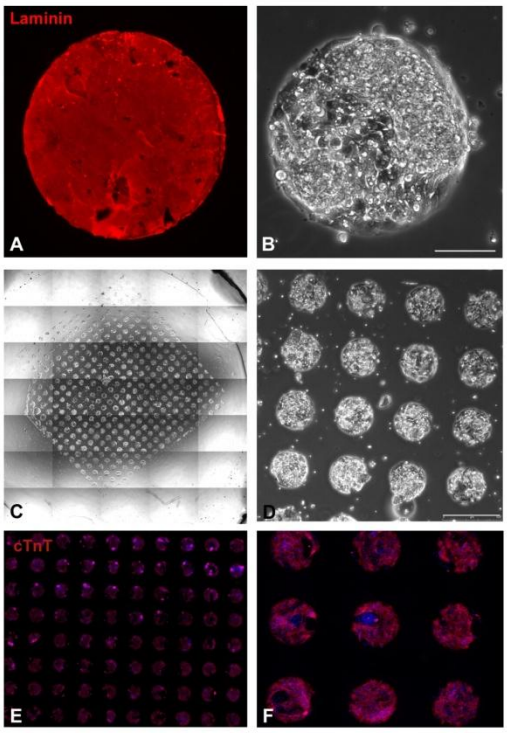


Figure 1. Microstructured hCMS culture. A: laminin immunofluorescence of a micropatterned spot. B, C, D: microstructured hCMs culture. E, F: cTnT immunofluorescence of the hCMs culture, nuclei were counterstained with hoechst.

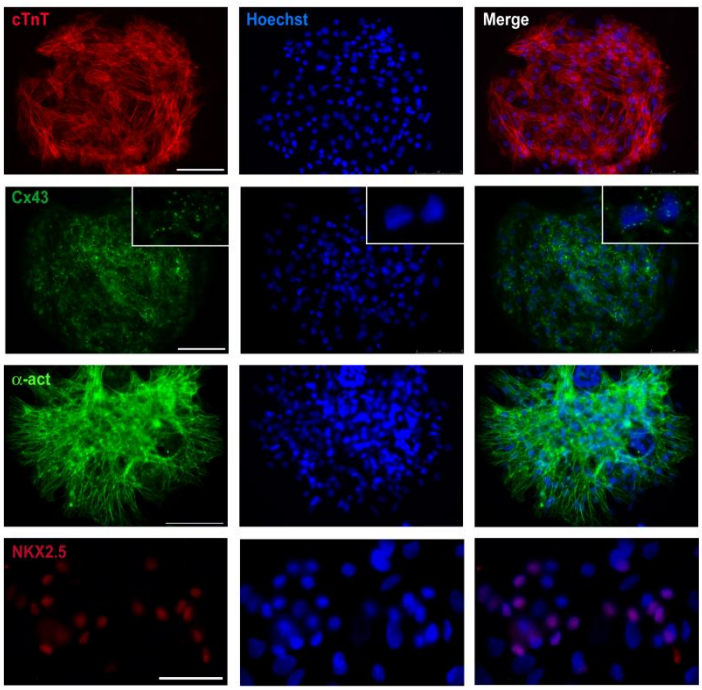


Figure 2. Characterization of microstructured hCMS culture. A: cardiac Troponin T, B: connexin 43, C: α-actinin, D: NKX2.5. Nuclei were counterstained with hoechst.

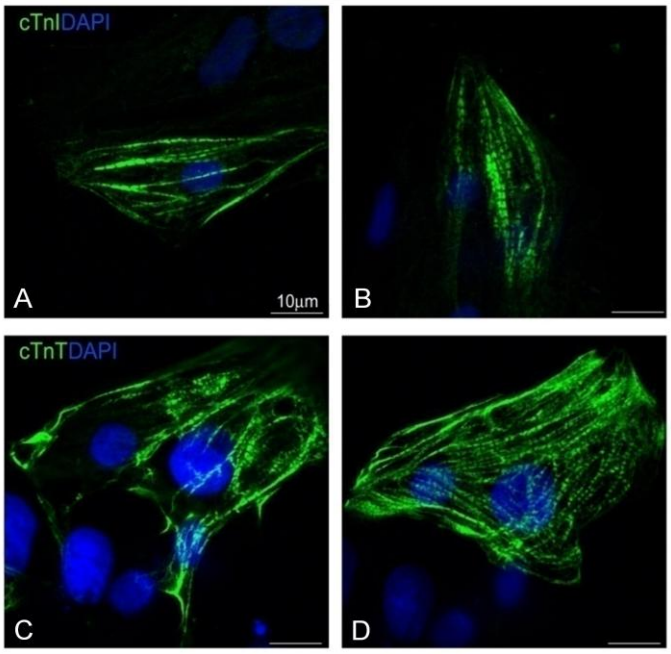
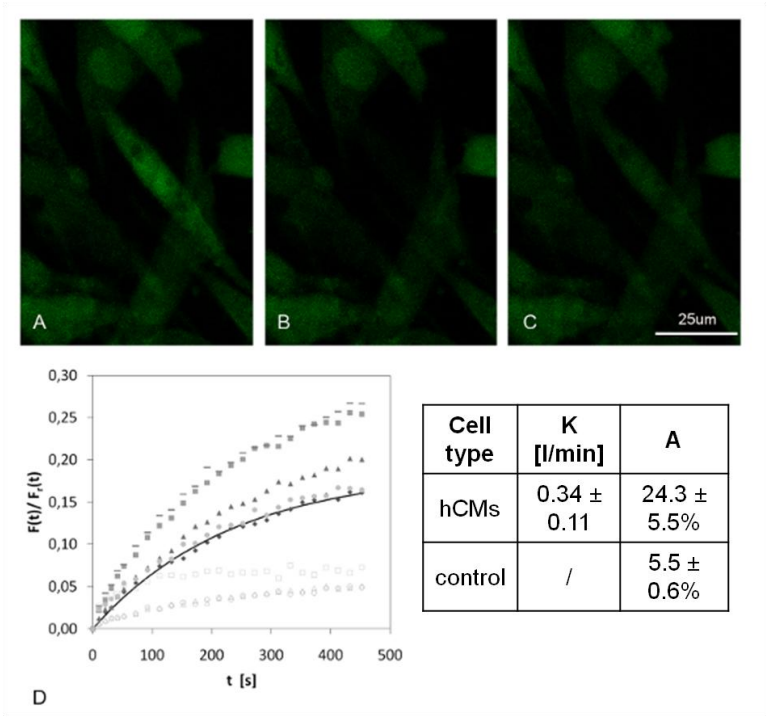


Figure 3. microstructured hCMs characterization. A-B: Immunofluorescence against and adult isoform of cardiac Troponin I of T39 hCMs, C-D: Immunofluorescence against and adult/fetal isoform of cardiac Troponin T of T39 hCMs, nuclei were counterstained with hoechst.



Cell type	K [l/min]	A
hCMs	0.34 ± 0.11	24.3 ± 5.5%
control	/	5.5 ± 0.6%

Figure 4. gap FRAP analysis. A-C: representative images of fluorescence restoration in hCMs, target cell is indicated by an arrow, scale bar: 50 µm. A: intensity of calceine AM fluorescence before photobleaching, B: fluorescence right after photobleaching, C: fluorescence recovery after 7.5 minutes. D: graph representing the kinetic profiles of raw and fitted recovery data.

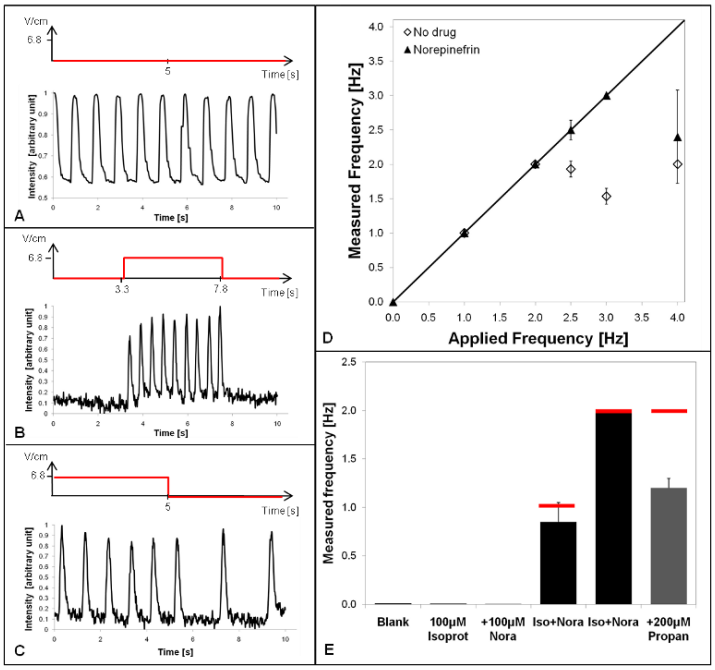


Figure 5. Experimental validation of morphometric analysis. In each panel, the graph with the red line represents the exogenous electrical stimulation, the graph underneath shows the displacement of hCMs. A: morphometric analysis of spontaneous contractions, B: morphometric analysis of hCMs induced to contract with exposed to electrical stimulation, C: morphometric analysis of spontaneously contracting hCMs, whose contraction frequency was captured by exogenous electrical stimulation. D: morphometric analysis of microstructured hCMs culture treated with culture medium (No drug) and 100µM norepinephrine. E: graph representing the contraction frequency of control hCMs (blank) and hCMs exposed to norepinephrine (Nora) and propranolol (propan). Red squares represent the frequency of the applied electrical stimulation.

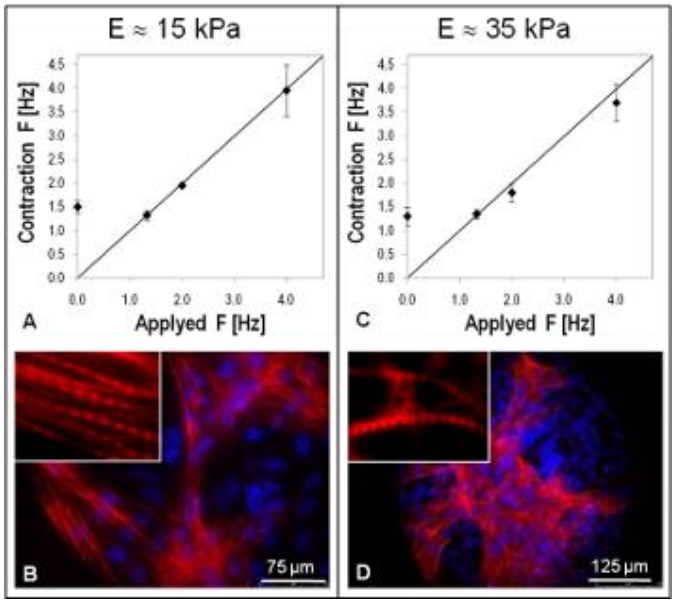


Figure 6. Effects of susbtrate stiffness on hCMs. *A, B: mophometric analisis and of cTnT immunofluorescence of hCMs cultured onto 15 kPa, B, D: mophometric analisis and of cTnT immunofluorescence of hCMs cultured onto a 35 kPa hydrogel. Nuclei were counterstained with hoechst.*

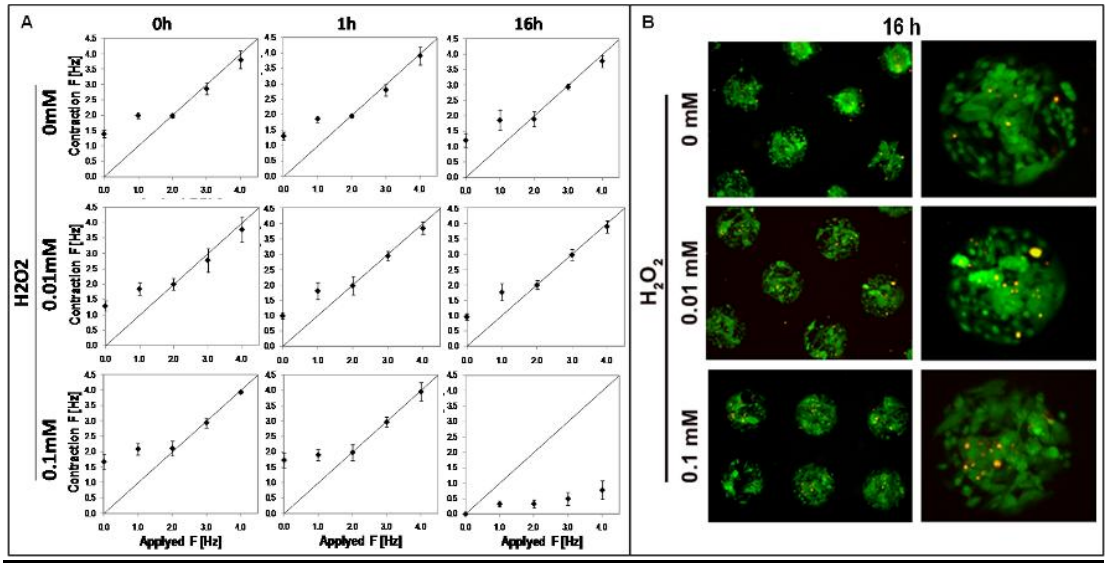


Figure 7. Effects of oxidative stress on hCMs. A: mophometric analisis of hCMs exposed to 0 mM, 0.1 mM and 0.1 mM H_2O_2 for 0, 1 and 16 hours. B: live and dead analysis after 16 hours of exposure to 0 mM, 0.1 mM and 0.1 mM H_2O_2.

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