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Study of the application potential of synthetic and natural polymeric coatings in stem cell research

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Study of the application potential of synthetic and natural polymeric coatings in stem cell research

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Dedicated to my family and friends who supported me in this challenging journey.

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RESUMO

A investigação em células estaminais embrionárias tem permitido grandes avanços na pesquisa em ciências biomédicas. Este é o evidente reflexo de estas serem células não especializadas que são capazes de se auto-renovar indefinidamente, e assim dar origem a novas células estaminais num estado indiferenciado, mas também têm a possibilidade de se diferenciarem em um ou múltiplos tipos de células especializadas sob condições definidas ou estímulos intrínsecos e/ou extrínsecos.

Linhas de células estaminais embrionárias de ratinho foram inicialmente derivadas e cultivadas há cerca de 30 anos e, desde então, têm sido uma ferramenta inestimável na compreensão dos processos de desenvolvimento embrionário dos mamíferos, bem como no estabelecimento das bases moleculares da pluripotência e da auto-renovação celular. Para além disso, por permitir a criação de geneticamente modificados (e.g. *knock-outs*) através de recombinação homóloga, estas são determinantes no estudo da função genética *in vivo*. Não menos importante, os estudos das células estaminais embrionárias de ratinho abriram o caminho à investigação das células estaminais embrionárias humanas que, dada a sua capacidade de auto-renovação e diferenciação, têm suscitado muito interesse pelo seu elevado potencial para uso em terapia celular. As células estaminais embrionárias têm sido co-cultivadas geralmente com células de suporte tais como fibroblastos embrionários de ratinho. Os fibroblastos embrionários de ratinho servem de células de suporte para cultura de células estaminais embrionárias desde a derivação das mesmas. Estes fibroblastos continuam a ser ainda hoje os mais usados como células de suporte para cultura e manutenção de células estaminais de ratinho e humanas, uma vez que proporcionam um substrato que aumenta a eficiência de adesão das células estaminais, promove a manutenção da sua pluripotência e capacidade de auto-renovação e facilita a sua sobrevivência e crescimento. Os fibroblastos embrionários usados para cultura de células estaminais produzem uma complexa mistura de fatores de crescimento, citoquinas e outros componentes da matriz extracelular tais como o fator inibidor de leucemia, proteínas morfogenéticas ósseas, activina, laminina e vitronectina, que mantêm as células estaminais embrionárias indiferenciadas e pluripotentes mesmo quando cultivadas a longo prazo.

Sabe-se que o comportamento das células estaminais embrionárias *in vitro* é fortemente influenciadas pelas condições de cultura. Estudos recentes demonstram que os métodos convencionais de cultura de células estaminais não reúnem os requisitos mínimos para regular e controlar com rigor a função das células estaminais. Além disso, os vários protocolos existentes para cultura de células estaminais envolvem o uso de matrizes de origem animal, tais como a gelatina e os fibroblastos de ratinho. De modo a ultrapassar esta limitação, vários cientistas têm-se empenhado em desenvolver matrizes artificiais no sentido de eliminar os componentes de origem animal dos sistemas de cultura de células, mas mantendo o controlo da capacidade de auto-renovação e diferenciação das células estaminais. O avanço no desenvolvimento de biomateriais tem-se revelado uma ferramenta fundamental para a criação de microambientes artificiais que visam mimetizar o microambiente *in vivo* das células estaminais, influenciando e controlando a expressão dos genes que definem o destino e as propriedades estaminais, ou seja, a auto-renovação ou a diferenciação. No entanto, apesar dos grandes avanços que têm ocorrido no desenvolvimento de biomateriais, a maioria são dispendiosos e poucos são apropriados para manter as células estaminais embrionárias num estado indiferenciado. Por isso, o objetivo principal deste trabalho foi o de encontrar ou desenvolver um biomaterial capaz de dar suporte ao crescimento de células estaminais embrionárias indiferenciadas, evitando os custos de uma série de procedimentos experimentais trabalhosos e morosos da cultura paralela de fibroblastos com a cultura de células estaminais, e que fosse de fácil manuseamento tal como a gelatina. Visto que o ambiente que envolve as células estaminais tem um papel determinante na seleção do destino das mesmas, o objetivo deste trabalho consistiu então em otimizar as condições de cultura de células estaminais embrionárias de ratinho através da substituição da monocamada de fibroblastos ou da gelatina por um suporte artificial que mimetizasse a matriz extracelular e que servisse como substrato para adesão celular, proliferação e diferenciação. No âmbito deste trabalho desenvolveu-se e testou-se a aplicabilidade de um revestimento de goma de semente de alfarroba e de uma matriz de nanofibras sintéticas na manutenção da pluripotência e capacidade de diferenciação de células estaminais embrionárias de ratinho em cultura.

A estabilidade química e a biocompatibilidade dos biomateriais de origem natural tem contribuído para a sua utilização cada vez mais frequente na medicina

moderna. A goma de semente de alfarroba é um polímero natural que tem uma vasta gama de aplicações desde a indústria alimentar e cosmética, passando também pela farmacêutica devido à sua biocompatibilidade e às suas propriedades de adesão e de espessante. Nós quisemos testar este composto natural na cultura de células estaminais pluripotentes. Numa primeira abordagem, células estaminais embrionárias de ratinho foram cultivadas em poliestireno revestido com goma de semente de alfarroba e avaliou-se o seu potencial de manutenção da pluripotência através da análise da morfologia das colónias, do ensaio da fosfatase alcalina, da expressão de marcadores de pluripotência (*Nanog*, *Sox2* e *Oct4*) e da capacidade de diferenciação nos três folhetos embrionários (endoderme, mesoderme e ectoderme) após cultura a longo prazo. De acordo com os resultados, as células estaminais embrionárias cultivadas em goma de semente de alfarroba apresentaram-se sob a forma de colónias regulares, semelhantes às colónias obtidas quando as células estaminais foram cultivadas em fibroblastos, o suporte *standard* para a manutenção da pluripotência. As células estaminais embrionárias cultivadas em goma de semente de alfarroba mantiveram a sua viabilidade e tiveram um crescimento semelhante às células cultivadas em gelatina. Por outro lado, as células estaminais embrionárias cultivadas em goma de semente de alfarroba apresentaram níveis de expressão de marcadores de pluripotência e de atividade da fosfatase alcalina equivalentes e em alguns pontos mais elevados do que as células cultivadas em gelatina. Após 30 dias de cultura em goma de semente de alfarroba, as células estaminais embrionárias mantiveram a sua capacidade de se diferenciar nos três folhetos embrionários.

Apesar das várias vantagens apresentadas pelos polímeros naturais, a sua composição pode variar consoante o lote e o produtor. O principal componente da goma de semente de alfarroba é o polímero galactomanano que se encontra no endosperma das sementes e funciona como reserva energética. Neste trabalho testou-se também a utilização de goma de semente de alfarroba de diferentes origens (Roeper e Industrial Farense) e purificada *versus* não purificada, para cultura de células estaminais embrionárias. Em geral, confirmou-se que o revestimento do poliestireno tratado para cultura de células com goma de semente de alfarroba promove o crescimento de células estaminais embrionárias num estado indiferenciado e viável, o que tem potencial para ser um suporte de origem vegetal para cultura de células alternativo à gelatina.

Tendo em conta que a matriz extracelular nativa é maioritariamente constituída por fibras, e que no caso da cultura sobre fibroblastos estas são produzidas pelos fibroblastos, desenvolveu-se um suporte de nanofibras poliméricas de poli (etilenoglicol-*β*-metacrilato de trimetilsilício-*β*-ácido metacrílico), conjugado com péptido glicina-arginina-glicina-aspartato-serina. Esta matriz é então resultante da conversão de co-polímeros anfifílicos em nanofibras através de um processo de organização espontânea (*self-assembly*). Considerando que um suporte ideal deve conter locais que proporcionem a adesão celular, os co-polímeros anfifílicos foram desenhados e sintetizados de modo a incorporar uma sequência de péptidos termoestáveis que correspondem a uma sequência biológica integrante da matriz extracelular. Com o objetivo de determinar o potencial de aplicação futura do suporte de nanofibras para cultura de células estaminais, células estaminais embrionárias indiferenciadas de ratinho foram cultivadas no suporte de nanofibras e em condições convencionais. O potencial de aplicação das nanofibras em investigação de células estaminais foi avaliado através da morfologia, proliferação, viabilidade, auto-renovação e pluripotência das células estaminais embrionárias indiferenciadas de ratinho comparativamente com os resultados obtidos para as condições de cultura *standard*. De acordo com os resultados, as nanofibras promoveram o crescimento celular e um aumento da expressão dos genes de pluripotência em comparação com as metodologias convencionais de cultura de células estaminais. Além disso, as células estaminais embrionárias cultivadas a longo prazo em nanofibras conservaram a capacidade de diferenciação. Portanto, neste trabalho foi desenvolvido uma nova matriz de nanofibras sintéticas que permite a eliminação de matrizes de origem animal e providencia um método económico para cultura de células constituído por uma rede de nanofibras. Esta matriz de nanofibras possui uma escala semelhante à matriz extracelular nativa onde as células podem ser mantidas e manipuladas.

Paralelamente ao estudo da utilização de biomateriais na cultura e manutenção de células estaminais, a última parte do meu trabalho foi dedicada ao estudo da função da proteína CCBE1. A proteína CCBE1 contém domínios putativos de colagénio e de fator de crescimento epidermal (EGF). O gene *Ccbe1* foi identificado num ensaio de expressão diferencial efetuado para revelar novos genes expressos nos percursores cardíacos realizado pelo nosso laboratório e está envolvido em processos de linfangiogénese, cardiogénese e carcinogénese. No entanto, apesar

de putativamente CCBE1 ser uma proteína secretada, a sua função e mecanismo de ação continuam a ser desconhecidos. Dados experimentais publicados e outros realizados no nosso laboratório revelaram que o ratinho mutante para o gene *Ccbe1* morre durante o desenvolvimento embrionário. Outros resultados indicam que *Ccbe1* é muito expresso em fibroblastos embrionários de ratinho. Por estas razões, foram realizados estudos de função de *Ccbe1* utilizando culturas primárias de fibroblastos embrionários de ratinho como modelo. Realizaram-se ensaios de adesão, proliferação, viabilidade e migração. De acordo com os resultados, verificou-se que os fibroblastos isolados do ratinho mutante para *Ccbe1* a E13.5 apresentam uma morfologia típica de fibroblastos senescentes, a sua proliferação é mais lenta e têm expressão de *Bax* aumentada, um inibidor de proliferação celular. Por outro lado, quando CCBE1 é adicionado a fibroblastos *Ccbe1*$^{-/-}$, a sua proliferação e viabilidade aumentam.

O papel de *Ccbe1* em migração foi também testado. Os fibroblastos mutantes para *Ccbe1* apresentaram um carácter mais migratório do que os fibroblastos normais e dependente do substrato. Contrariamente, este carácter migratório é reduzido quando o ensaio de migração é feito na presença de CCBE1 no meio de cultura. Apesar de o mecanismo de ação de *Ccbe1* continuar a ser desconhecido, os resultados aqui apresentados sugerem que *Ccbe1* coordena a adesão celular, migração e proliferação, o que sugere que *Ccbe1* deverá desempenhar um papel fundamental na matriz extracelular.

Palavras-chave: *Ccbe1*, células estaminais embrionárias, cultura de células, goma semente alfarroba, nanofibras, suporte de crescimento substituto da gelatina

ABSTRACT

Self-renewability and the ability to differentiate into various functional cells are characteristics of embryonic stem cells (ESCs) that make them attractive for applications in biomedical field, namely in restoring the function of damaged cells/tissues. In research, ESCs are usually cultured in gelatin or over a monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFsi). The latter is the gold standard to maintain pluripotent ESCs in culture. A variety of alternative technologies have been suggested to control stem cell fate and function, but examples of versatile, non-animal derived and inexpensive materials able to support pluripotent ESCs are limited. To circumvent this, we aimed to find a biomaterial able to support pluripotent ESC cultures that would avoid the laborious and time consuming parallel culture of MEFsi and as simple to handle as gelatin. There is an increasing interest in regulating stem cells under a specific microenvironment using biomaterials as artificial extracellular matrices (ECMs) to control their self-renewability and differentiation capacity. In the present work we developed and tested the applicability of two biomaterials, one natural and one synthetic polymer, to support mouse ESC (mESC) culture. Accordingly, undifferentiated mESCs were cultured in coatings of Locust Bean Gum (LGB) and of a new synthetic nanofiber (nf) material based on the self-assembly of a triblock copolymer, poly (ethyleneglycol-*β*-trimethylsilyl methacrylate-*β*-methacrylic acid), conjugated with the peptide Glycine-Arginine-Glycine-Aspartate-Serine. According to our data, compared to conventional stem cell culture methodologies, ESCs grown in LBG and nanofiber coatings maintained their self-renewability and tri-lineage differentiation capacity even after long term culture.

In parallel, this work also comprises the functional study of collagen and calcium-binding EGF domains 1 (*Ccbe1*) using primary fibroblasts as a tool. It has been shown that *Ccbe1* is involved in lymphangiogenesis, cardiogenesis and carcinogenesis, however, its function and molecular action remains unknown. The data presented here suggests that *Ccbe1* coordinates cell adhesion, migration and proliferation, thus playing a key role in the ECM.

Keywords: *Ccbe1*, cell culture, embryonic stem cells, gelatin substitute growth support, locust bean gum, nanofibers.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

2D - two dimensional
3D - three dimensional
ºC - degree Celsius
x *g* - times gravity

A

7-AAD - 7-aminoactinomycin D
αMhc - α-myosin heavy chain
αSma - α-smooth muscle actin
Afp - α-fetoprotein
ALP - Alkaline Phosphatase
AIBN - Azo*bis*isobutyronitrile

B

Bax - BCL2-associated X protein
Bcl2 - B cell leukemia/lymphoma 2
BrdU - bromodeoxyuridine

C

Ccbe1 - collagen and calcium-binding EGF domains 1
cDNA - complementary DNA
Col1α1 - collagen type I α1
CPD - cumulative population doubling
cTnT - cardiac troponin T

D

DAPI - 4',6-diamidino-2-phenylindole
DMEM - Dulbecco's modified eagle medium
DNA - deoxyribonucleic acid
dNTP - deoxyribonucleotide
DP_n - degree of polymerization

E

EB - embryoid body
ECM - extracellular matrix
EDTA - ethylenediaminetetraacetic acid
ERK - extracellular signal-regulated kinase

δ - ^{1}HNMR chemical shift, in parts per million
δ - bending vibration in IR
ν - stretching vibration in IR

eq - equivalent
ESC - embryonic stem cell

F

FAK - focal adhesion kinase
FBS - fetal bovine serum
FDA - American agency for public health Food and Drug Administration
FE-SEM - field emission scanning electron microscopy
FGF - fibroblast growth factor

G

Gapdh - glyceraldehyde 3-phosphate dehydrogenase
GFP - green fluorescent protein
GMEM - Glasgow Minimum Essential Medium
GMP - Good manufacturing practice
GRGDS - Glycine-Arginine-Glycine-Aspartate-Serine

H

h - hour
HA - hyaluronic acid
hESCs - human embryonic stem cells
^{1}H NMR - Proton nuclear magnetic resonance

I

ICM - inner cell mass
iPS - induced pluripotent stem cells
IR - infrared spectroscopy

K

KSR - KnockOut™ Serum Replacement

L

LBG - locust bean gum
LECs - lymphatic endothelial cells
LIF - Leukemia inhibitory factor

M

MEFs - mouse embryonic fibroblasts
MEFsi - inactivated mouse embryonic fibroblasts
mESCs - mouse embryonic stem cells
min – minutes
Mmp2 - matrix metallopeptidase 2

N

NTC - Non-template controls
NEC - Non-enzyme controls

P

PBS - phosphate buffered saline
PCL - poly (ε-caprolactone)
PCR - polymerase chain reaction
PD - population doubling
PEG - poly (ethylene glycol)
PEG-PTMSMA-PMAA - poly (ethyleneglycol-*β*-trimethylsilyl methacrylate-*β*-methacrylic acid)
PEO - poly (ethylene oxide)
Pgk1 - phosphoglycerate kinase 1
pHEMA - poly(2-hydroxyethyl methacrylate)
PI - propidium iodide
PIPAAm - Poly(*N*-isopropylacrylamide)
PLGA - poly (lactic-co-glycolic acid)
PLLA - poly(lactic acid)
PS – polystyrene

Q

qPCR - quantitative PCR

R

RNA - Ribonucleic acid
rpm - revolutions per minute
RQI - RNA quality indicator
RT - room temperature

S

s - singlet signal in ^{1}H NMR spectrum
SDS - sodium dodecyl sulfate
SE - secondary electron

T

Tbp - TATA binding protein
TCPS - tissue culture polystyrene
te - elution time
TEM - Transmission Electron Microscopy
TGFβ - Transforming growth factor beta
THF - tetrahydrofuran

U

uPa - urokinase-type plasminogen activator
UV - ultraviolet

V

VEGF-C - vascular endothelial growth factor-C
v/v - volume per volume

W

w/v - weight per volume

Chapter I

General Introduction

1. INTRODUCTION

In 1981, a major breakthrough occurred in science, especially in the field of developmental biology, with the isolation of embryonic stem cells from the inner cell mass of the blastocyst (Evans and Kaufman, 1981; Martin, 1981). Although the research on adult stem cells began about 60 years ago with the discovery of stem cells in the bone marrow, the techniques for culturing mouse embryonic stem cells were first reported only 30 years ago (Evans and Kaufman, 1981; Martin, 1981) and versions of these seminal procedures are still used today as standard procedures.

ESCs have capacity to self-renew and to differentiate, theoretically, into all tissues types that constitute the living organism. Therefore, stem cells are a potential cell source for tissue engineering and cell therapy applications (Gepstein, 2002; Nelson et al., 2010; Weiss, 2013; Wollert and Drexler, 2005). Despite that the major advances in cell culture techniques occurred since 1981, almost all tissue cells have been studied in Petri dishes, multi-well plates or glass slides coated with different substrates. However, all of these could differ radically from the three-dimensional (3D) microenvironment in the body and, consequently, cells isolated from tissues of higher organisms frequently modify their metabolism, morphology and gene expression profile as an adaptation process to the culture conditions (Gelain et al., 2006; Hutmacher et al., 2009; Ouyang et al., 2007; Petersen et al., 1992; Santos et al., 2012; Von Der Mark et al., 1977). Furthermore, up to date the existing two-dimensional (2D) and 3D culture systems formed from animal-derived biomaterials contain residual growth factors and undefined constituents that make it difficult to use them in human regeneration or replacement therapies.

1.1. Cell culture

1.1.1. Concepts and Historical background

Tissue culture (a generic term to include organ culture and cell culture) was first defined at the beginning of the twentieth century as a method for studying the behavior of animal cells free of systemic variations that might arise in vivo both during normal homeostasis and under the stress of an experiment (Freshney, 2005). The culture of cells from such primary explants of tissue dominated the

field for more than 50 years, so it is not surprising that the term ''*tissue culture*'' has remained in use as a generic term despite the fact that most of the evolution or expansion in this area in the second half of the twentieth century was made possible by the use of dispersed cell cultures (Freshney, 2005). Indeed, the term *cell culture* is applied when the cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relationships with neighboring cells, are cultured in aseptic laboratory in similar environmental conditions to *in vivo* (Freshney, 2005). Disaggregation of explanted cells or cells that were surgically removed from an organism and subsequent plating out of the dispersed cells so they can attach, divide and grow, was first demonstrated by Rous and was called *primary culture* (Rous and Jones, 1916). The concept of *passaging*, also known as *subculture* or *splitting* cells, which involves transferring a small number of cells into a new vessel, become more evident in 1950s with the generalization of trypsin use, following procedures described by Dulbecco (Dulbecco and Vogt, 1960).

Although animal cell culture was first successfully undertaken by Ross Harrison in 1907 with culture of frog nerve cells (Harrison, 1907), it was only until from the late 1940's to early 1950's that several developments occurred that made cell culture widely available as a tool for scientists. Since the 1950s, further optimization of the culture conditions consisted on the use of antibiotics in the culture media, which facilitated long-term cell line propagation (Perlman, 1979) and avoid many of the contamination problems that plagued earlier cell culture attempts. The development of the techniques, such as the use of trypsin to remove cells from culture vessels was fundamental to obtain continuously growing *cell lines* and colonies of animal cells derived and developed subcultures from a primary cell culture. The 1950s were also the years of the development of standardized and chemically defined cell culture media, which led ultimately to the development of serum-free media (Jayme et al., 1997). Recently, the efforts made to develop alternative supports, scaffolds and coatings provide a widespread increase of quality and a promising future for laboratories and industries that use cell culture as a tool. The stimulus from medical science carried out interest into warm-blooded animals. In warm-blooded animals both normal and pathological development are closer to that found in humans, which encouraged scientists to perform substantial efforts towards the development of *in vitro* biomimetic environments. The accessibility to different tissues, many of which grew well in culture, made the

embryonated hen's egg a favorite choice; but the development of experimental animal strains, particularly with genetically pure strains of rodents, brought mammals to the forefront as the favorite material. The development of transgenic mouse technology, together with the well-established genetic background of the mouse, has added further impetus to the selection of this animal as a favorite species (Freshney 2005).

1.1.2. Cell culture applications

Cell culture has become one of the major tools used in the life sciences today. There are two major advantages in cell culture. One is the control of the physiochemical environment (pH, temperature, osmotic pressure, and O_2 and CO_2 tension), which can be controlled very precisely, and the physiological conditions like the control of hormone and nutrient concentration in the culture medium. The other main advantage is the characterization and homogeneity of sample. After one or two passages, cultured cell lines tends to assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tend to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture, replicate samples are theoretically identical to each other, and the characteristics of the cell line may be perpetuated over several generations (Freshney 2005). In addition, the possibility to perform *in vitro* modeling of *in vivo* conditions avoids moral and ethical questions of animal or human experimentation. Furthermore, in terms of costs, scale and mechanization, the cultures may be exposed directly to a reagent at a lower and defined concentration, so fewer reagents are required in comparison to the use *in vivo*.

The fields of research that lend themselves particularly to cell and tissue culture are summarized in Fig. 1.1:

- *Intracellular activity*, e.g., the replication and transcription of deoxyribonucleic acid (DNA), cell cycle, protein synthesis, energy metabolism, and drug metabolism; Ribonucleic acid (RNA) flux, the translocation of hormone receptor complexes and resultant signal transduction processes, and membrane trafficking;

- *Microenvironmental interaction*, e.g., nutrition, infection, cytotoxicity, carcinogenesis, drug screening and its metabolism, ligand–receptor interactions, therapeutics and diagnostics;
- *Cell–cell interaction*, e.g., morphogenesis, paracrine control, cell proliferation kinetics, metabolic cooperation, cell adhesion and motility, matrix interaction, and organotypic models for medical prostheses and invasion;
- *Genetics*, including genome analysis in normal and pathological conditions, gene therapy, genetic manipulation, differentiation, prenatal diagnosis and genetic counseling;
- *Cell products and secretion*, biotechnology, proteomics, bioreactor design, product harvesting and downstream processing for production of pharmaceutical drugs, vaccines (viruses), recombinant or genetically engineered proteins, monoclonal antibodies.

Despite all the advantages, the cell culture techniques imply a high level of skills and understanding on the part of the operator in order to recognize the requirements of the system and to diagnose problems as they arise.

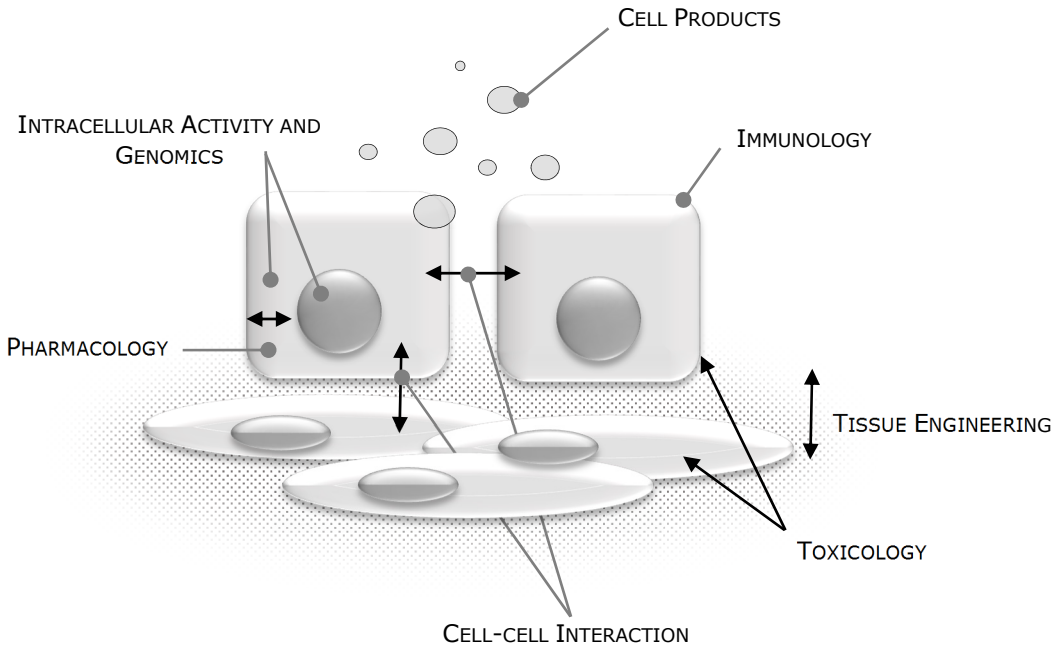

Figure 1.1: Cell culture applications. Cell culture techniques are used in cell and molecular biology research and studies. Some of the important areas where cell culture plays an important role are toxicity testing, cancer research, virology, gene therapy, drug discovery and tissue constructs.

The higher limitations of cell culture is the cost in effort and in materials that goes into the production of relatively little amount of cells and the loss of the phenotypic characteristics typical of the tissue from which the cells had been isolated (dedifferentiation). Furthermore, regarding origin of cells, if differentiated properties are lost it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived (Freshney 2005).
Another common problem observed in continuous culture of cell lines is chromosomal instability. Even with short-term cultures of untransformed cells, heterogeneity in growth rate and the capacity to differentiate within the population can produce variability from one passage to the next (Freshney 2005).
In sum, animal cell culture technique has played and still plays an important role in research and in drug discovery, production of antiviral vaccines and ultimately help improving the health and quality of life of patients suffering from life threatening diseases like cancer or genetic disorders.

1.1.3. Embryonic stem cell culture

At present, a lot of effort has been made to establish and improve the culture of stem cells, in particular culture of embryonic stem cells (ESCs), mainly due to its enormous potential. Stem cells can differentiate into more than one kind of cell depending on whether it originates from an embryo (tissue precursor cells), embryonic stem cells, or from an adult organism also called adult stem cells.
Embryonic stem cell is an unspecialized cell type that is characterized by their ability to generate identical copies of itself (in an undifferentiated state) for indefinite periods in culture (self-renewal), and their capacity to give rise to mature or specialized cells of a variety of cell types (pluripotency; Lanza 2006; Fig. 1.2). Mammalian ESCs are isolated exclusively from the inner cell mass (ICM) of blastocysts in a 5 and 3.5-day pre-implantation human and mouse embryo, respectively (Kaufman et al., 1983; Thomson, 1998). The blastocyst is a structure formed during early embryogenesis of mammals, after the formation of the morula, but prior to implantation. It possesses an inner cell mass, or embryoblast, which subsequently forms the embryo, and an external layer of cells, or trophoblast, which later forms the placenta (Fig. 1.2). The first embryonic stem cells explored were mouse embryonic stem cells (Evans and Kaufman, 1981).

Mouse derived ESC lines have been cultured and maintained in a feeder cell layer of mouse embryonic fibroblasts (MEFs) because they provide a complex mixture of nutrients and substrata for the long term growth and proliferation of undifferentiated pluripotent ESCs. Since mouse ESCs derivation in 1981 mouse ESCs continue to be one of the most used in research as its use does not raise as many legal and ethical issues like human ESCs (hESCs).

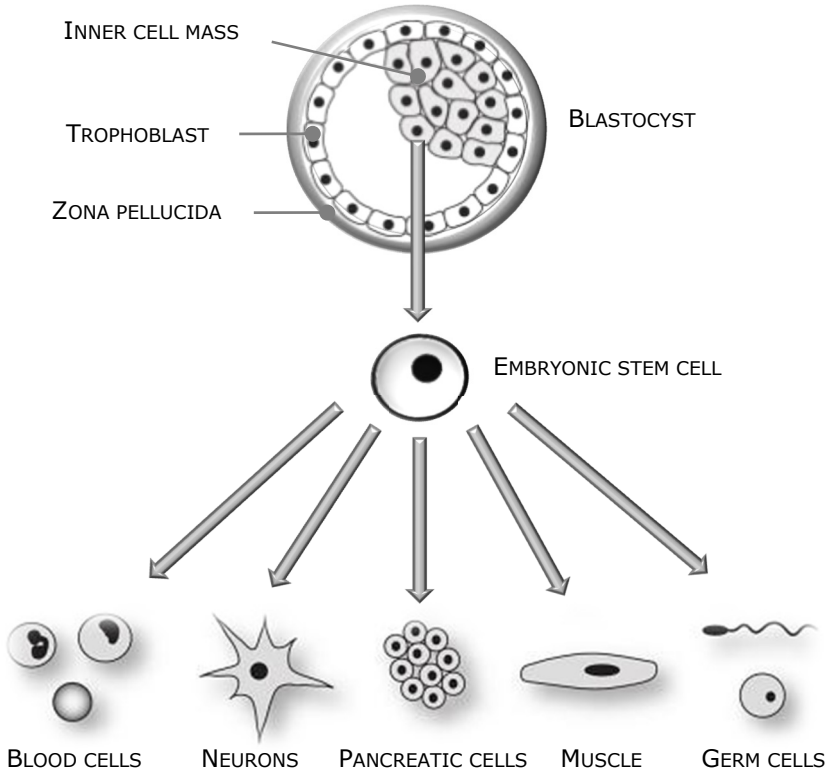

Figure 1.2: Differentiation of embryonic stem cells. Embryonic stem cells are obtained from the inner cell mass of a blastocyst. When cultured, these cells can differentiate into many different kinds of cells, representing the three germ layers. Adapted from (Panno, 2005).

1.1.4. The potential of embryonic stem cells

The importance of ESCs for living organisms is unquestionable. The inner cell mass gives rise to the entire body of the organism, including all of the many specialized cell types and organs, such as the heart, lungs, kidney, pancreatic, skin and germ cells e.g. sperm or oocytes. Later in adulthood, stem cells function as a sort of internal repair system in many tissues, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. Stem cells allow

blood, bone, gametes, epithelia, nervous system, muscle, and myriad other tissues to be replenished by fresh cells throughout life. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells that lie dormant can be activated at particular life cycle stages or following injury, to replace cells that are lost through normal wear and tear, injury, or disease.

Taking into account their potential, embryonic stem cells are a powerful tool for the study of gene function and regulation, the generation of cellular and animal models for human diseases, and the investigation of cell differentiation (Kokron et al., 1997). Successful derivation of murine ESCs from ICM of mouse blastocysts and subsequent development of a precise gene targeting strategy by homologous recombination in ESCs has led to the development of highly sophisticated techniques for generation of genetically altered animal models (Keskintepe et al., 2007). Genetically altered mice represent powerful models to dissect and understand complex biological processes, as well as to manipulate gene expression towards development of therapeutic strategies for a variety of diseases including cancer, inflammatory and infectious diseases, and neurogenetic and cardiovascular disorders (Huijbers et al., 2011). Initially, scientists were interested in embryonic stem cells because they provided an approach to improve our understanding of how the body develops from a fertilized egg, the normal embryo development, so to study totipotency and the plasticity of cells during embryonic development. Nevertheless, it is clear now that as ESC research the understanding of how tissues are specialized, maintained and repaired in health and allow us to understand how diseases occur and develop, ESCs may be used to treat and cure a wide variety of diseases (Fig 1.3 and 1.4). Furthermore, the most serious diseases, such as cancer or congenital defects, are the result of problems in the process of stem cell differentiation towards more specialized cells. Therefore, as soon as scientists understand better normal cell/tissue development, we will be able to understand and perhaps prevent or correct the errors the causes of these medical conditions. Stem cells are emerging as one of the fundamental foundations in the field of tissue biology. The most powerful potential application of stem cells is in the growth organ specific cell type(s) and consequent construction of tissues and organs for medical therapies as an alternative to the donated organs and tissues. This application of stem cells would solves a medical and social problem because today the number of people needing a transplant far exceeds the number of organs donated for transplantation. The process of replacing or regenerating human cells,

tissues or organs to restore or establish normal function, is refereed as regenerative medicine (Mason and Dunnill, 2008). Depending on the source of cells, regenerative medicine can potentially solve the problem of organ transplant rejection if the organ's cells are derived from the patient's own tissue or cells. In that field, the induced pluripotent stem cell (iPS) technology recently developed by the Nobel laureate Shinya Yamanaka (Takahashi and Yamanaka, 2006) may allow the generation of cells/organs genetically tailored to a patient, thus eliminating the concern of immune rejection. Up to date, there are several successful examples of the use of regenerative medicine, such as the injection of stem or progenitor cells (cell therapies), the induction of regeneration by biologically active molecules administered alone or as a secretion by infused cells to stimulate resident adult stem cells (immunomodulation therapy) and transplantation of *in vitro* grown organs and tissues (tissue engineering).

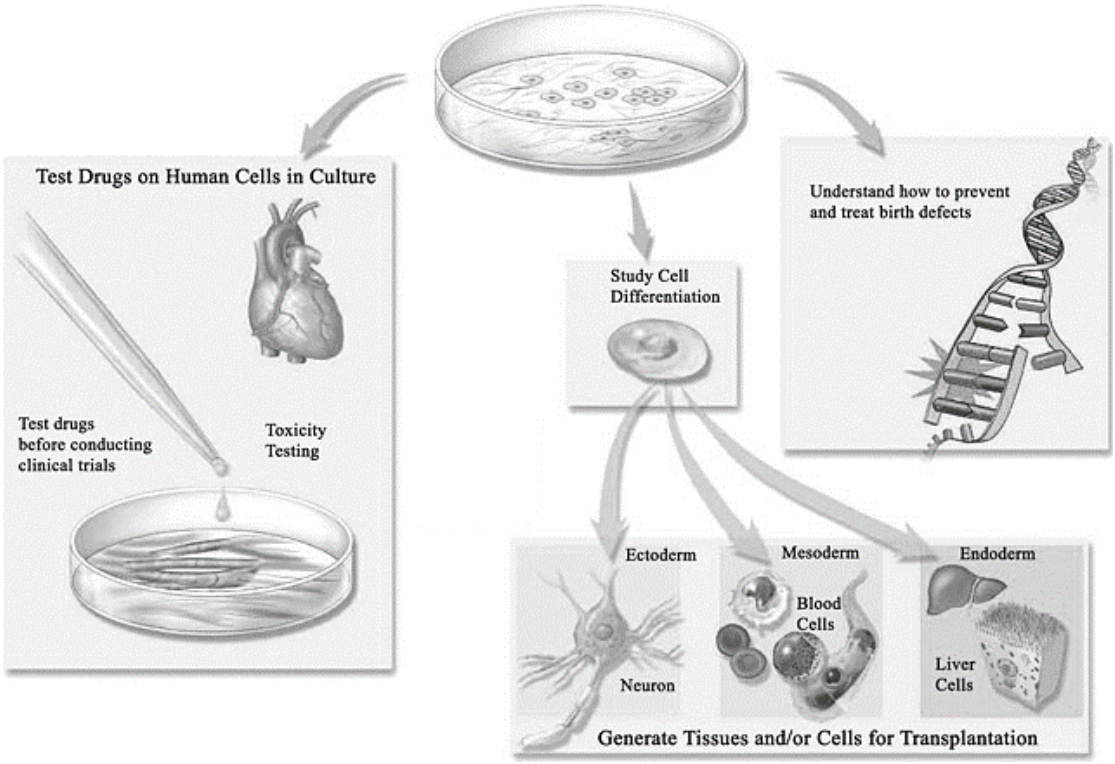

Figure 1.3: The promise of stem cell research. Stem cell research offers a useful tool for unravelling the molecular mechanisms that determine the differentiation fate of a pluripotent cell and for understanding the gene expression properties and epigenetic modifications essential to maintain the pluripotent state. This knowledge may be used to generate cells for transplantation therapies, whereby a specific cell population compromised by disease is replaced with new and functional cells. Differentiated derivatives of human pluripotent cells are also useful as models for understanding the biology of disease and developing new drugs, particularly when there is no animal model available for the disease to be studied. Adapted from (Yu and Thomson, 2006).

The combined use of pluripotent stem cells and regenerative medicine offers the possibility of a renewable cell source of replace cells and tissues in order to treat numerous diseases, conditions, genetic and degenerative disorders (Fig. 1.4). Among them, Parkinson's disease, amyotrophic lateral sclerosis, spinal cord injury, multiple sclerosis, age-related functional defects, hematopoietic and immune system disorders, heart failures, chronic liver injuries, burns, lung disease, diabetes and arthritis can be treated trough stem cell-based therapies and are currently being developed (Fig. 1.4.) (Diekman et al., 2012; Hsu et al., 2012; Tewarie et al., 2008; Weiss, 2013). The culture of stem cells makes possible the production of tissues and cells for transplantation, for example, pancreatic islet cells for diabetes, heart muscle cells for heart disease, nerve cells for Parkinson or Alzheimer's disease and hematopoietic stem cells or bone marrow for leukemia or other blood disorders and chemotherapy (Fig. 1.4). In general, ESCs can be applied in both scientific research and medical fields, such as in discovery science (human development, pathobiology, genotype/phenotype and molecular mechanisms), diagnostics (regenerative potential, drug toxicology, individualized medicine) and therapeutics (congenital or acquired heart disease, regenerative biologics, immunity and innate rejuvenation).

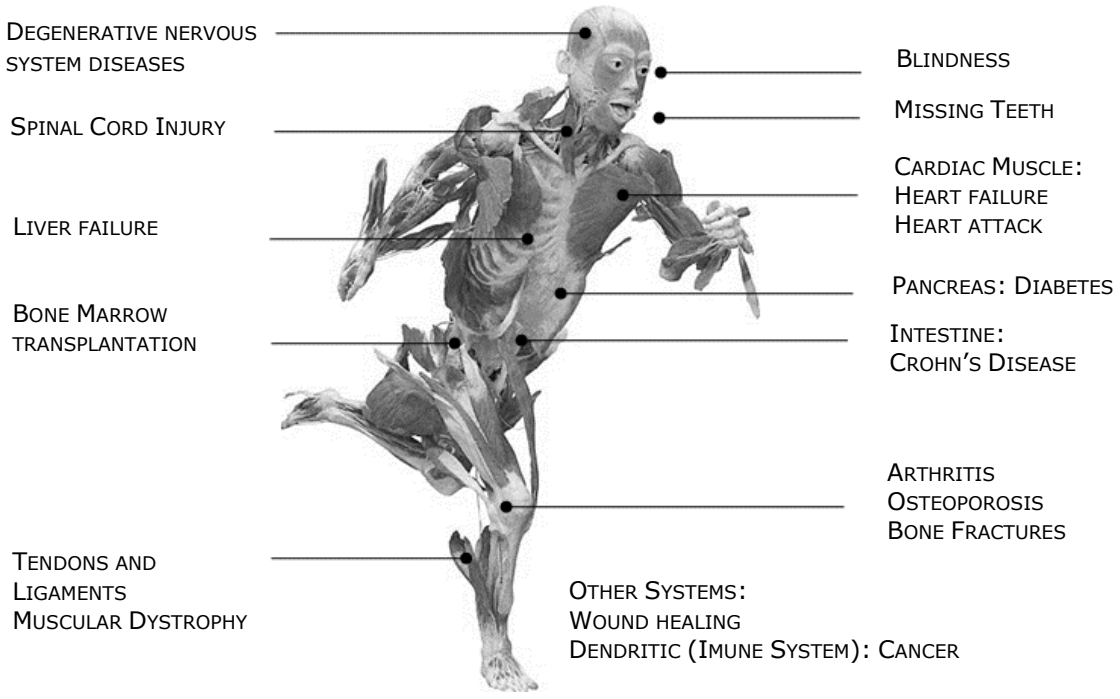

Figure 1.4: Potential medical applications of stem cells. Diseases and conditions where stem cell treatment is promising or emerging. Human model from "Bodies – The Exhibition".

The most attention-grabbing breakthroughs in stem cell science involve the promise of its use in regenerative medicine (cell and tissue transplantation), as well as, application in pre-clinical models for drug development, and as "disease in a culture dish" models for testing drug efficacy and mechanistic studies. Other significant benefit of stem cell technologies is the possibility to develop *in vitro* models using human-derived stem cells, that hold a great potential for the development of biologically relevant models for evaluating the toxicity of substances and perform drug screening. Recent progress at incorporating stem cell niches into a bioengineered human tissue model is perhaps the most interesting development in stem cell field.

1.2. Stem cell niche

The stem cell niche concept was firstly introduced in 1978 (Schofield, 1978) and represents the natural microenvironment that surrounds stem cells, i.e. the local tissue microenvironment capable of housing and maintaining one or more stem cells, which in combination with extrinsic and intrinsic factors, determine the behavior and fate of stem cells (Morrison and Spradling, 2008). Until recently, niches were a theoretical concept supported by the observation of that transplanted stem cells survived and grew only in specific locals. However, such structures were recently characterized in diverse tissues of invertebrates and allow the establishment of principles that probably coordinate the behavior of niches in other organisms. In adult organisms, is thought that the majority of stem cells are in a quiescent state, but can be activated by exogenous factors, starting division and differentiation and thus contributing to the renewal or repair of tissues or organs. Therefore, stem cell niches play a crucial role in maintaining tissue homeostasis and tissue repair and regeneration in case of injuries (Walker et al., 2009). For instance, neuronal stem cells from central nervous system are located in the lateral ventricles in the subventricular zone after embryonic development and in the dentate gyrus of the hippocampus in adult brain (Conover and Notti, 2008). In other cases, the local of stem cells niche can be dynamic, namely the localization of cells from hematopoietic system that in steady-state conditions reside in bone marrow and occupy facultative niches, distributed in the trabecular surface of bone (Morrison and Spradling, 2008). Indeed, hematopoietic stem cells

are constantly in circulation from a compartment of bone marrow to other (for instance, from femur to tibia). The capacity of activating facultative niches is not limited to the hematopoietic system, namely, in adult skin, new hair follicles can be formed after injury when nascent follicles arise from epithelial cells outside of the hair follicle stem cell niche, which suggests that epidermal cells in the wound assume a hair follicle stem cell phenotype (Ito et al., 2007). Furthermore, it was also verified that the complexity of spatial organization of ESC in culture, in combination with exogenous factors [leukemia inhibitory factor (LIF), fibroblast growth factor (FGF) and transforming growth factor beta (TGFβ), supporting cells] created niches or heterogeneous microenvironments that influence ESC fate (Peerani, 2009).

In vivo, resident adult stem cells are located in tissue-specific anatomically defined clusters called "niches". The stem cell niche is highly complex and dynamic, including both cellular and acellular components, which provides spatial and temporal cues to support and coordinate stem cell activities, regulating their cell fate (Lund et al., 2009). Extensive studies in numerous laboratories have begun to elucidate the critical components of many stem cell niches, which include mesenchymal, vascular and inflammatory cell types, diffusible and cell surface-associated signaling molecules, and physical parameters such as matrix rigidity, shear stress, oxygen tension, and temperature (Fig. 1.5). Within the stem cell niche, cell fate is thought to be controlled both spatially and temporally, as well as through cell-cell and cell-matrix interactions (Nava et al., 2012). The cell-cell and cell-ECM interactions not only provide structural support, regulate adhesion and produce soluble signals that can control stem cell function, but also provide mechanical signals, based on substrate rigidity, which allow stem cells to respond to external physical forces (Choi et al., 2011; Sun et al., 2012). In addition, acellular or physic elements, such as pH, temperature and shear forces, and chemical signals provided by the niche also modulate stem cell behavior in response to the external environment (North et al., 2009) (Fig. 1.5).

In sum, these cellular and acellular components appear to be integrated by stem cells to inform their fate decisions, including choices between quiescence or proliferation, self-renewal or differentiation, migration or retention, and cell death or survival (Wagers, 2012).

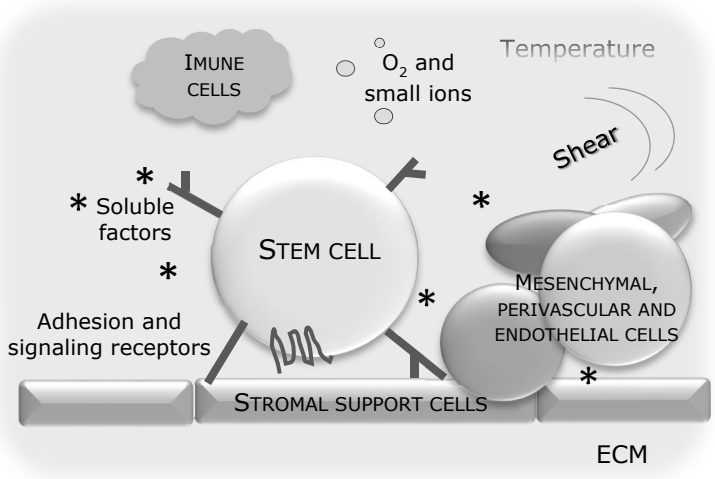

Figure 1.5: Constituents of a stem cell niche. Stem cell niches are highly complex and dynamic, including both cellular and acellular components. In the niche, stem cells are exposed to ECM, soluble and immobilized molecules (cytokines, metabolic products), support cells, and physical cues (topography, structure, stiffness, static and dynamic forces).

In vitro, the microenvironment or niche of a cell, comprises the culture substratum, i.e. the appropriate culture dish or specifically coated surface, which allow attachment and spreading of cells (*the contact environment*) and the culture medium, which represents *the diffuse environment*. The latter comprises all types of soluble molecules – nutrients and salts, hormones and growth factors (Gstraunthaler, 2003). Cell-matrix and cell-cell junctions crosstalk, and these two junctions cooperatively regulate cell movement, proliferation, adhesion and polarization (Sakamoto et al., 2006).

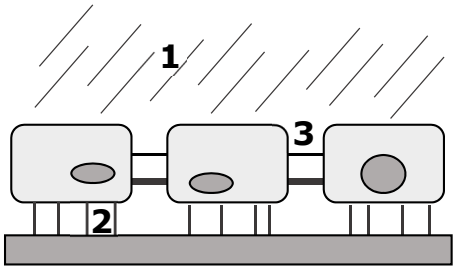

Figure 1.6: Parts of the microenvironment of the mammalian cell regulating its behavior *in vivo* and *in vitro*. (**1**) The diffuse environment, which in cell cultures is solely provided by the culture medium, (**2**) the contact environment (cell-matrix adhesion), and (**3**) the junction connections between neighboring cells (cell-cell adhesion). Adapted from (Gstraunthaler, 2003).

1.3. Biomaterials as structural support for ESC culture

As mentioned above, the extracellular microenvironment plays a pivotal role in controlling stem cell fate responses (Kshitiz et al., 2013). Therefore, to enable precise control of the ESC niche it is necessary to control of cell-cell contacts, cell-ECM interactions, cell-soluble factor interactions as well as mechanical and electrical stimuli in a spatiotemporal manner (Khademhosseini et al., 2006b). Consequently, identification of appropriate environmental stimuli that support cellular proliferation, pluripotency or lineage specific differentiation, is critical for the clinical application of the stem cell therapies.

Even though the first mouse embryonic stem cell lines were derived more than 3 decades ago and standard protocols for ESC cultivation are widely used today, many efforts are being made to mimic the endogenous microenvironment of cells in a body. The ultimate goal is to find an approach that is closest to the *in vivo* situation, but where the functional properties of cells can be easily observed and manipulated (Gelain et al., 2006). To the researcher, a suitable culture environment is one that does more than just allow cells to survive in culture. Instead it usually means an environment that at the very least allows cells to increase in number by undergoing cell division. Indeed, when conditions are the adequate, some cultured cells will express their "wellness" with their environment by carrying out important *in vivo* physiological or biochemical functions, such as muscle contraction or the secretion of hormones and enzymes. Therefore, a number of engineering approaches such as surface modifications, coatings and scaffolds have been applied in an attempt to control the ESC niche (Edalat et al., 2012).

ESCs are anchorage-dependent cells and hence they require a good substrate for attachment and growth. Feeder cells such as xenogenic MEFsi are commonly used and necessary for the *in vitro* maintenance and growth of many cell types, in particular ESCs (Hu et al., 2012; Khademhosseini et al., 2006a) or induced pluripotent stem cells. In this approach, MEFsi are a niche-supporting cells providing a suitable microenvironment for maintenance and growth of undifferentiated ESCs due to their ability to supply growth factors like LIF, activin, bone morphogenetic proteins (BMPs), insulin-like growth factor, and cytokines and extracellular matrix proteins, such as laminin, fibronectin and vitronectin (Peerani, 2009). Nevertheless, animal-derived feeder cell-based culture methods can cause

unexpected disadvantages such as uncertain data outcomes, xenotransmission of unknown pathogens and may impede the establishment of stable culture conditions. Consequently, the development of non-cellular niches for defined stem cell culture systems has become a priority. Several approaches have been developed to control the morphology and function of cultured ESCs by the design of the culture surface. In 1987, Prof. David F. Williams and a group of experts defined biomaterial as "all substances (other than drugs) designed to become in contact with biological systems with the aim to evaluate, treat, raise or replace any tissue, organ or function of the organism" (Williams, 1987). Since then, a high diversity of biomaterials have been developed for tissue engineering and to serve as support to a stem cell niche *in vitro*. These materials function as platforms for cell attachment, migration, proliferation and differentiation.

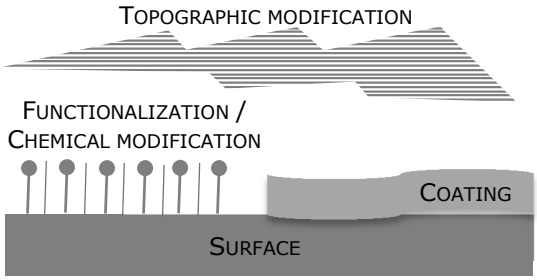

Figure 1.7: Surface modifications as a general approach for controlling ESC fate. The growth surface can be improved by chemical modifications, immobilization of bioactive molecules or attachment factors, coating with an attractive matrix regarding the cells under study and by introducing three-dimensionality to cell culture.

Biomaterials can present a variety of structures depending on their composition and processing methods. In general, the modification of culture surfaces comprises chemical modifications, biofunctionalization and geometric or topographic modifications (Fig. 1.7) (Mashayekhan and Miyazaki, 2011).

Concerning the *chemical modifications*, treated plastics are the most commonly used substrates. Plasma-modified tissue culture polystyrene (TCPS) is widely used in cell culture for an ample range of different cell types (Biazar et al., 2011; Sasai et al., 2009). On the other hand, Salgado *et al* proposed a new concept of miniaturized device for combinatorial screening of chemical composition and cytocompatibility of biomaterials. For that, they demonstrated that polystyrene (PS) can be used to produce superhydrophobic surfaces and further UV/ozone irradiation modify the wettability of such substrates up to the superhydrophilic regime, using hollow photomasks to generate spot regions in which biomaterials

can be then dispensed and tested (Salgado and Oliveiraz, 2012). Furthermore, hydrophobic surfaces were also used for enhanced differentiation of embryonic stem cell-derived embryoid bodies (Valamehr et al., 2008). On the other hand, *immobilization of bioactive molecules or attachment factors*, such as collagen, gelatin, fibronectin and laminin, can be used depending on the main purpose of the culture as substrate coatings to improve growth and function of normal cells. These biomolecules can be simply adsorbed onto the material's surface or covalently linked via chemical groups previously created on the surface.

Biomaterials from animal sources like gelatin, collagen gels, polyglycosaminoglycans and Matrigel™ are examples of surfaces widely used for stem cell culture that, besides the good results in terms of cell growth, cannot be used for transplantation therapies because of the possibility of containing residual undefined components or impurities and consequently promote immunity responses (Greenlee et al., 2005; Mallon et al., 2006). In addition, despite that animal-derived biomaterials present multiple ligands which enhances surface functionality, they do not completely mimic the highly complex embryonic stem cell niche. In most cases, the immobilization or adsorption of a coating on the vessel growth area is not enough to create a suitable ESC culture microenvironment. *In vivo*, topographical structures such as grooves and ridges at the nano and microscale level are present, such as in the fibrous ECM proteins (Fig. 1.8) and the rough mineralized bone, that are difficult to reproduce *in vitro*.

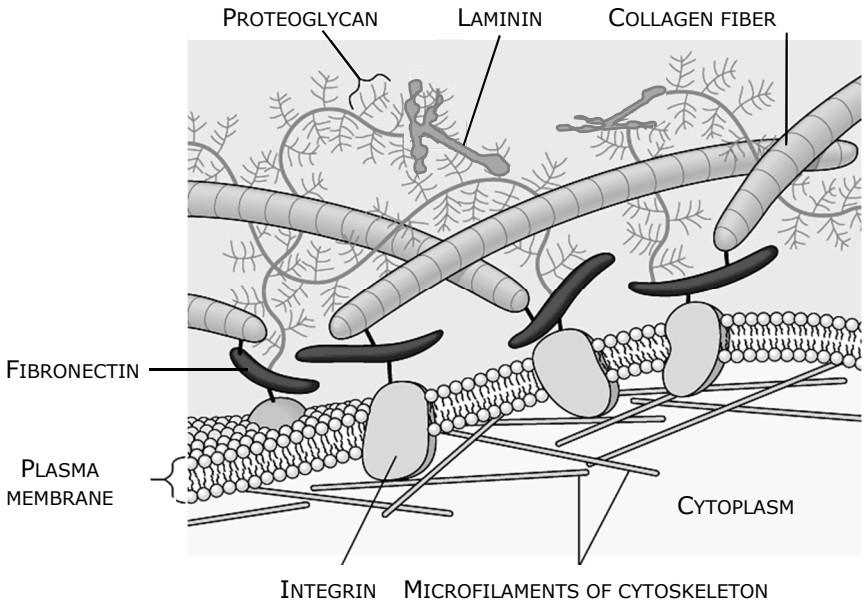

Figure 1.8: An overview of the macromolecular organization of the extracellular matrix. ECM is a highly dynamic and complex structure that surrounds and supports cells.

Structural ECM proteins consist primarily of the collagen and elastin families of proteins. Collagen fibers strengthen and organize the matrix; elastin fibers provide flexibility and resilience. Nonstructural ECM proteins, such as fibronectin, laminin, and tenascin have an adhesive or integral role; these proteins allow for cell attachment and form crosslinks within the matrix gel. Finally, numerous proteoglycans and heparan sulfate-containing proteins form the hydrated, gel-like mixture that stabilizes the matrix within its aqueous environment. Adapted from (Campbell and Reece, 2005).

Many efforts have been made in the last decade to engineer substrate materials' topography to promote stem cell responses, at both the cell and tissue levels (Park et al., 2007). Numerous methods can be used to fabricate or engineer materials, however the most widely used techniques are those that allow the development of nanofibrous systems, tubes or meshes, which have the potential to provide enhanced cell adhesion by virtue of the similarity of their 3D architecture to natural ECM. Currently, there are several techniques available for the synthesis of nanofibers: electrospinning, melt-blow, self-assembly, injection molding, template synthesis and phase separation. Of these techniques, electrospinning, followed by self-assembly, are the most widely studied techniques and has also demonstrated the most promising results in terms of tissue engineering applications (Vasita and Katti, 2006). Accordingly, the results reported in Alam Nur-E-Kamal *et al* study constitute the first demonstration that a 3D nanofibrillar surface composed of electrospun polyamide nanofibers (Ultra-Web®) can promote the proliferation and self-renewal of mESCs. The data highlight the role of dimensionality in maintaining stemness in proliferating mESCs and suggest that nanofibers may provide an important new tool for promoting stem cell proliferation for applications in regenerative medicine (Nur-E-Kamal et al., 2006). In another report, Anli Ouyang *et al* concluded that the smaller-pore polyethylene terephthalate fibrous matrix produced by electrospinning technique is favorable for growing and maintaining undifferentiated ESCs (Ouyang et al., 2007). Another easy and versatile technique to produce fibers (and other structures) is by self-assembly of block copolymers. Self-assembly is a "bottom up" approach, that refers to the spontaneous association of numerous individual entities into a coherent organization and well-defined structures to maximize the benefit of the individual parts without external instruction; it is reversible, and can be controlled by the proper design of the components, the environment, and the driving force (Pelesko, 2007). A block copolymer is a polymer derived from more than one species of monomer and is formed when the different monomers cluster together giving rise to 'blocks' of

repeating units. Because of the block architecture, the copolymers can self-assemble into a variety of different morphologies (Fig. 1.9). Variation of block length allows the adjustment of the typology and size of these structures on a scale between 1 nm and 10 µm (Förster and Plantenberg, 2002).

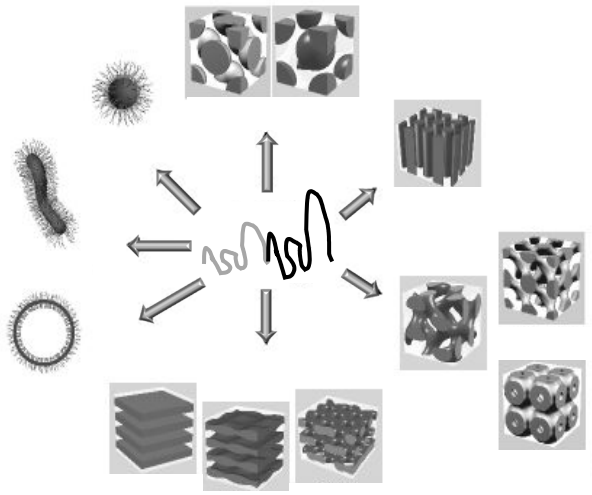

Figure 1.9: Block copolymer microstructure formed in bulk and solutions by self-assembly. Self-organization of block copolymers give rise to structures with different shapes, such as spherical micelles, cylindrical micelles, vesicles and simple lamellae. Adapted from (Förster and Plantenberg, 2002).

Several factors have to be considered when designing the appropriated cell or tissue culture support, i.e. fabrication cost and easiness, porosity, biocompatibility, cytotoxicity, fluid transport for the exchange of gases and nutrients, biochemical and mechanical properties and biodegradability, depending on the intended degree of cell-materials interaction. A wide range of biomaterials have been developed for different cell and tissue culture applications. These biomaterials can be divided into three major classes: polymers (natural or synthetic), metals and ceramics (Bhat and Kumar, 2012).

1.4. Natural biomaterials for ESC culture

For several decades, ECM from natural sources has served as an important tool for biologists. In fact, the development of natural biomaterials has existed for centuries and natural polymers are considered the first biodegradable biomaterials used clinically (Ige et al., 2012). Biopolymers or natural polymers are an attractive class of polymers since they are derived from natural sources, relatively easily available and cheap and can be chemically modified. Natural polymers can be

classified as proteins, polysaccharides or polynucleotides. Proteins and polysaccharides play several roles *in vivo* and thus make such materials attractive for cell or tissue engineering applications. Additionally, due to their natural origin, usually these polymers presented components containing sites for cellular adhesion and tend to be biocompatible.

The use of natural products as a biomaterial is currently undergoing a resurgence in the biomedical field and biopolymers have received particular attention as tissue engineering substrates for both 2D and 3D cell culture (Jana et al., 2011). Furthermore, natural polymers are widely used because they inherently possess adhesive ligands and their cellular origin, similarities with the ECM and their biological activity properties promote cellular adhesion, interaction and further improvement of cell' performance in biological system. For this same reason, natural polymers can be disadvantageous in isolating certain cell responses. Other limitations of natural polymers include the lot-to-lot variability in molecular structure depending on the source and producer, as well as the need to ensure the purity of the polymer in case of replacement therapies to avoid immunogenic response.

1.4.1. Protein-based biomaterials

One of the main functions of proteins is to provide structure to tissues and this property suggests that protein-based biomaterials would be suitable for tissue engineering applications involving stem cell differentiation and transplantation. Protein-based biomaterials such as collagen, elastin, gelatin and fibrin are widely used as cell culture coatings or matrices as they make up much of the body's native ECM (Sell et al., 2010).

1.4.1.1. Gelatin and Matrigel®

Gelatin and Matrigel®, commonly used as support of ESC culture (Greenlee et al., 2005), are heterogenous materials composed of complex protein mixtures. Gelatin is an animal-derived material produced by partial hydrolysis of collagen extracted from boiled bones, connective tissues and organs of animals, and this poses problems for use in cell replacement therapies (Gorgieva and Kokol, 2011). On the other hand, Matrigel® matrix is a reconstituted basement membrane preparation

that is extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM proteins. This material, once isolated, is approximately 60% laminin, 30% collagen IV, and 8% entactin, but also contains uncharacterized number of growth factors that varies substantially between batches (Hughes et al., 2010). Therefore, these biomaterials have limited applications for cell or tissue replacement therapies due to viability and to the immunogenic response that can occur following implantation associated with their animal-derived source.

1.4.1.2. Fibrin and Collagen

Fibrin and collagen are two major components of ECM microenvironment commonly used as biopolymers in tissue engineering due to their availability, scaffolding function, and bioactive qualities (Beier et al., 2009; Couet et al., 2007). Fibrin biopolymer has a structural role and is a biochemical stimulant of the wound healing response (Janmey et al., 2009). On the other hand, collagen is characterized by a high stiffness and tensile strength that imparts resistance to tensile and shear loads in soft tissues (Viguet-Carrin et al., 2006). Therefore collagen, fibrin or collagen-fibrin composites are used clinically in drug delivery (Nishimura et al., 2013) and as a surgical sealant (Moench et al., 2010), as well as supportive materials for cell culture (Lee et al., 2010), skin (Han et al., 2010) and vascular tissue engineering (Cummings et al., 2004). An advantage of using solubilized collagen and fibrin in tissue engineering is that they can be molded and reconstituted into essentially any desired geometry, however some authors alerted that mechanic properties of collagen-fibrin gels may not be plastic enough to mimic some tissues (Lai et al., 2012).

1.4.2. Polysaccharide-based biomaterials

Polysaccharides, polymeric carbohydrate molecules composed of long chains of monosaccharide units bound by glycosidic bonds, are extensively used in recent years in biomedical and pharmaceutical applications. Polysaccharides represent one of the most abundant industrial raw materials. Their sustainability and intrinsic properties such as biodegradability and biodegradability, make them an interesting subject of intensive research. In addition, polysaccharides have interesting physical properties (film-forming, gelling and thickening properties) that allow

their use in different applications and its processing in different forms such as beads, films, capsules and fibers (Rinaudo, 2008). Some of the most commonly used polysaccharide-based scaffolds and coatings used for stem cell culture and differentiation include agarose, alginate, hyaluronan, starch, carrageenans, guar and xanthan gum and chitosan.

1.4.2.1. Agarose and Alginate

Agarose, which is isolated from red algae and seaweed, is commonly used as a medium for cell culture in the form of agar and as a platform for motility (Mousseau et al., 2007) or chemotactic invasion assays (Wiggins and Rappoport, 2010). One of the attractive properties of agarose is that its stiffness can be altered, allowing for tuning of the mechanical properties of the scaffold. Therefore, agarose scaffolds have been used to culture eukaryotic cells (Gordeev et al., 2012), in combination with mesenchymal stem cells for differentiation in chondrocytes (Schmitt et al., 2012) or with induced pluripotent stem cells (iPS) for cartilage defect repair (Diekman et al., 2012). Furthermore, Sigma Life Science developed the 3D Petri Dish®, composed of agarose, which maximizes cell-to-cell interactions. It comprises a broad selection of micro-molds used to form applicable culture formats for the creation of spheroids, gliomaspheres, hepatospheres, chondrospheres, osteospheres, cell aggregates, neurospheres, cardiospheres and embryoid bodies. Alginate is one of the most studied and applied polysaccharidic polymers in tissue engineering and drug delivery field, mainly due to its pH-sensitive property. In general, alginate is modified or combined with other polymers to form hydrogel matrices, microspheres or micelles beads (H. Liu et al., 2008) and used for cartilage (Dobratz et al., 2009), bone (Suárez-González et al., 2010) and vascular tissue engineering (Lee et al., 2003), and also to obtain platforms for toxicology screenings (Lan and Starly, 2011). AlgiMatrix™ is an animal-free product and a ready-to-use sponge that allows cells to invade the pores and secrete endogenous ECM components that support in vivo-like morphologies, structures and behaviors. AlgiMatrix™ provides formation of vascularized embryoid bodies (EBs) (Gerecht-Nir et al., 2004) and is applicable for development of cardiac co-cultures (Dar et al., 2002) (Fig. 1.10).

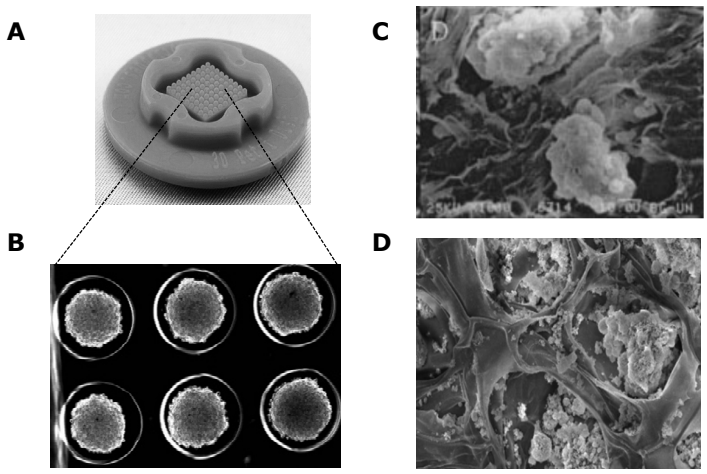

Figure 1.10: Cell culture in agarose and alginate. A-B. MicroTissues® 3D Petri Dish® micro-mold for spheroids. **A.** Autoclavable precision micro-mold to cast 3D Petri Dish for forming larger spheroids. **B.** Human mesenchymal stem cells seeded on the 3D Petri Dish used to form large EBs or spheroids. **C.** Scanning electron microscopy (SEM) of cardiac cells morphology within alginate scaffolds at day 3 postseeding (Dar et al., 2002). **D.** SEM of LF120 alginate scaffolds seeded with hESCs and cultured for 1 month with hEBs developed mainly within the confining scaffold pores (Gerecht-Nir et al., 2004).

1.4.2.2. Starch

Starch, which is the major dietary source of carbohydrates, is used in medicine based on its adhesive, thickening, gelling, swelling and film-forming properties, as well as its ready availability, low cost and controlled quality. Starch chemical structure can be variable concerning its origin. Most starches are composed of two kinds of polysaccharides, a linear α-(1→4) linked glucan, called amylose, and an α-(1→4) linked glucan with 4.2 to 5.9% α-(1→6) branch linkages, called amylopectin (Fig 1.11 A). Starch is mainly used in pharmaceutical applications as excipient, tablet disintegrant, hydrogel, microcapsules, nanoparticles and a few reports describe the use of starch as a biomaterial for bone-tissue engineering (Salgado et al., 2004) or for culture of fibroblasts (Reddy and Yang, 2009) (Fig. 1.11 A, B).

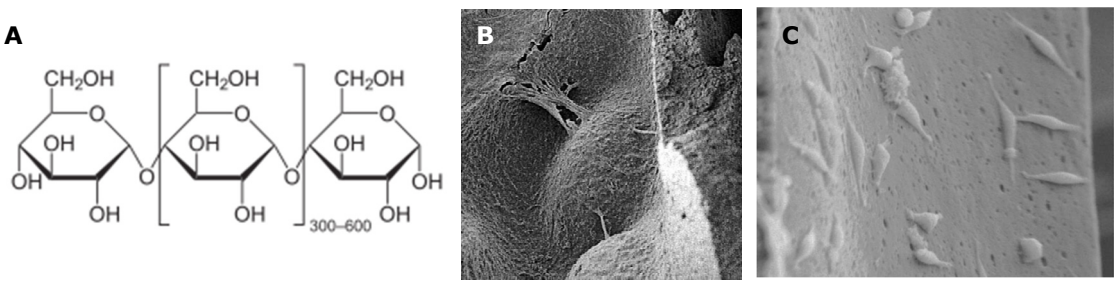

Figure 1.11: Starch chemical structure and culture of fibroblasts and osteoblasts in starch-derived supports. A. Chemical structure of a segment of amylose (simple or

unbranched starch) adapted from (Robyt, 2008). **B.** SEM images showing the attachment of mouse fibroblasts on the starch acetate fibers, 5 days after seeding (Reddy and Yang, 2009). **C.** SEM of starch-based scaffolds showing human osteoblast-like cells colonizing the inner regions of the scaffolds and collagen fibril deposition, indicating the possible deposition of bone extracellular matrix (Salgado et al., 2004)

1.4.2.3. Chitosan

Chitosan is an animal derived linear polysaccharide, composed of glucosamine and N-acetyl glucosamine units linked by β (1–4) glycosidic bonds (Fig. 1.12 A). Chitosan, the fully or partially deacetylated form of chitin, has also attracted much attention in tissue engineering and drug delivery research fields with a wide variety of applications ranging from skin (Hilmi et al., 2013), bone (B Malafaya et al., 2005), cartilage (Yang et al., 2011) and vascular grafts (Qiu et al., 2009) to fibrous substrates for fibroblasts (Nie et al., 2012), mesenchymal stem cells (Yang et al., 2011), adipose tissue derived-stem cells (B Malafaya et al., 2005), osteoblasts and chondrocytes (Lahiji et al., 2000), cardiomyocytes (Karp et al., 2006) and neuronal (Wang et al., 2009) cell culture (Fig. 1.12 B).

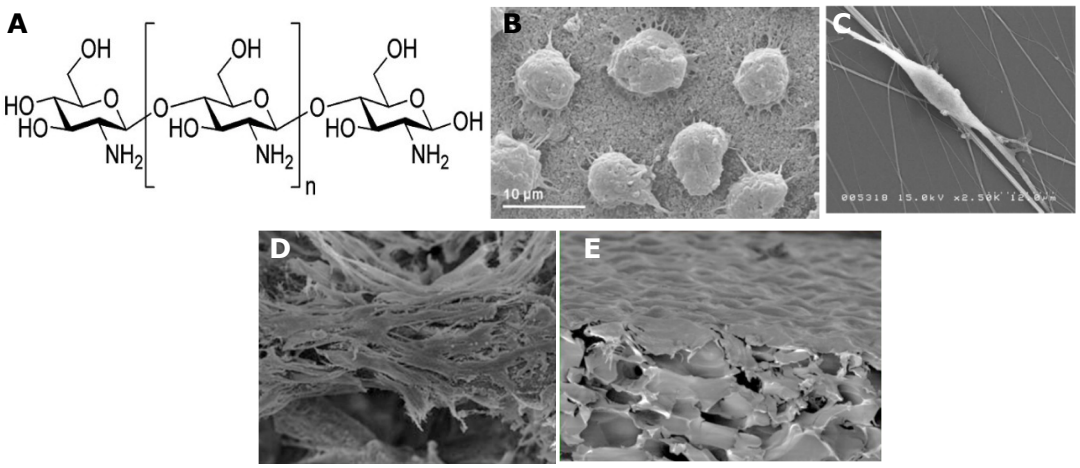

Figure 1.12. Chitosan chemical structures and its applications in cell culture. A. Chemical structure of chitosan. **B.** SEM microphotographs showing the morphology of cells isolated from adipose tissue seeded on chitosan particle agglomerated scaffolds and cultured for 2 weeks under chondrogenic conditions (B Malafaya et al., 2005). **C.** The scanning electron micrograph of Schwann cells cultured on the oriented chitosan nanofiber mesh sheet. (Wang et al., 2009) **D.** SEM microphotographs of mesenchymal stem cells attached on PLCL/chitosan scaffold taken 16h postseeding (Yang et al., 2011). **E.** SEM micrograph of human dermal fibroblasts covering the pores of chitosan at day 14 (Hilmi et al., 2013).

1.4.2.4. Hyaluronan

Hyaluronan, also known as hyaluronic acid (HA), is a polymer of disaccharides composed of D-glucuronic acid and D-N-acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds (Fig. 1.13 A). HA is one of the major components of the extracellular matrix; it is present in connective tissue, in the synovial fluid of articular joints and in the vitreous humor of the eye. HA is important in many biological processes such as tissue hydration, cell differentiation, cell behavior and tissue repair. HA contains sites for cell adhesion and its upregulation during embryogenesis, suggest its suitability as a scaffold material for the culture of ESCs (Gerecht et al., 2007; Ramírez et al., 2011). Other approaches have combined HA derivated scaffolds with keratinocytes, fibroblasts, and endothelial cells for engineering skin (Tonello et al., 2005). HA has been also used for adipose tissue engineering (Halbleib et al., 2003) and for cartilage (Lee et al., 2012) and bone regeneration (Kim et al., 2007). Hyaluronan is available for several applications, such as, for lubrication and mechanical support for the joints in osteoarthritis (Hyalgan® and Hyalubrix®), as well as, culture media for *in vitro* fertilization (EmbryoGlue® from Vitrolife, USA)

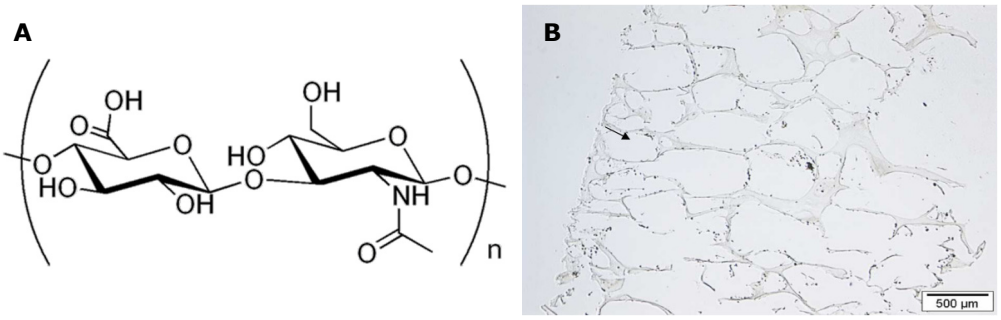

Figure 1.13: Chemical structure and application of hyaluronan. A. Chemical structure of hyaluronan. **B.** Distribution of human adipocyte precursor cells inoculated in a Hyaffs® scaffold for 36 days (Halbleib et al., 2003).

1.4.2.5. Xanthan, Gelan and Locust bean gum

Xanthan gum, a microbial desiccation-resistant polysaccharide prepared commercially by fermentation of *Xanthomonas campestris*, was firstly used as a solidifying agent for plant tissue culture media (Jain and Babbar, 2006). Nowadays,

xanthan gum has been successfully used in fabrication of matrices with uniform drug release characteristics and also carboxymethyl xanthan gum polysaccharide has been investigated as an artificial matrix for the encapsulation of chondrocytic cells (Mendes et al., 2012). On the other hand, gellan gum, a polysaccharide manufactured by microbial fermentation of the *Sphingomonas paucimobilis* microorganism, was used primarily as a gelling agent, alternative to agar, in microbiological and plant tissue culture (Jain et al., 2005). Nowadays, gellan gum has been presented as a biomaterial for culture of mammalian cells in hydrogel systems (Smith et al., 2007) and for cartilage tissue engineering (Oliveira et al., 2010).

Locust bean gum, or LBG, is a galactomannan extracted from the seeds of the carob tree, and displays a number of appealing characteristics for biopharmaceutical applications, among which its high adhesiveness and gelling capacity. LBG was the first galactomannan used both industrially (paper, textile, pharmaceutical, cosmetic and other industries) and in food products (ice cream and other preparations). LBG is widely used in drug delivery systems (Manjanna et al., 2013) and in biopharmaceutical applications (Dionísio and Grenha, 2012), however its potential use in ESC culture remained unexplored.

In sum, natural polymers offer the advantage of being very similar, often identical, to macromolecular substances present in the human body. Therefore, the biological environment is prepared to recognize and interact with natural polymers favorably in use in pharmaceutical, cell and tissue engineering applications (Table 1.1). Despite the wide variety of natural polymers commonly used for culture of stem cells for tissue engineering applications few are applicable or tested for culture of ESCs.

Table 1.1: Summary of natural biomaterials and their applications in cell and tissue engineering applications**.** A, animal; V, vegetal; B, bacterial; S, seaweed

Origin	Type of material	Cultured cells and (Future) applications	References
		Protein-based biomaterials	
A	Fibrin	Culture of mesenchymal cells (vascular tissue engineering)	(O'Cearbhaill et al., 2010)
		Cell migration and invasion assays	(Doillon et al., 2004)
	Collagen	Culture vascular smooth muscle cells	(Cummings et al., 2004)
		Culture of dermal fibroblasts (skin engineering)	(Hu et al., 2010)
		Cell invasion assays	(Kikuchi et al., 2011)
	Fibrin-Collagen	Culture of Vascular smooth muscle cells (vascular engineering)	(Hong and Stegemann, 2008)
		Culture of Myoblasts (engineering of skeletal muscle tissue)	(Beier et al., 2009)
		Neuronal stem cell culture	(Lee et al., 2010)
	Gelatin	Mouse and human ESC culture	(Li et al., 2009)
	Matrigel (laminin, collagen IV,entactin)	Mouse and human ESC culture	(Greenlee et al., 2005; McElroy and Pera, 2008)
		Cell motility and invasion assays	(Hulkower and Herber, 2011)
		Cardiac tissue engineering	(Li and Guan, 2011; Zhou et al., 2013)

Table 1.1 (Cont.): Summary of natural biomaterials and their applications in cell and tissue engineering applications. A, animal; V, vegetal; B, bacterial; S, seaweed

Origin	Type of material	Cultured cells and (Future) applications	References
Polysaccharide-based biomaterials			
S	Agarose	Culture of eukaryotic cells (HeLa, HEK293, H1299 and SC-1)	(Diekman et al., 2012; Gordeev et al., 2012)
		Differentiation of mesenchymal cells in chrondrocytes and repair of cartilage defects	(Schmitt et al., 2012)
		Cell motility and invasion assays	(Mousseau et al., 2007; Wiggins and Rappoport, 2010)
S, B	Alginate	Platform for drug screening	(Lan and Starly, 2011)
		Growing of stem cell spheroid culture (embryoid bodies) and cardiac co-cultures	(Dar et al., 2002; Gerecht-Nir et al., 2004)
V	Starch	Drug delivery	(Santander-Ortega et al., 2010)
		Culture of fibroblasts	(Reddy and Yang, 2009)
		Bone tissue engineering	(Salgado et al., 2004)
A	Chitosan	Cell and tissue culture	(B Malafaya et al., 2005; Hilmi et al., 2013; Karp et al., 2006; Lahiji et al., 2000; Nie et al., 2012; Qiu et al., 2009; Wang et al., 2009)
		Drug delivery	(Hu et al., 2013)
B	Xanthan gum	Drug delivery	(Mendes et al., 2012)

Table 1.1 (Cont.): Summary of natural biomaterials and their applications in cell and tissue engineering applications. A, animal; V, vegetal; B, bacterial; S, seaweed

Origin	Type of material	Cultured cells and (Future) applications	References
Polysaccharide-based biomaterials			
A	Hyaluronan (Glycosminoglycan)	ESC culture	(Gerecht et al., 2007; Ramírez et al., 2011)
		Mesenchymal and endothelial cell culture for skin, adipose, cartilage and bone engineering	(Halbleib et al., 2003; Kim et al., 2007; Lee et al., 2012; Tonello et al., 2005)
B	Gellan gum	Culture of mammalian cells in hydrogel	(Smith et al., 2007)
		Cartilage engineering	(Oliveira et al., 2010)
V	Locust bean gum	Drug delivery	(Manjanna et al., 2013)
		Biopharmaceutical applications	(Dionísio and Grenha, 2012)

1.5. Synthetic biomaterials for ESC culture

The development of synthetic gels has rapidly advanced during the past decade, and has been motivated by the desire to provide better control over the materials and their biological properties (Baker and Chen, 2012). Synthetic biomaterials offer many advantages, including reproducibility due to their defined chemical composition and the ability to control the mechanical properties, degradation rate, and shape independently. Synthetic biomaterials include polymer-, peptide- and ceramic-based biomaterials (Willerth et al., 2008). Polymer- and peptide-based biomaterials are the most commonly used in cell culture.

1.5.1. Synthetic peptides

An example of a peptide-based biomaterial applied in cell culture was presented in Klim *et al*; a substrate of heparin-binding peptide GKKQRFRHRNRKG derived from vitronectin, which is recognized by cell-surface glycans, supported long-term propagation of multiple hESCs in fully defined conditions (Klim et al., 2010). In another study, Kolhar et al demonstrated that both RGD and cyclic RGD (CRGDC) can support the culture of hESCs, with CRGDC increasing the adhesion of the ESCs over the linear RGD peptide (Kolhar et al., 2010). The use of a self-assembled layer of RGD was also reported in Yea *et al* in a fabrication of a mouse ESC chip (Yea et al., 2008).

1.5.2. Synthetic polymers

Synthetic polymer-based biomaterials represent the largest class of biomaterials (Bhat and Kumar, 2012). Synthetic polymers typically possess a structural backbone, cell-binding ligands, and a 'cell-friendly' crosslinking mechanism. In general, synthetic polymers are attractive because they can be fabricated into various shapes and are easier to design and manipulate to obtain a desired pore, morphologic features and chemical functional groups. Furthermore, the geometrical and specific mechanical modifications of synthetic polymers make them more suitable to use as a support for ESC culture and further differentiation. For instance, a moldable polymer is easily adapted to acquire the specifications of the tissue where it will be implanted. Moreover, the possibility of incorporation of synthetic polymers with specific degradation is advantageous over the natural polymeric systems as e.g. in the scope of drug delivery applications where control of release rate is mandatory. However, several synthetic materials are unattractive for cell adhesion. In those cases, polymers can be chemically modified or easily designed to incorporate specific proteins or ligands such as fibronectin or RGD peptides to coat and promote stem cell adhesion and spreading (Bellis, 2011). On the other hand, there are still some issues concerning the biocompatibility of synthetic polymers and its suitability for transplantation *in vivo*, as well as whether or not the material and its byproducts resultant of degradation can trigger an immune response.

A wide variety of synthetic polymers has been explored to produce nanofibers and hydrogels for culture and tissue engineering. Among the numerous synthetic polymers, the most commonly used polymer supports for culture of ESCs are poly (lactic-co-glycolic acid) (PLGA) and poly (ethylene glycol) (PEG). Other polymers, such as poly (2-hydroxyethyl methacrylate) (pHEMA) and poly (ε-caprolactone) (PCL) have been also explored as a coating for ESC culture.

1.5.2.1. Poly (lactic-co-glycolic acid)

Poly (lactic-co-glycolic acid), or *PLGA*, has been approved by the American agency for public health Food and Drug Administration (FDA), and one of the most commonly used polymers to fabricate nanofibers for bone (Chen et al., 2013; Curran et al., 2013) and cartilage (Uematsu et al., 2005) tissue engineering and controlled drug delivery (Kirby et al., 2011) due its biocompatibility and the ability to modulate the degradation rate. In the presence of cells, PLGA scaffolds degrade in to their monomers, which are natural metabolites but that can have negative effects due to their acidic nature. PLGA scaffolds have been used for engineering a wide range of tissues. Several studies have been conducted using orderly PLGA fibers to manipulate adhesion (Salamian et al., 2013) and orientation of fibroblasts (Hwang et al., 2009) and endothelial cells (Hsueh et al., 2009) for further application in recreation of striated tissues, such as cardiac muscle.
PLGA has also been used to develop *in vitro* models that mimic *in vivo* conditions. One of those systems consisted in porous PLGA microsphere that was used both as a cancer cell culture substrate to expand cells and as a cancer cell transplantation vehicle for tumor construction in mice. This 3D tumor model that mimics the *in vivo* environment is ideal for screening anticancer drugs and their formulations, and presented an alternative for animal models developing tumors (Kang and Bae, 2009). In other study, porous PLGA was also used to produce sponges to grow hepatic cells (Hep3B cells) (Zhu et al., 2008). Study of the disorders of nervous system is another area where PLGA scaffolds seeded with stem cells can be used as a model to study them and develop further possible therapies. In a preclinical test, PLGA scaffolds were designed to mimic the spinal cord and its culture with murine neural stem cells produced an increase in functional recovery after traumatic spinal cord injury (Teng et al., 2002). An additional study demonstrated that hESCs seeded inside of PLGA scaffolds could

be directed to differentiate into neurons when treated with the appropriate cues (Levenberg et al., 2003). PLGA has also been demonstrated to be a suitable scaffold to culture Schwann cells and differentiate neuronal stem cells for further transplantation of functional neural tissue in spinal cord injury (Xiong et al., 2012).

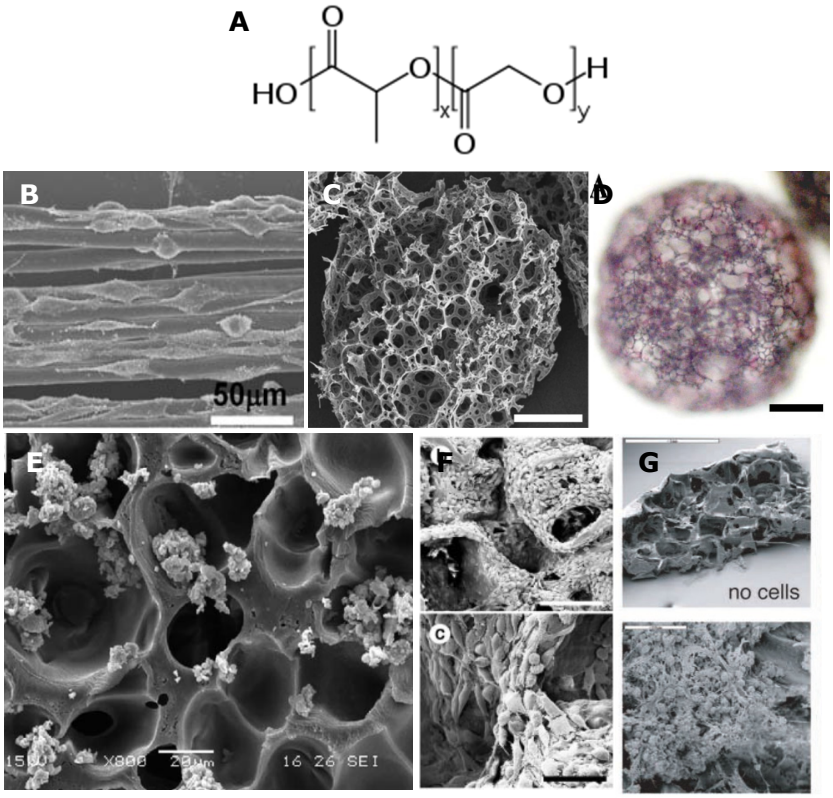

Figure 1.14: Structure and cellular applications of PLGA. A. Structure of poly(lactic-co-glycolic acid), x= number of units of lactic acid; y= number of units of glycolic acid. **B.** Cell morphology of mouse L929 fibroblasts on PLGA fibers shows different orientations with respect to the long axis of fibers (Hwang et al., 2009). **C.** SEM micrographs of cross-section of porous PLGA microsphere (Kang and Bae, 2009). **D.** H&E staining of MCF-7 cells cultured on porous PLGA microsphere in spinner flask for 5 days and (Kang and Bae, 2009). **E.** SEM images of Hep3B cells grown on the PLGA sponges after 1 week of culture (Zhu et al., 2008). **F.** Inner PLGA-derived scaffolds seeded with NSCs. (Teng et al., 2002) **G.** SEM of PLGA scaffolds without and with differentiating hESCs (Levenberg et al., 2003)

1.5.2.2. Poly(ethylene glycol)

Poly(ethylene glycol), *PEG*, with high molecular weight versions being referred to as poly (ethylene oxide) (PEO), is a commonly used polymer for biomaterial applications because they can be chemically modified to contain bioactive molecules. PEG has been used as a support tool in cell and tissue culture in various shapes, e.g. as coating and as a 3D structure. Sugiura *et al* reported the

development of dynamically controlled 3D micropatterned cellular co-cultures of mESCs and human hepatocellular carcinoma cells, within photocurable and chemically degradable PEG-hydrogels, to study a range of cell-culture applications related to cell differentiation, tissue engineering and regenerative medicine (Sugiura et al., 2013). On the other hand, the PEG-microwell array system developed for stem cell culture is a potentially versatile tool for mESC differentiation studies and high-throughput stem cell experimentation as it demonstrated an improved degree of homogeneity of the resulting aggregate populations, therefore establishing a robust protocol for eliciting high EB formation efficiencies (Moeller et al., 2008). Furthermore, 3D PEG-based hydrogel matrix niche can be used to support expansion and self-renewal of multiple hESC lines (Jang et al., 2013). Moreover, in combination with stem cells, PEG-based matrices have been evaluated for their suitability as potential replacement for bone, cartilage, nerve, liver and vasculature tissue. In one study, a nanostructured array of a PEG hydrogel was developed to use as a cell culture platform to guide the growth of primary rat cardiomyocytes for potential tissue engineering applications (D. H. Kim et al. 2006). Furthermore, the synthesis of polyethylene glycol dimethacrylate (PEGdma) from PEG allows the formation of a 3D nanofibrous matrix that directs rat mesenchymal stem cell differentiation to vascular cells (endothelial and muscle cells) (Wingate et al., 2012). Moreover, PEG scaffolds have been investigated in combination with human mesenchymal stem cells for adipose tissue engineering and with mouse embryonic liver cells to generate hepatocytes (Underhill et al., 2007) for further liver tissue engineering, showing the versatility of such scaffolds.

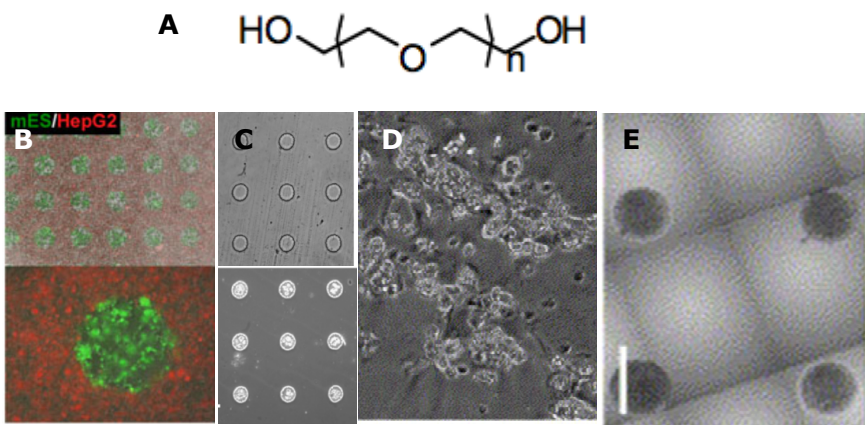

Figure 1.15: Structure and cellular applications of PEG. A. Chemical structure of poly(ethylene glycol). **B.** Dynamic 3D micropatterned co-culture of mESCs (green) and

HepG2 (red) cells in the photocurable PEG and chemically degradable alginate hydrogel (Sugiura et al., 2013). **C.** EBs grown in microwell array for EB culture until they were constrained by the size of the well, yielding a homogeneous culture (Moeller et al., 2008). **D.** Co-culture of mouse embryonic liver cells and fibroblasts encapsulated in RGDS-PEG hydrogel randomly and in highly defined architecture and cellular organization (**E.**) (Underhill et al., 2007).

1.5.2.3. Polycaprolactone

Polycaprolactone (*PCL*) has been used extensively in the biomaterials field and in a large range of implants, devices and drug-delivery devices since the 1970s, due to its easy manufacturing process and manipulation of its rheological and viscoelastic properties (Woodruff and Hutmacher, 2010). Indeed, PCL drew attention due to its versatile properties, including tailorable degradation kinetics and mechanical properties, ease of shaping and manufacture enabling appropriate pore sizes conducive to tissue in-growth, and the controlled delivery of drugs contained within their matrix. In addition, functional groups can also be added to render the polymer more hydrophilic, adhesive, or biocompatible enabling favorable cell responses. An extensive amount of research has been conducted using PCL matrices for cell culture and *in vitro* models applications. Namely, PCL nanofiber scaffolds revealed to be suitable for culture, attachment and proliferation of rat parenchymal hepatocytes (Lubasová et al., 2010) and kidney epithelial cells (Ghasemi-Mobarakeh, 2008). In work done by the Meiners' lab, fibroblasts, normal rat kidney cells and breast epithelial cells were seeded on a PCL nanofibrillar matrix. Fibroblasts and normal rat kidney cells displayed the morphology and characteristics of their counterparts in vivo and, interestingly, breast epithelial cells underwent morphogenesis to form multicellular spheroids containing lumens, highlighting the considerable value of PCL scaffold for applications in cell-based therapies and studies of cell/tissue function and pathology (Schindler et al., 2005). In another study, biofunctionalized PCL scaffold was used to create a 3D pharmacokinetic cancer model using a prostate cancer cell line (Hartman et al., 2010). Several companies develop and commercialize now PCL nanofibers for cell culture. These include tissue culture plates with 3D PCL nanofiber scaffold inserts from Biotek and CELLTREAT Scientific Products, and there are also aligned or randomly oriented PCL nanofibers integrated into standard multi-well cell culture dishes for high-throughput cell culture, cancer research, stem cell, and regenerative medicine from Nanofiber Solutions™.

Scaffold-based tissue engineering aims to promote the repair and/or regeneration of tissues through the incorporation of cells and/or biomolecules within a 3D scaffold system which can be maintained *in vitro* culture conditions until implantation. So, after established that PCL supports cell attachment, migration, growth and differentiation in *in vitro* models, several studies proceed with the implantation of PCL scaffolds containing differentiated cells, to repair defects and guide tissue development into a mature and healthy state, using different animal models (Martinez-Diaz et al., 2010; Sawyer et al., 2009).

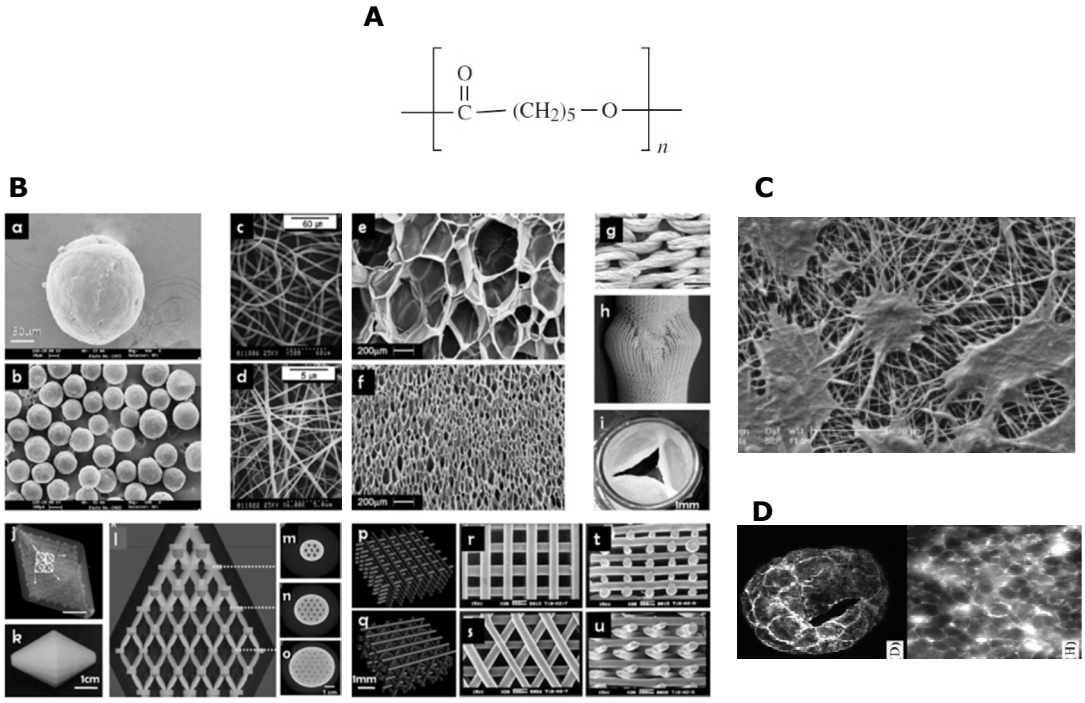

Figure 1.16: Chemical structure and applications of PCL. A. Chemical structure of polycaprolactone (Gunatillake and Adhikari, 2003) **B.** Structures made from PCL: nanospheres (a,b), nanofibers (c,d), foams (e,f), knitted textiles (g,h,i). Selective laser sintered scaffold (j-o). Fused deposition modeled scaffolds (p–u). Adapted from (Woodruff and Hutmacher, 2010). **C.** kidney epithelial cells on the electrospun nanofibrous after 7 days of culture (Ghasemi-Mobarakeh, 2008). **D.** Confocal sections of a multicellular spheroid composed of T47D breast epithelial cells grown on nanofibers. Note the lumen extending through the spheroid (Schindler et al., 2005).

1.5.2.4. Poly (2-hydroxyethyl methacrylate)

Poly (2-hydroxyethyl methacrylate), *PHEMA*, a polymer that forms a hydrogel in water, was developed by Drahoslav Lim and Otto Wichterle and patented in 1953 (Wichterle and Lím, 1960). PHEMA has been used in many biomedical devices since the twentieth century and is probably one of the best known biomaterial used

today to transplant undifferentiated ESCs or their differentiated derivatives and repair an injured part of the organ or tissue (Kopecek, 2009). However, HEMA hydroxyl groups often prevent adhesion and spreading of cells, making PHEMA poorly recognized by osteoblasts and adherent cells. Therefore, the polymer has been modified by blending with hydrophobic materials, such as poly(methyl methacrylate) or with natural substrates normally present in the ECM such as collagen, to improve cell attachment and proliferation. An extensive amount of research has been conducted using PHEMA-based slabs, coatings and hydrogels to grow undifferentiated mouse and human ESCs in 2D systems (Horák et al., 2004; Kroupová et al., 2006) and to culture neuronal cells in 3D (Shepherd and Parker, 2011).

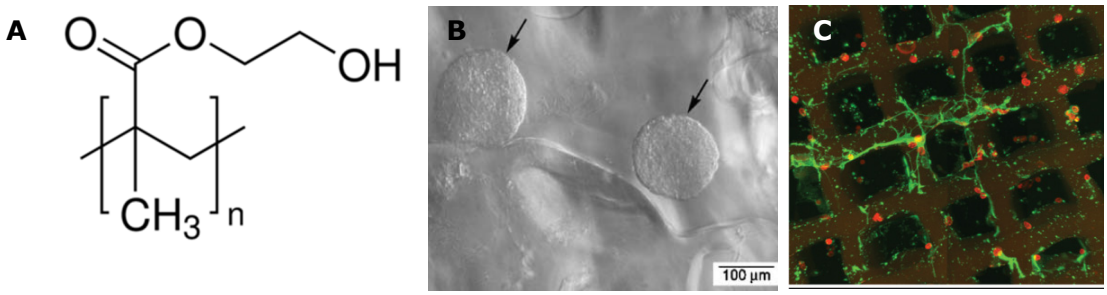

Figure 1.17: Chemical structure and applications of PHEMA. A. Chemical structure of poly (2-hydroxyethyl methacrylate). **B.** Compact colonies of mESCs cultivated for 4 days on the surface of P(HEMA-co-EDMA) slab in transmitting light (Horák et al., 2004). **C.** Confocal images of primary rat hippocampal cells distributed within pHEMA scaffold processes actin (green) nuclei (red) (Shepherd and Parker, 2011)

The development of PHEMA solved a critical piece of the puzzle in tissue engineering approaches, which was to find a suitable polymeric material for seeding cells and for subsequent growth of tissues. The mechanic and thermo-responsive properties of Poly(*N*-isopropylacrylamide) (PIPAAm) and PHEMA allows fabrication of functional tissue constructs from designed three-dimensional structure of cells using layered method of cultured cells and rapid recovery of cell sheets. Therefore, the biological functions and viability of recovered cell sheets is maintained, thus allowing practical assembly of tissue structures (Tang et al., 2012). In fact, the grafting of PHEMA and PIPAAm onto cell culture surface enables rapid cell sheet recovery (Kim et al., 2013).

1.5.2.5. Co-polymers

In cell and tissue culture, the use of co-polymeric materials is frequent. In fact, the development of copolymers improve the properties of scaffolds or coatings increasing the number and type of applications (Table 1.2). Therefore, several authors combined the use of PLGA, PEG, PCL, poly(lactic acid) (PLLA) and methacrylate monomers to form biomaterials. The use PEG-PCL of is one of the most frequent combinations of copolymers. Fibrous hydrogels of PEG and PCL have been used for vascular applications (Crowder et al., 2012), to culture human neuroblastoma cells (Han et al., 2012) and dermal fibroblasts (Grafahrend et al., 2008). On the other hand, PLGA-PCL blends have been used for culture of osteoblastic cells (Lucchesi and Barbanti, 2010) and human adipose stem cells for further use in small-caliber vascular grafts (Diban et al., 2013). Several copolymeric compounds such as poly(lactic acid)–PEG (PLLA-PEG) and PLLA-PCL have been used as a biomimetic ECM for culture of cardiomyocytes (Zong et al., 2005) and culture of periosteal tissue (Kouya et al., 2013), respectively. In another work, Anderson et al identified a combinatorial library of biomaterials formed from acrylate and methacrylate monomers that support appropriate cellular attachment, proliferation and differentiation of hESC (Anderson et al., 2004)

Table 1.2: Summary of the synthetic biomaterials most common used and their applications in cell and tissue engineering applications.

Type of material	Cultured cells and (Future) applications	References
	Synthetic peptides	
	Culture of mouse and human ESCs	(Yea et al., 2008) (Klim et al., 2010) (Kolhar et al., 2010)

Table 1.2 (Cont.): Summary of the synthetic biomaterials most common used and their applications in cell and tissue engineering applications.

Type of material	Cultured cells and (Future) applications	References
	Synthetic polymers	
PLGA	Cartilage and bone tissue engineering	(Uematsu et al., 2005) (Chen et al., 2013; Curran et al., 2013)
	Drug delivery	(Kirby et al., 2011)
	Neural stem cell culture and differentiation	(Levenberg et al., 2003; Teng et al., 2002; Xiong et al., 2012)
	Culture of fibroblasts and endothelial cells (recreation of striated muscles)	(Hsueh et al., 2009; Hwang et al., 2009; Salamian et al., 2013)
	Porous microsphere model to study developing tumors	(Kang and Bae, 2009)
	Culture of hepatic cells in sponges	(Zhu et al., 2008)
PEG	Co-culture of mESCs and human hepatocellular carcinoma cells to study cell differentiation	(Sugiura et al., 2013)
	Microwell array for EB culture	(Moeller et al., 2008)
	Expansion and self-renewal of multiple hESC lines	(Jang et al., 2013)
	Nanopatterned culture of rat primary cardiomyocytes	(D. H. Kim et al. 2006)
	Rat mesenchymal stem cell differentiation to vascular cells (endothelial and muscle cells)	(Wingate et al., 2012)
	Mouse embryonic liver cells to generate hepatocytes	(Underhill et al., 2007)

Table 1.2 (Cont.): Summary of the synthetic biomaterials most common used and their applications in cell and tissue engineering applications.

Type of material	Cultured cells and (Future) applications	References
	Synthetic polymers	
PCL	Rat parenchymal hepatocytes kidney epithelial cells	(Lubasová et al., 2010) (Ghasemi-Mobarakeh, 2008)
	Culture of prostate cancer cell line (3D pharmacokinetic cancer model)	(Hartman et al., 2010)
	Cartilage and bone repair animal models	(Martinez-Diaz et al., 2010; Sawyer et al., 2009).
PLLA	Culture of cardiomyocytes	(Zong et al., 2005)
	Culture of periosteal tissue (bone regeneration)	(Kouya et al., 2013)
	Human umbilical vein endothelial cells	(Can et al., 2011)
PGA	Culture of human smooth muscle cells and vascular-derived cells (vascular tissue engineering)	(Brugmans et al., 2013) (Hajiali et al., 2011)
PMMA	Culture of human stromal cells	(Patel et al., 2006)
	Cell culture chip	(Petronis et al., 2006)
	Culture of Human osteosarcoma cells (bone regeneration)	(Son et al., 2013)
PHEMA	Culture of undifferentiated mouse and human ESCs	(Horák et al., 2004; Kroupová et al., 2006)
	Culture of neuronal cells	(Shepherd and Parker, 2011)

In 1987, a group of experts defined the word biomaterial as "a non-viable material used in a medical device, intended to interact with biological systems" (European Society of Biomaterials Conference, 1987) and since then, the field has advanced considerably resulting in the development of implantable scaffolds that consist entirely of specific biomaterials. A wide range of biomaterials have been developed for different applications and current knowledge of using biomaterials in combination with stem cells has increased. Natural or synthetic biomaterials comprise advantages and drawbacks that can be easily overcome by taking advantage of composite compounds. Indeed, there are several biomaterials available that could be adapted or directly used depending on the application, either for culture and expansion of undifferentiated stem cells or for directed differentiation of stem cells into mature phenotypes. Here, we will explore the use of natural and synthetic polymers to support the culture and maintenance of pluripotent mouse ESCs.

1.6. Collagen and calcium binding EGF domains 1

State of the art

Collagen and calcium binding epidermal growth factor domains 1 (*Ccbe1*) is a still poorly characterized gene, whose function is thought to be related to ECM remodeling and migration because its domains are found in several of the ECM proteins. *Ccbe1* gene has been identified in several vertebrate systems. In chick, a differential screening Affymetrix GeneChip® Chicken Genome arrays conducted in our laboratory identified *Ccbe1* as one of the upregulated genes present in the heart/hemangioblast precursors (Bento et al., 2011) and might be responsible for alterations in cell migration events during chick heart development. In a zebrafish genetic screening, *Ccbe1* was found to be indispensable for embryonic lymphangiogenesis and venous sprouting (Hogan et al., 2009). In mice, *Ccbe1* null mutants die in utero with malformations such as severe edema and *Ccbe1* has also shown to be essential for budding and/or migration of lymphatic endothelial cells (LECs), maintenance of their proliferation and subsequently for lymphatic vasculature formation (Bos et al., 2011). Bos and collaborators reported *Ccbe1* as a factor critically required for budding and migration of $Prox1^+$ and $Lyve\text{-}1^+$ LECs (fate-specified LECs) from the cardinal vein to form the lymph sacs and give rise

to the lymphatic vasculature (Bos et al., 2011). However, accordingly to the presented results, the observation of less migration seemed to be related to absence of signaling or absence of a required number or pool of cells to migrate, as LEC numbers are reduced in mutants, and not due to less ability of cells to initiate migration. Indeed, the results indicated an overall reduction in the number of Lyve-1^{+} and Lyve-1^{+} LECs between E10.5 and E12.0 in Ccbe$1^{-/-}$ embryos, resulting in a primary defect before the formation of lymph sacs. Furthermore, the authors revealed that CCBE1 has little lymphangiogenic effect on its own, but dramatically enhances the lymphangiogenic effect of vascular endothelial growth factor-C (VEGF-C), an inducer of lymphangiogenesis and a growth factor active in stimulation of endothelial cell growth and proliferation, *in vivo.* Those results suggest that in the absence of VEGF-C interaction, *Ccbe1* seems to be essential but not sufficient for lymphangiogenesis. In that study, it is also hypothesized whether CCBE1 might be part of the extracellular ECM, as suggested by the CCBE1 domain structure (Bos et al., 2011). Furthermore, CCBE1 mutations in human lead to clinical manifestations of severe lymphedema or Hennekam syndrome, a disorder characterized by abnormal lymphatic system and mild to moderate levels of growth and mental retardation (Alders et al., 2013; Connell et al., 2012; Marchiò et al., 2013). Moreover, gene expression analyses using a high-throughput systematic multiplex qPCR revealed that CCBE1 was downregulated in the majority of clinical cases of breast cancer and CCBE1 also modulates cell migration and survival in human ovarian cancer cells (Barton et al., 2010; Yamamoto and Yamamoto, 2007).

The function of *Ccbe1* is however still contradictory and poorly understood. While the importance of *Ccbe1* for the development of the lymphatic system appears to be unquestionable (Bos et al., 2011; Hägerling et al., 2013), unlike other lymphangiogenesis associated genes, CCBE1 is not expressed in endothelial cells, but is spatially and temporally expressed along the migration routes of lymphatic endothelial cells and may be a guidance molecule involved in lymphangioblast budding and migration (Hogan et al., 2009). On the other hand, CCBE1 has also been reported to be highly down-regulated in primary breast carcinomas as compared with matched normal breast tissue (Yamamoto and Yamamoto, 2007). Furthermore, it was shown that CCBE1 is highly expressed in normal ovary, as compared with other tissue types (Shyamsundar et al., 2005), but is low expressed in ovarian cancer cell lines and primary carcinomas (Barton et al., 2010). In

Barton's study, qPCR analysis of 78 primary ovarian carcinomas revealed that CCBE1 was significantly down-regulated in ovarian cancers of all histological subtypes as compared with normal ovarian surface epithelium. In addition, CCBE1 was frequently down-regulated in ovarian and breast cancer cell lines as compared with normal ovarian and breast epithelial cell lines. These data indicated that CCBE1 may be a tumor metastasis suppressor gene. Indeed, CCBE1 overexpression in breast cancer cells inhibited cell migration (Barton et al., 2010). Therefore, loss-of-function of CCBE1 in ovarian cancer context confers migratory and survival advantage to cancer cells.

As in Bos *et al* study mentioned above (Bos et al., 2011), the work conducted by Barton also hypothesized that the structural motifs of CCBE1, including the presence of collagen repeats and an Asp/Asn hydroxylation motif, together with its expression in the surface epithelium and stroma of the ovary, are indicative of a function in the cross-talk between the surface epithelial cells and the ECM affecting cellular migration (Barton et al., 2010).

Recently, another work suggests an additional role for CCBE1 outside the lymphatic vasculature. The study reveals that loss of *CCBE1* in mice results in severe mid-gestation anemia, likely due to reduced fetal liver erythropoiesis. Zou and co-workers have identified CCBE1 as a required secreted factor that plays an unexpected and critical role in fetal erythropoiesis, process by which red blood cells (erythrocytes) are produced. Like the well-characterized secreted factors erythropoietin and stem cell factor, CCBE1 functions cell non-autonomously to promote erythroid survival, maturation and proliferation. Fetal liver erythroid precursors of *Ccbe1* null mice exhibit reduced proliferation and increased apoptosis. As such, fetal livers were normal or mildly reduced in size in Ccbe1$^{-/-}$ embryos at E12.5 and markedly smaller and pale by E14.5 and E16.5. In addition, the number of hematocrit in E15.5 *Ccbe1*$^{-/-}$ embryos was severely reduced compared with that of heterozygous littermates. Furthermore, staining for phospho-histone H3 revealed reduced numbers of proliferating cells in *Ccbe1*$^{-/-}$ fetal liver and staining for activated caspase 3 confirmed an increased number of apoptotic cells. Those findings identify a primary and specific defect in definitive erythropoiesis within the Ccbe1$^{-/-}$ fetal liver as the cause of anemia. Thus, secreted CCBE1 by non-hematopoietic cells is required to maintain a unique and specific erythropoietic environment which support erythroblastic island formation specifically within the fetal liver erythroid niche.

Despite all the controversy that rose about the function of *Ccbe1*, according to the literature *Ccbe1* seems to be involved in lymphangiogenesis and venous sprouting, cardiogenesis and (anti-) carcinogenesis events. *Ccbe1* is thought to act as a modulator of cell migration, specification, proliferation and ECM remodeling, but its function remains mostly uncharacterized.

1.7. Specific aims

Basic and clinical research carried out during the last few years on embryonic stem cells has constituted a revolution in regenerative medicine and cancer therapies by providing the possibility of generating multiple therapeutically useful cell types. However, much work remains to be performed in the laboratory and in the clinic to understand how to use these cells for cell-based therapies to treat disease. One of the important issues to overcome is related to the culture of cells in a support suitable for further cell-based therapies, thus a support which does not induce immune response. Therefore, in order to use ESCs in those applications, they must be cultured in animal-free derived matrices. Several authors have been searching and developing synthetic and natural alternative supports that could be used to maintain ESC pluripotency, enabling a subsequent application in basic stem cell biology and regenerative medicine. However, so far the feeder-dependent and gelatin-based cultures are still standard protocols to culture pluripotent mouse ESCs.

In the present work, Chapter III and IV refers to the application of natural and synthetic polymeric coatings in stem cell research. The main goal was to develop new supporting biomaterials that could be used both to grow pluripotent stem cell and allow their differentiation. Ideally, the supports should lead to production of high quality of undifferentiated pluripotent stem cells; provide better control of cell proliferation and differentiation; build a realistic micro-environment where the functional properties of stem cells can be observed and manipulated; and be cheap, easy to use and time-efficient for stem cell research. Taking this into consideration, in Chapter III and IV, a set of successive experiments were performed to characterize the selected natural and synthetic matrices and to validate their suitability in mouse ESC cultures. In Chapter III - *Locust Bean Gum*

as an alternative polymeric coating for embryonic stem cell culture and in Chapter III.I - *Comparative study of LBG from different sources*, it was explored the potential use of LBG, a natural vegetal-derived polysaccharide, in pluripotent mouse ESC culture. In Chapter IV - *Novel triblock copolymer nanofiber system as an alternative support for embryonic stem cells growth and pluripotency*, is reported the synthesis of poly(ethyleneglycol-β-trimethylsilyl methacrylate-β-methacrylic acid)–glycine–arginine–glycine–aspartate–serine (PEG–PTMSMA–PMAA–GRGDS)-based nanofibers and the capability of this new artificial nanofiber network to support pluripotent mouse ESC culture.

According to literature and data from our lab suggest that *Ccbe1* modulates cell migration and survival. However, the function and the mechanism whereby *Ccbe1* modulates both proliferation and migration is unknown.
In Chapter V, *Characterization of Ccbe1 mutant MEFs – Ccbe1 and its effect in proliferation and migration*, we took advantage that conventional *Ccbe1* mutant mice existent in our animal facility and of the primary cell culture approach, as they provide effective tools to investigate the basic principles of *in vivo* mammalian development. *Ccbe1* is highly expressed in mouse embryonic fibroblasts so, here, we aimed to explore the autonomous proliferative capacity of $Ccbe1^{+/+}$ and $Ccbe1^{-/-}$ primary MEFs in culture. Also, to test it's role in fibroblasts for stem cell differentiation support. The analysis of cell morphology, adhesion, proliferation and cell cycle was complemented with an apoptosis assay. With those assays we aimed to clarify if *Ccbe1* has a role in cell survival. Furthermore, we also intended to evaluate the motility properties of $Ccbe1^{-/-}$ MEFs, using different matrices that may interact with CCBE1. The study of Ccbe1 role in proliferation and migration of primary mouse embryonic fibroblasts was complemented with the rescue of $Ccbe1^{+/+}$ phenotype by introducing CCBE1 in the culture system.

Chapter II

Materials and Methods

2. MATERIALS AND METHODS

2.1. Cell Culture

Handling of the cells, medium preparation and other techniques that require aseptic conditions were performed in a laminar flow cabinet (Hera safe Heraerus) after irradiation for 15 minutes (min) with ultraviolet (UV) light. Mammalian cells were cultured in an humidified incubator (Hera cell 150 Heraerus) at 37 degrees Celsius (ºC) with 5% of CO_2 in 95% air and media and culture solutions were pre-warmed to 37 ºC in a water bath prior to use. Microscopic examination of cell growth was preformed frequently by using an inverted light microscope (Leica).

2.1.1. Isolation of primary mouse embryonic fibroblasts

Embryos to isolate MEFs were collected from pregnant female mice at embryonic day 13.5 to 14.5. Briefly, the pregnant females were sacrificed by CO_2 inhalation and the ventral surface was swabbed liberally with 70% alcohol and placenta was dissected out following the protocol described elsewhere (Freshney, 2006) and transferred to a clean dish with fresh phosphate buffered saline (PBS). In case of the *Ccbe1* mutant embryos, each individual embryo with its involving placenta was handled separately. Internal organs, head and blood system cells were removed from the free membranes and placenta. The remainder was then transferred to another fresh Petri dish. Next, in a laminar flow hood, the remaining of the embryos, consisting of the carcass, was washed five times with PBS, in five Petri dishes. Subsequently, the carcasses were transferred to sterile 10 cm plates with 15 ml of 0.05% of Trypsin / 0.53 mM ethylenediaminetetraacetic acid (EDTA) (Gibco) where it was finely cut with a razor blade. The resulting suspension was resuspended and homogenized by pipetting up and down six to seven times to help breaking up the tissue and then the plate was placed in the incubator for 15 min. After that, 5 ml of Trypsin/EDTA was added to the plate and again pippeted up and down followed by a 10 min incubation in the incubator. The digested tissue was dissociated, homogenized and next the cellular suspension was passed through a 100 µm cell strainer of nylon to remove the large pieces of undigested tissue and the cells collected in another plate. The cell strainer was rinsed with 5

ml of PBS and the permeated was placed in the same plate. The collected cellular suspension was transferred to a 50 ml falcon and centrifuged at 200 x *g* (times gravity) for 8 min at room temperature (RT). The supernatant was discarded and the cell pellet resuspended in MEFs culture medium (described in 2.1.2). The cells were platted to obtain the cellular content of one embryos by one 75 cm^2 T-flask and incubated at 37 ºC with 5% CO_2. The medium was changed after six hours and after one day. When a confluence of 70% was achieved, MEFs were subcultured and at passage 1 cells were ready for cryopreservation at about 1 million cells per vial.

2.1.2. Culture of Mouse Embryonic Fibroblasts

MEFs were cultured in tissue culture plate with 10 ml of growth medium consisting of Dulbecco's modified eagle medium (DMEM) Glutamax with D-glucose and without sodium pyruvate (Gibco) supplemented with 10% volume per volume (v/v) of heat inactivated fetal bovine serum (FBS; Sigma), 2 mM of L-glutamine (Gibco), 0.1 mM mercaptoethanol (Gibco), Penicillin (100 U/ml) / Streptomycin (100 µg/ml) (Gibco). Medium was changed every two days.
MEFs were split 1:3 or 1:5 (maximum) as soon as they became 70% confluent. For passage, cells were washed with 5 ml of PBS two times and trypsinized with 2 ml of trypsin/EDTA followed by 8 min incubation at 37 ºC. The trypsin-EDTA was inactivated by adding 5 ml of MEFs medium. The suspension was pipetted up and down to ensure a single-cell suspension and collected in a falcon for centrifugation at 200 x *g* for 8 min at RT. The cell pellet was resuspended in fresh MEFs medium and the cells were then plated in a 100 mm dish and incubated at 37 ºC with 5% CO_2.

2.1.3. Preparation of MEF feeder layers

When a confluence of 90% was reached or when MEFs forms a good feeder mesh for ESC culture, 1.5 µg/ml mitomycin C (Sigma) was added in the medium and cells were incubated for 2 hours (h) at 37 ºC. Then, cells were washed 3 times with 10 ml of PBS to completely remove the mitomycin, and then trypsinized with 2 ml of trypsin/EDTA for 8 min in the incubator. MEFs medium was added to stop the trypsinization, and the cells were then centrifuged at 200 x *g* for 8 minutes.

The cell pellet was resuspended in 1 ml of fresh MEFs medium and the number of inactivated MEFs was counted. Finally, the cells were frozen or replated to make the feeder layer. About $1x10^6$ of inactivated MEFs were used to cover the bottom of a 100 mm plate and approximately $4x10^5$ cells per well in a six well plate.

2.1.5. Traditional Mouse Embryonic Stem Cell Culture

E14GPF8 mESCs, that constitutively express green fluorescent protein (GFP), were kindly provided by Dr. Tristan Rodriguez. E14GPF8 mESCs were thawed and grown on 0.1% of gelatin (w/v) or on top of MEFsi at 37ºC and 5 % CO_2 in a 10 cm diameter - plate with 10 ml of a growth medium consisting of Glasgow Minimum Essential Medium (GMEM) without pyruvate and glutamine (Gibco) supplemented with 10% (v/v) of ES screened and defined FBS (HyClone), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 50 µM β-mercaptoethanol (Gibco), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco), 0.1mM non-essential aminoacids (Gibco) and 1000 U/ml of LIF (ESGRO®, Millipore). mESCs were subcultured every 2 to 3 days through incubation of 0.05 % trypsin/EDTA solution for 5 min and the culture medium was replaced daily.

2.1.4. Total and Viable cell count

Cells were washed with PBS and incubated with trypsin/EDTA at 37ºC and 5% CO_2. The cellular suspension was resuspended in growth medium, centrifuged and ressuspended in 50 to 1000 µl of medium depending on the predicted cellular concentration. One volume of 0.4% of trypan blue solution (w/v; Sigma) was added to the suspension and incubated for 1 to 2 min. The suspension was homogenized again and 10 µl of the mixture was introduced on a hemocytometer or Improved Neubauer chamber (VWR). Cells were counted using an inverted light microscope. The number of cells were counted in at least five 1 mm^2 squares of one chamber and determined the average number of cells per square. If a minimum of 20-50 cells per 1 mm^2 square could not be counted, the cells were centrifuged, resuspended and counted again. While viable cell do not absorb the dye, dead or non-viable cells absorb the dye and, hence viable cells are colorless and non-viable cells are stained blue. To estimate the number of cells of original

suspension the following formula was used: *Nº of cells = Nº of cells counted x 10^4 x Cell suspension volume (ml) x Dilution factor*

Cells were also counted by flow cytometry and the viability determined using propidium iodide (PI). For flow cytometry, the maintenance medium was removed, cells were washed with PBS and trypsinized. Cells were collected to a flow cytometry tube (BD Biosciences) and incubated with PI (5 µg/ml; Sigma). The cell suspensions were analyzed by flow cytometry upon excitation with the red laser (488 nm) being the emission signals measured in the FL2 channel (585/42 nm). Data was analyzed with BD CellQuest™ Pro software (BD Biosciences). Viability ratio was obtained as the ratio between the number of viable cells and total number of cells. Cell proliferation was calculated as the ratio between viable cells and initial cell number.

In case of cell passaging, population doubling (PD) per passage was calculated as *log(nf/n0)/log2*, where *n0* is the initial and *nf* the final number of cells at each passage. Cumulative population doubling (CPD) at each passage was calculated by adding population doubling per passage. When n0 was greater than nf, the population doubling was defined as 0.

2.1.6. Subculture of Mouse Embryonic Stem Cells

mESCs were routinely split 1:5 every 2 to 3 days or when 60 % confluence had been achieved. Culture medium was aspirated off and cells were rinsed twice with 5 ml of PBS, 2 ml of 0.05% trypsin/EDTA were added and incubated for 5 min at 37 ºC and 5% CO_2. 5 ml of pre-warmed ES cells medium were added to stop trypsinization and suspension was pipetted up and down to achieve a single-cell suspension. The suspension was transferred to a 15 ml centrifuge tube, centrifuged at 200g for 5 minutes, and the pellet carefully resuspended in fresh ESC medium and plated.

2.2. Polymeric coatings

2.2.1. Purification of LBG

1 L of deionized water was heated until it reached 85ºC, then, 5 g of LBG were added gradually to avoid formation of clusters, and stirred for 1h. After letting the solution cool down to RT, it was centrifuged at 22 000 x *g* for 1h at 20ºC. The

supernatant was precipitated by pouring into an equal amount of ethanol, gently stirring with a glass rod. The precipitate formed was transferred to a new beaker and washed with fresh ethanol. Then, vacuum filtration was used to remove the excess ethanol by compressing the precipitate against the walls of the Buckner funnel. Finally, the precipitate was cut in small pieces and residues of ethanol were removed by drying at 30 ºC for 3 days in a vacuum oven.

2.2.2 Characterization of natural polymeric coatings by Scanning Electron Microscopy (SEM)

Natural polymer coatings were characterized using a field emission scanning electron microscope (FE-SEM; FESEM Ultra Plus, Zeiss, Germany) at 3 kV of voltage and a secondary electron (SE) detector. In brief, solutions of natural polymers were incubated in TCPS coverslips o.n., then the excess of solution was removed and the remaining coating analyzed. The coverslips were placed onto metal plates and a 5 nm thickness iridium film was sputter-coated on the samples (Q150T S/E/ES Sample Preparation System, Quorum Technologies) before visualization.

2.2.3. Synthesis and characterization of nanofibers

2.2.3.1. Materials and instruments

All reagents and solvents for synthesis were reagent grade and were used without further purification, unless stated otherwise. Trimethylsilyl methacrylate (TMSMA) (Sigma-Aldrich) and methacrylic acid (MAA) (Fluka) were distilled at low pressure in a Büchi Glass Oven B-585 micro distiller before use. Azo*bis*isobutyronitrile (AIBN) (Fluka) was recrystallized from methanol and dried under vacuum at RT. GRGDS (Sigma) was dissolved in miliQ water to a concentration of 5 mg/mL. Polymer isolation and identification was performed as described above.

2.2.3.2. Polymer synthesis

PEG-PTMSMA-PMAA was obtained from the diblock copolymer poly(ethyleneglycol-*β*-trimethylsilylmethacrylate) (PEG-PTMSMA), which synthesis is described

elsewhere (Mouffouk et al., 2011). In short, to synthetize PEG-PTMSMA-PMAA, to 1.83 g of PEG-PTMSMA dissolved in 5 mL of tetrahydrofuran (THF), 1.8 mL (106 eq) of methacrylic acid and 0.033 g of AIBN were added. After three freeze-pump-thaw cycles, the mixture was incubated in an oil bath at 60 ºC, under stirring, for 24 h. The flask was then cooled down to RT and 20 mL of THF added to dilute the mixture, which had become very viscous, and its content precipitated by pouring into diethyl ether. The polymer was separated from the supernatant by centrifugation at 15344 x *g* for 10 min followed by decantation. This procedure was repeated once more, providing a white solid that was vacuum dried, at 40 ºC overnight, yielding 3.5 g (96%). ^{1}H NMR (CD_3OD): δ0.06 [(s), $(CH_3)_3Si$], 0.10 [s, $(CH_3)_3Si$], 1.09 (s, $CH_3C–C=O$), 1.19 (s, $CH_3C–C=O$), 1.86 (s, $CH_2C–C=O$), 1.99 (s, $CH_2C–C=O$), 3.35 [s, $CH_3O–(CH_2CH_2O)_n$], 3.64 [s, $(CH_2CH_2O)_n$], 3.72 [s, $(CH_2CH_2O)_n$]. DP_n (NMR) = 94. IR (KBr): 1706 [ν (C=O)], 1255 [δ(Si$(CH_3)_3$], 1181 and 846 [ν(Si$(CH_3)_3$] cm^{-1}. Gel permeation chromatography (GPC) analysis revealed a monomodal molecular weight distribution; elution time (t_e) = 16.00 min.

2.2.3.3. Polymeric fiber formation and bioconjugation with the peptides

Twenty mg of the polymer were added to 1 mL of miliQ water containing Pen/Strep (100 U/mL) and sonicated for 30 min. The solution became opaque white as the polymer dissolves and self-assembled into nanostructures that were confirmed by Cryo-TEM. Then, 15 µL of GRGDS peptide were added to the previous solution (corresponding to a 1:10 peptide/polymer molar ratio), and the resulting solution sonicated for an additional 5 min.

2.2.3.4. Cryo-Transmission Electron Microscopy (TEM)

The structure of the self-assembled nanofibers was observed on a Fei Titan Krios™ Cryo-Transmission Electron Microscope (TEM). Nanofibers were suspended in a fluid staining medium (1% uranyl acetate) and applied to a standard pre-treated support film. Then, the specimen grid was blotted with filter paper to remove excess fluid and rapidly plunged into liquid ethane that had been cooled to liquid nitrogen temperature (freezing rate on the order of 1.000.000 K/sec) to prevent

the formation of ice crystals. Images were recorded under low electron dose conditions (10 – 25 $e^-/Å^2$).

2.3. Stem cell culture in natural and synthetic polymeric coatings

2.3.1. Preparation of polymeric coatings and mESC culture

LBG has limited solubility in water and, hence, LBG solutions were prepared by dissolving the powder (Roeper, Germany) in sterile milliQ water and stirring for 2h at 60 ºC. LBG amount was adjusted to obtain a final concentration of 0.1% weight per volume (w/v). The solution was then transferred to a tube, sonicated o.n. at 60-70 ºC and filtered using a 0.45 µm pore size syringe filter. A 0.1% (w/v) solution of gelatin type B (Sigma, Switzerland) was also prepared in sterile milliQ water. The polymeric nanofiber solutions were prepared as described above (2.2.3.3. Polymeric fiber formation and bioconjugation with the peptides). Polymeric nanofiber and LBG solutions (LBG from Roeper, LBG from Industrial Farense, purified LBG) were placed into 6-well culture plate to cover the entire growth area, which allowed the nanofibers to form a mesh and adsorb to TCPS surface and the polymers to adsorb to the TCPS surface. Upon 12 h incubation at 4ºC, in case of using nanofibers the wells were sterilized for 30 minutes in UV light. Subsequently, the excess of nanofibers and natural polymers solution was removed and mESCs resuspended in growth medium were seeded directly onto polymer covered wells, in triplicate and at an approximate density of 20 000 cells/well. Half of the medium was changed every day.

2.3.2. Alkaline Phosphatase assay

The pluripotency of ESCs was tested by an Alkaline Phosphatase (ALP) staining, using an Alkaline Phosphatase staining kit (86R; Sigma) according to the manufacturer's instructions. In brief, the cells were fixed with the citrate:acetone:formaldehyde solution for 2 min, washed twice with water, stained with the ALP staining solution for 30 min and counterstained with Hematoxylin for 2 min. The morphology of the colonies and ALP positive cells were observed in an inverted light microscope (Leica DMIL) and photographed by the coupled digital camera (Leica DC 500).

2.3.3. Differentiation potential *in vitro*

Differentiation potential of ESCs cultured on nanofibers and in natural polymers over multiple passages was compared to cultures under standard conditions. Determination of the differentiation potential was assayed by the quantification of the expression of gene markers from the three primary germ layers in EBs collected at day 10 of differentiation. To do so, undifferentiated ESCs were seeded and passaged every 3 days at the same cell density for 10 passages. At day 30, correspondent to day 0 of differentiation, cells were resuspended in differentiation medium, which consists of growth medium with 15% (v/v) FBS and without LIF supplementation, and a three-dimensional environment consisting of embryoid bodies (EBs) was created to induce spontaneously differentiation of undifferentiated ESCs (Fig. 2.1). For that, cells were dissociated in ESC culture differentiation medium and drops of 20 µl of ESC suspension (22 cells/µl) plated onto the base of a bacteriological Petri dish. Cells were placed in hanging droplets by inverting the base of a Petri dish. After 48 hour incubation, mESC differentiation medium was added so that the cells were cultured in suspension for 4 days. The EBs were then plated on 0.1% gelatin coated 6-well plates at day 6 and cultured up to day 10 of differentiation. Culture medium was replaced every two days.

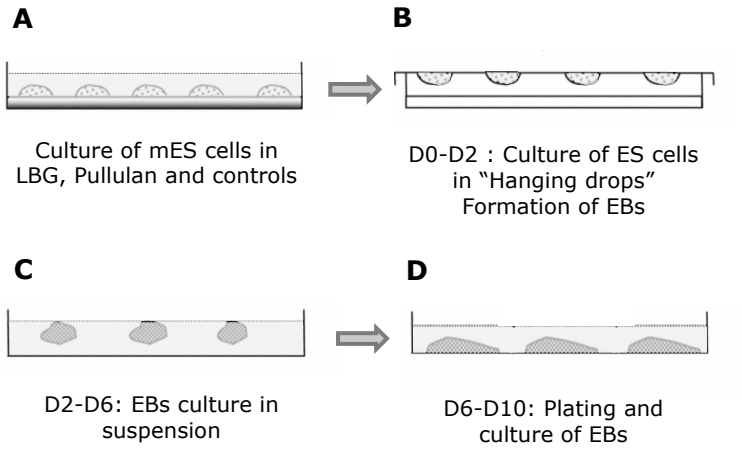

Figure 2.1: Schematic representation of the hanging droplet method used to induce differentiation of mESCs. A. Undifferentiated ESCs in culture. **B.** ESCs were dissociated and drops of 20 µl of ESC suspension (440 cells) were plated onto the base of a Petri dish (day 0). Cells were placed in hanging droplets by inverting the base of a Petri dish into the lid containing 2 ml of PBS. **C.** After 48 h incubation (days 1 to 2), the cells were cultured in suspension for a further 4 days. **D.** EBs were then plated and cultured up to day 10 of differentiation. Adapted from (Pandur 2005).

2.4. Characterization of *Ccbe1* mutant MEFs

2.4.1. Mice

The *Ccbe1* mutant mice used in the present work were ordered from "The Mutant Mouse Regional Resource Center" (MMRRC) at the University of California, in which coding exon 1 and exon 2 of the Ccbe1 gene was targeted (NCBI accession AK028377.1) by homologous recombination (Bos et al., 2011; Tang et al., 2010). MEFs derived from wild type (*Ccbe1*$^{+/+}$) and *Ccbe1* mutant (*Ccbe1*$^{-/-}$) embryos were used in the following methods at passage 0 to 3.

2.4.2. Cell Adhesion and Spreading

Ccbe1$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 0 were seeded sparsely in a 6-well plate at a density of 100 000 cells per well (10 400 cells/cm^2) previously marked with horizontal and vertical reference lines. Cell adhesion was evaluated at 0, 20, 40, 80 and 240 min (4h) by microscopy and photography. After 4 h, the detached cells were washed with PBS and the adherent cells were collected and counted. In addition, 130 000 MEFs at passage 1 were plated in wells of a 6-well plate (13 500 cells/cm^2) and cultured for 48h to reach confluence. Then, cells were washed with PBS and the adhesive capacity was determined by incubating the cells with 0.01% of trypsin for 0, 1, 2, 3, 5 and 8 min and monitored by microscopy and photography.

2.4.3. Proliferation and viability of *Ccbe1* KO mouse embryonic fibroblasts

2.4.3.1. Cell proliferation

MEFs were isolated separately from littermate embryos at 13.5 and 14.25 days of gestation. After 24 to 48h, adherent fibroblasts were collected, some were used for genotyping and the majority was split once before the proliferation assay. Subsequently, *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ primary MEFs were plated at a density of 144 000 cells per well of a 6-well plate (15 000 cells/cm^2), in technical triplicate, and subcultured or passaged every 3 days at the same cell density, in parallel and under identical cell culture conditions. The medium was changed 24h after seeding to remove cell debris. Passage 0 corresponds to the seeding day.

2.4.3.2. Cell cycle analyses by Bromodeoxyuridine labeling assay

The bromodeoxyuridine (BrdU) labeling assay started with the seeding of 80 000 *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ primary mouse embryonic fibroblasts per well, in triplicate, in a 6-well plate. Cells were cultured for 24 h and incubated with 10 µM of BrdU (BrdU Labeling and Detection Kit I - Roche) diluted in growth medium in last 14 h before flow cytometry acquisition. After BrdU incubation, BrdU labeling medium was aspirated and MEFs were washed twice with PBS to eliminate unincorporated BrdU. MEFs were then trypsinized and centrifuged at 300 x *g* in flow cytometry round-bottom tubes (BD Biosciences) for 5 min at 20 ºC. The pellet was resuspended and fixed and permeabilized by incubating with Cytofix/Cytoperm solution (BD Cytofix/Cytoperm™ Kit) for 15 min at RT. 3 ml of PBS were added to wash fixative solution and tubes were centrifuged for 5 min at 300 x *g* and 20 ºC. After fixation, MEFs were incubated with anti-BrdU working solution (mouse monoclonal antibody clone BMG 6H8 IgG1 containing nucleases, Roche) for 30 min at 37ºC / 5% CO_2, washed with PBS, followed by incubation in anti-mouse-Ig-fluorescein working solution (sheep Ig, Roche) for further 30 min at 37ºC / 5% CO_2. The BrdU antibody and the anti-mouse antibody were diluted at a ratio of 1:10 in incubation buffer and PBS respectively (Roche), according to the manufacturer's instructions. Finally, cells were washed with PBS and stained with 7-aminoactinomycin D (7-AAD; 1 µg/ml of 7-AAD; Biolegend). Nuclear uptake of BrdU and 7-AAD was quantitated on a cell analyzer cytometer (BD FACSCalibur™ cell analyzer, BD Biosciences) and analyzed with BD CellQuest™ Pro software (BD Biosciences). For BrdU evaluation by flow cytometry, was used excitation with the red laser (488 nm) and detection in the FL1 channel (green, 530/30 nm) and 7-AAD was excited by 488 nm laser light and detected in the FL 3 channel (>670 nm).

2.4.3.3. Cell Proliferation Dye eFluor® 670

Cultured *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs were trypsinized followed by two washes with PBS to remove any serum. Cells were resuspended in 1 ml of PBS at RT and counted. 1 ml of a 10 µM solution of Cell Proliferation Dye eFluor® 670 (eBioscience) in PBS (at RT) was added drop by drop to the cell suspension and incubated for 10 min at 37 ºC in the dark. The labeling was stopped by adding 10 ml of cold growth medium and incubation on ice for 5 min. Finally, labeled *Ccbe1*$^{+/+}$

and *Ccbe1*$^{-/-}$ MEFs were washed 3 times with 5 ml of growth medium and seeded at a density of 140 000 cells per plate of a 6 cm diameter culture plates (5 000 cells/cm^2). A portion of non-labeled and eFluor® 670 labeled MEFs was acquired on a cell analyzer cytometer (BD FACSCalibur™ cell analyzer, BD Biosciences) to define the population of undivided cells corresponding to day 0. Flow cytometry was carried out upon excitation with the red laser (633 nm) being the emission signals measured in the FL4 channel (661/16 nm). The fluorescence intensity of the dye was measured along the time of culture, at day 2, 4 and 7 by flow cytometry and data analysed using the BD FACSDiva™ software (version 6.1.3, BD Biosciences). The growth medium was changed at day 1, 3 and 5.

2.4.3.4. Apoptosis assay

Ccbe1$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs were seeded at a density of approximately 200 000 cells per plate of a 6 cm diameter culture plate (7 000 cells/cm^2) and cultured for 48h in growth medium (10% FBS) and in starvation medium (2% FBS) to induce cell stress. Then, cells were detached from culture plates and collected to a flow cytometry tube and incubated PI (5 µg/ml, Sigma) immediately before FACS acquisition by BD FACSCalibur™ cell analyzer. Flow cytometry was carried out upon excitation with the red laser (488 nm) being the emission signals measured in the FL2 channel (585/42 nm). Data was analyzed with BD CellQuest™ Pro software (BD Biosciences).

2.4.4. Immunofluorescence for Cleaved Caspase3

100 000 *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 3 were seeded per well of a 6-well plate in TCPS coverslips and cultured for 24 h. Then, the culture medium was removed, the coverslips rinsed with PBS and fixed with 1% paraformaldehyde (Sigma) for 30 min followed by 30 min of permeabilization with 1% Triton-X100 at RT. The samples were incubated in blocking solution (3 g Glycine, 800 g Bovine Serum Albumin, 80 g sodium azide, 400 ml PBS) for 30 min at 37 ºC. Cells were incubated with rabbit polyclonal anti-cleaved caspase 3 antibody (1:200; R&D Systems) in blocking solution o.n. at 4 ºC. Next, the cells were washed 3 times for 5 min with PBS, followed by incubation with goat anti-rabbit Alexa 594 (1:1000; Sigma) for 1h at 37 ºC in dark. Cells were then washed 3 times with PBS and

coverslips were mounted with Mowiol-DAPI (4',6-diamidino-2-phenylindole) and sealed. Images were captured using Axio Imager Z2 Fluorescence microscope (Zeiss).

2.4.5. Wound-induced migration assay

0.1% gelatin (Sigma), 10 µg/ml of fibronectin (Sigma) and vitronectin (Invitrogen) solutions were incubated o.n. at 4 ºC in a 6-well plate previously marked with an horizontal reference line. *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs were mitotically inactivated with mitomycin C incubation for 2 h and then seeded into the pre-coated 6-well culture plates at a concentration of 500 000 cells/well, in triplicate, and cultured in growth medium for 24 h. The confluent monolayers of cells were "wounded" by scraping the bottom of the dish with a plastic P1000 pipette tip. Three wounds per well were done perpendicularly to the reference line (Fig. 2.2 A). Cellular debris were removed by washing with 1 ml PBS and the growth medium was added. In the wound-healing assay using antibodies to block specific subtypes of integrins, cells were seeded on gelatin, scratches were performed and MEFs were incubated with growth medium supplemented with antibodies against β1-integrin (1:100; Santa Cruz) and β4-integrin (1:100; Abcam) and with 10 µg/ml of CCBE1 protein. The migration of the cells was monitored by microscopy and photography at 0, 6, 12 and 24 h time points. 10 measurements were performed per picture at 0 and 6h using ImageJ 1.45 Software (Fig. 2.2 B). The pictures were captured using the 10x objective of Leica DM IL microscope, in which, 1 mm was correspondent to 944 pixels.

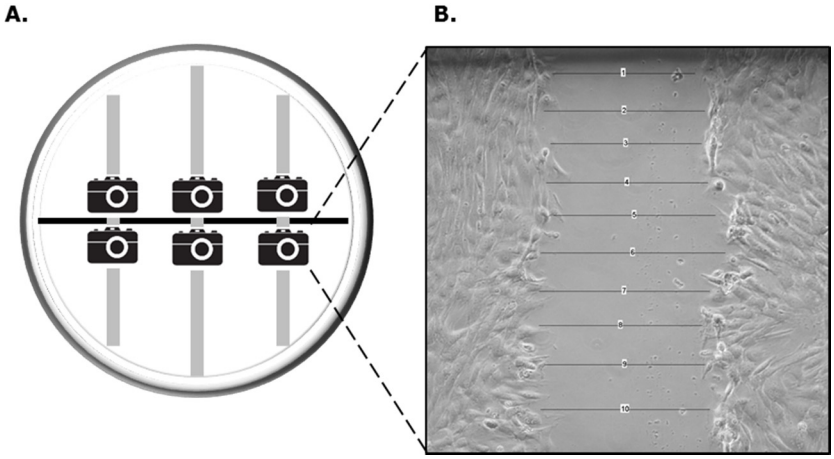

Figure 2.2: Representation of wound-induced migration assay. A. Schematic representation of one well from a 6-well plate during the migration assay. Pictures were

taken under and above the guideline at 5 time points (0, 6, 12, 16 and 24h). **B.** Representative picture of length measurements using ImageJ Software.

2.5. DNA extraction from MEFs and mouse tissues

A small portion of tail or head of the embryos was cut and mixed with 375 µl of lysis buffer [50 mM Tris-HCl pH 8, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.5 mg/ml Proteinase K] for 1h at on ThermoMixer Compact (Eppendorf) at full speed [1400 revolutions per minute (rpm)] until reaching complete digestion of the tissue. To pellet the proteins, 125 µl of saturated NaCl (6M) was added, the suspension was vortexed until tissue or cells particulates were dissolved and centrifuged at 16.1 x *g* for 10 min. The 475 µl of the supernatant (containing the DNA) was then transferred into a new 1.5 ml centrifuge tube and 375 µl of isopropanol was added and mixed 1 to 2 min on an eppendorf mixer at 750 rpm. The solution was centrifuged at 16.1 x *g* for 2 min at 4 ºC. Then, the DNA pellet was washed with 375 µl of 70% ethanol by inverting the tube several times. The solution was centrifuged one more time at 16.1 x *g* for 5 min at 4 ºC. The pellet was allowed to dry at RT. Finally, the DNA was dissolved in 50 µl nuclease free H_2O though incubation at 37 ºC for 15 min on an ThermoMixer Compact (Eppendorf) at 800 rpm and stored at 4 ºC.

2.6. RNA extraction

The RNA isolation was performed using TRI Reagent (Sigma) and was basically constituted by four steps: homogenization, phase separation, RNA precipitation and wash. The RNA was extracted after cells counting. The cellular suspension was centrifuged at 200 x *g* and 4 ºC and the pellet was collected to an RNAse free tube. The tube was quickly placed in ice and 1000 µl of TRI reagent was added with the objective of homogenizing the pellet and incubated at -80ºC o.n. After that, 200 µl of chloroform (Merck) was added to the homogenized and shaken vigorously. The resultant homogenized is centrifuged for 10 min at 16.1 x *g* and 4 ºC. Following centrifugation, three phases (pink, white and colorless) were visible within the tube. The colorless upper aqueous phase is thoroughly collected (avoiding transfer any interface) into a new tube containing 200 µl of chloroform and mixed. The mixture was centrifuged again and upper phase was collected to

a tube where 250 µl of isopropanol were previously added. The tube is mixed well by hand and placed at -20 ºC for 1 h or o.n., depending on the initial amount of cells. After RNA precipitation, the sample is centrifuged for 15 min at 16.1 x *g* and 4 ºC, the supernatant was removed and the pellet is carefully washed twice with 1000 µl of 75% ethanol and centrifuged for 5 min at 16.1 x *g* and 4 ºC. Subsequently, the supernatant was discharged and the pellet was left to dry for few minutes at RT. Finally, the pellet was dissolved in 20 µl of nuclease free H_2O and stored at -80 ºC.

2.7. Reverse Transcription PCR

The Reverse Transcription was performed using First strand cDNA synthesis kit in which the complementary DNA (cDNA) was synthesized from a volume equivalent to 0.5 or 1 µg of RNA sample, RiboLock RNase Inhibitor, Reaction Buffer, dNTP Mix, Oligo$(dT)_{18}$ Primer, RevertAid^{TM} M-MuLV Reverse Transcriptase and water nuclease-free up to 20 µl, following the manufacturer's instructions (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific). Non-template controls (NTC) containing no RNA and non-enzyme controls (NEC) were included. For quantitative PCR, cDNA was diluted at a ratio of 1:10. The resultant cDNA was stored at – 20ºC or at -80 ºC for longer storage.

2.8. Semi-quantitative PCR

The PCR mix was prepared with Dream Taq buffer (1x, Thermo Scientific), deoxyribonucleotides (dNTPs; 0.2 µM, Sigma), primer reverse (1.25 µM, Sigma), primer forward (1.25 µM, Sigma), Dream Taq Polymerase (0.06 U/µl, Thermo Scientific), 1 µl of each DNA sample and filled with nuclease free water to reach a total volume of 25 µl. In Table 1 there is a brief descript about the different primers and the PCR program.

2.9. Quantitative PCR

Quantitative PCR (qPCR) was performed using the Sso Fast Eva Green Supermix (Bio-Rad) on a CFX96 Real-Time PCR Detection System (Bio-Rad). Table 1 contains a brief description of the primers and qPCR programs used. The performance of

each qPCR reaction was determined using the standard curve, which consisted of a series of 10-fold dilutions of a positive control sample of cDNA. cDNA bands were cut from the gel and placed in 1.5 ml centrifuge tube. DNA was extracted with the GeneJET™ Gel Extraction Kit (Fermentas) according to manufacturer's instructions.

Reactions that performed efficiency upper than 90%, Pearson correlation coefficient close to 1 and melting curve without non-specific amplification were considered acceptable for current application. The relative level of expression of each target gene was calculated using ddCq method (Bustin, 2000). Co-culture of ESCs and MEFSi, cells cultured in gelatin or *Ccbe1*$^{+/+}$ MEFs were used as control and gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), TATA binding protein (*Tbp*) or phosphoglycerate kinase 1 (*Pgk1*) expression, depending on the experiment. Data were analyzed with Bio-Rad CFX Manager software.

2.10. Statistical analysis

Unless otherwise specified, results are expressed as mean ± standard deviation (SD). Data were statistically analyzed using One-way analysis of variance test (ANOVA) to test for significant differences between the experimental conditions and *t-student* for pairwise comparison, using Sigma Stat software. Differences were considered significant for $p<0.05$.

Table 2.1: Primer sequences, product size and program used for semi-quantitative and qRT gene expression analysis. *αMhc*, α-myosin heavy chain; *αSma*, α-smooth muscle actin; *Afp*, α-fetoprotein; *Bax*, BCL2-associated X protein; *Bcl2*, B cell leukemia/lymphoma 2; *Col1α1*, collagen type I α1; *cTnT*, cardiac troponin - T; *Mmp2*, matrix metallopeptidase 2; *uPa*, urokinase-type plasminogen activator.

Gene name	Forward sequence (5'–3')	Reverse sequence (5'–3')	Ta (ºC)	Amplicon size (bp)
Gapdh	GGGAAGCCCATCACCATCTTC	AGAGGGGCCATCCACAGTCT	59	356
Tbp	ACAGGAGCCAAGAGTGAAGAAC	GGAGAACAATTCTGGGTTTGA	56	244
Pgk1	ATGGATGAGGTGGTGAAAGC	CAGTGCTCACATGGCTGACT	59	118
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG	61	353
Oct4	AGTATGAGGCTACAGGGACA	CAAAGCTCCAGGTTCTCTTG	58	251
Sox2	CGAGATAAACATGGCAATCAAATG	AACGTTTGCCTTAAACAAGACCAC	56	236
Col1α1	GCAGACGGGAGTTTCTCCTC	TCAAGCATACCTCGGGTTTC	61	247
β1-integrin	AATGTGTTCAGTGCAGAGCC	TTGGGATGATGTCGGGA	53	261
βIII-tubulin	CCTGGAACCATGGACAGTGTT	CAGCACCACTCTGACCAAAGA	55	85
Sox1	CCAAGAGACTGCGCGCGCTG	GGGTGCGCCGGGTGTGCGTG	60	362
Nestin	AGGCTGAGAACTCTCGCTTGC	GGTGCTGGTCCTCTGGTATCC	58	111
Hand1	CCAGTTACATCGCCTACTTG	CCTGGTCTCACTGGTTTAGT	56	240
αSma	ATCGTCCACCGCAAATGC	AAGGAACTGGAGGCGCTG	56	89
Afp	ATGTATGCCCCAGCCATTCTGTCC	GAGATAAGCCTTCAGGTTTGACGC	60	442
Gata4	GAAAACGGAAGCCCAAGAACC	TGCTGTGCCCATAGTGAGATGAC	60	163
Isl1	CCTGTGTGTTGGTTGCGGCA	GGGCACGCATCACGAAGTCG	61	242
Nkx2.5	CCACTCTCTGCTACCCACCT	CCAGGTTCAGGATGTCTTTGA	60	107
cTnT	GGAAATCCAAGATCACTGCCTCC	GGGCACTGAGGGACAGACCA	60	168
αMhc	GATGGCACAGAAGATGCTGA	CTGCCCCTTGGTGACATACT	59	120
Ccbe1	GACACACGTGGACCTACCGAG	CCGTGCACTGCTGTTCACAGG	59	222
Ccbe1 WT	GGGGGCCGAGAAGAAGC	GAAGCACGTGGTGAGCTCG	57	652
Ccbe1 KO	GCAGCGCATCGCCTTCTATC	CACCCACAATCCAATCCGC	57	385
Mmp2	GGCTGGAACACTCTCAGGAC	CGATGCCATCAAAGACAATG	59	213
uPa	TTACTGCAGGAACCCTGACAACCA	TGCTAAGAGAGCAGTCATGCACCA	59	104
Bax	ATGCGTCCACCAAGAAGCTGAG	CCCCAGTTGAAGTTGCCATCAG	60	166
Bcl2	GTCCCGCCTCTTCACCTTTCAG	GATTCTGGTGTTTCCCCGTTGG	60	148

Results

Chapter III

Locust bean gum as an alternative polymeric coating for embryonic stem cell culture

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Author´s contribution:

The majority of the experimental work was performed by A.R. Perestrelo with the exception of the coating characterization by electronic microscopy performed by A. Grenha.

3.1. ABSTRACT

Pluripotent embryonic stem cells (ESCs) have self-renewal capacity and the potential to differentiate into any cellular type depending on specific cues (pluripotency) and, therefore, have become a vibrant research area in the biomedical field. ESCs are usually cultured in gelatin or on top of a monolayer of feeder cells such as mitotically inactivated mouse embryonic fibroblasts (MEFsi). The latter is the gold standard support to maintain the ESCs in the pluripotent state. Examples of versatile, non-animal derived and inexpensive materials that are able to support pluripotent ESCs are limited. Therefore, our aim was to find a biomaterial able to support ESC growth in a pluripotent state avoiding laborious and time consuming parallel culture of MEFsi and as simple to handle as gelatin. Many of the new biomaterials used to develop stem cell microenvironments are using natural polymers adsorbed or covalently attached to the surface to improve the biocompatibility of synthetic polymers. Locust beam gum (LBG) is a natural, edible polymer, which has a wide range of potential applications in different fields, such as food and pharmaceutical industry, due to its biocompatibility, adhesiveness and thickening properties. The present work brings a natural system based on the use of LBG as a coating for ESC culture. Undifferentiated mouse ESCs were cultured on commercially available LBG to evaluate its potential in maintaining pluripotent ESCs. In terms of morphology, ESC colonies in LBG presented the regular dome shape with bright borders, similar to the colonies obtained in co-cultures with MEFsi and characteristic of pluripotent ESC colonies. In short-term cultures, ESC proliferation in LBG coating was similar to ESC cultured in gelatin and the cells maintained their viability. The activity of alkaline phosphatase and *Nanog*, *Sox2* and *Oct4* expression of mouse ESCs cultured in LBG were comparable or in some cases higher than in ESCs cultured in gelatin. An *in vitro* differentiation assay revealed that mouse ESCs cultured in LBG preserve their tri-lineage differentiation capacity. In conclusion, our data indicate that LBG coating promotes mouse ESC growth in an undifferentiated state demonstrating to be a viable, non-animal derived alternative to gelatin to support pluripotent mouse ESCs in culture.

Keywords: embryonic stem cell culture, locust bean gum, natural polymers; gelatin substitute growth support, pluripotency.

3.2. INTRODUCTION

The large potential of ESCs for clinical applications, replacement therapies or tissue engineering is evidenced by their ability to self-renew and to differentiate into many cellular types (Evans and Kaufman, 1981). General practices of ESC growth usually rely on their culture on a feeder layer of mitotically inactivated primary mouse embryonic fibroblast to maintain them in a pluripotent state (Lanza et al., 2009). However, maintaining a parallel culture of MEFsi to the ESC culture is laborious and time-consuming, and certain procedures require additional steps for the separation of both cells. Therefore, many researchers culture ESCs in tissue culture polystyrene vessels coated with gelatin or Matrigel®, which are animal-derived protein solutions.

Gelatin, a translucent and colorless substance derived from collagen, is a cheap coating that has been vastly used to culture mouse ESC. As gelatin is an animal-derived material produced by partial hydrolysis of collagen extracted from the boiled bones, connective tissues and organs of animals (Gorgieva and Kokol, 2011), it poses problems for use in cell replacement therapies.

Nevertheless, in order to use ESCs in the aforementioned applications, they must be cultured in animal-free derived matrices. For that reason, several authors have been developing synthetic and natural alternative supports that could be used to maintain ESC pluripotency, enabling a subsequent application in basic stem cell biology and regenerative medicine (Jia et al., 2013; Kaivosoja et al., 2012; Meade et al., 2013; Perestrelo et al., 2013). Non-animal derived materials are less prone to induce problems for cell replacement therapies, namely immunogenic reactions, which is a clear advantage. Synthetic polymers are easily incorporated into a wide variety of materials, making their application very frequent. Nevertheless, replacing these polymers by natural counterparts is a parallel and cheaper approach, which further evidences high biocompatibility potential. Indeed, natural polymers are frequently used in nanocomposites or as coatings to improve the biocompatibility of metal implants, being highly recommended to make the bridge between synthetic devices and human tissue (Hauser et al., 2010).

Polysaccharides are the most common polymers in nature, consisting of monosaccharides bound together through glycosidic bonds (de Jong and van de Velde, 2007). The most abundant polysaccharides include starch, cellulose, chitin, glycogen, galactomannans and carrageenans (de Jong and van de Velde, 2007;

Jana et al., 2011). Among the galactomannans, Locust Bean Gum (LBG) is obtained from the seeds of the carob tree (*Ceratonia siliqua*) where it normally acts as storage carbohydrate during germination. LBG has a mannose backbone with single side chain galactose units in a mannose/galactose ratio of approximately 4:1 (Fig. 3.1 A). LBG is commercially available and is widely applied in different areas, from food to pharmaceutical industry, due to its gelling and thickening properties (Dionísio and Grenha, 2012). Additionally, it is also used as stabilizer and emulsifier. In this work a new application of LBG is described. The development of a LBG coating and its application in ESC culture as a support for undifferentiated and pluripotent mouse ESC growth is reported.

3.3. RESULTS

3.3.1. LBG solution forms an organized and patterned coating

To characterize the topography of the LBG coating, TCPS plates coated with the polymer were visualized by SEM. As described in the methodology section, the LBG coating was achieved by overnight incubation of LBG aqueous solution with the plates. SEM microphotographs revealed that the LBG coating induces a rough striated topography in a relatively well-organized pattern (Fig. 3.1 B).
TCPS (negative control) and gelatin coating were also characterized by SEM and revealed a completely smooth surface (data shown in *Supplementary Study*).

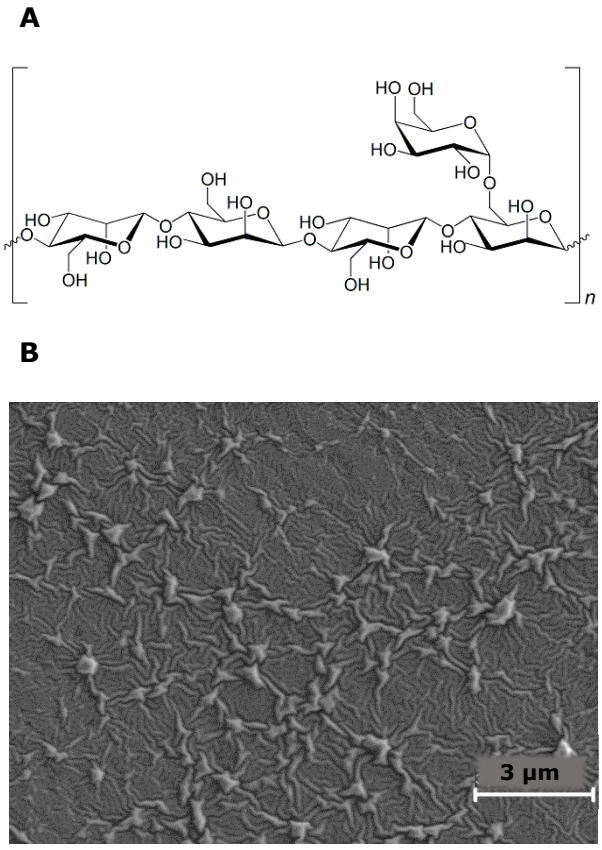

Figure 3.1: Chemical and morphological structure of LBG. A. Chemical structure of locust bean gum showing a linear polysaccharide (1-4)-β-linked backbone of mannose units with single (1-6)-α-D-galactose units attached. Adapted from (Dionísio and Grenha, 2012) **B.** SEM morphology of LBG coating. The polymeric film forms a striated and rough mesh.

3.3.2. LBG coating promotes ESC proliferation

To assess the ability of commercially available LBG to promote ESC adhesion, growth and self-renewal, undifferentiated mouse ESCs were cultured for 3 passages (9 days) in LBG coating or using standard conditions, such as in gelatin coating, on top of MEFsi and in the negative control TCPS. According to our data, the proliferation at days 3 and 6 of culture was significantly higher in ESCs cultured in LBG (12.71- and 11.18-fold) than in gelatin (10.37- and 9.27-fold) or TCPS (8.44- and 7.27-fold) (Fig. 3.2 A). After 9 days in culture, however, there were no significant differences in cell growth between ESCs grown in gelatin or LBG coatings, but ESCs cultured in LBG still presented significantly higher proliferation than ESCs cultured in TCPS, 10.93- and 8.25–fold respectively (Fig. 3.2 A). As expected, while ESCs cultured in TCPS had the lowest proliferation at all passages, the highest proliferating rate was observed in ESCs cultured on top of MEFsi, the gold standard support to growth ESCs (Fig. 3.2 A). These results suggest that in short-term culture, ESC proliferation in LBG coating was at least equivalent and for the first two passages higher than ESCs grown in gelatin.

Next, we evaluated cell viability by incubating ESCs with trypan blue, a dye which is taken up by dead cells but excluded from viable ones. The assay was performed at 3 and 9 days of culture and the percentage of viable cells was determined. As evidenced in Fig. 3.2 B, LBG coating maintains ESC viability more efficiently than gelatin, in the latter the percentage of viable ESCs being only approximately 89%. These data indicate that LBG coating is a suitable matrix to support proliferation and viability of ESCs in culture.

The culture conditions may play an important role in determining cell shape. Indeed, many cell cultures are capable of exhibiting multiple morphologies depending on the growth surface (Chen et al., 2012; Ji et al., 2012; D.-H. Kim et al., 2010; Solon et al., 2007). Morphologically, colonies in LBG presented a round, regular and well-defined dome-shape typical of pluripotent colonies (Fig. 3.2 C: a, a’), similar to the colonies obtained in cultures in MEFsi (Fig. 3.2 C: b, b’), even after 9 days of culture. In contrast, ESCs cultured in gelatin or TCPS are disorganized presenting a flat and irregular shape of the colonies, and with individual cells that develop cytoplasmic extensions which are characteristic of differentiated cells (Fig. 3.2 C: c, c’, d, d’). Therefore, simple morphological

analysis indicates that ESCs grown in LBG form colonies with the shape of pluripotent ESC colonies.

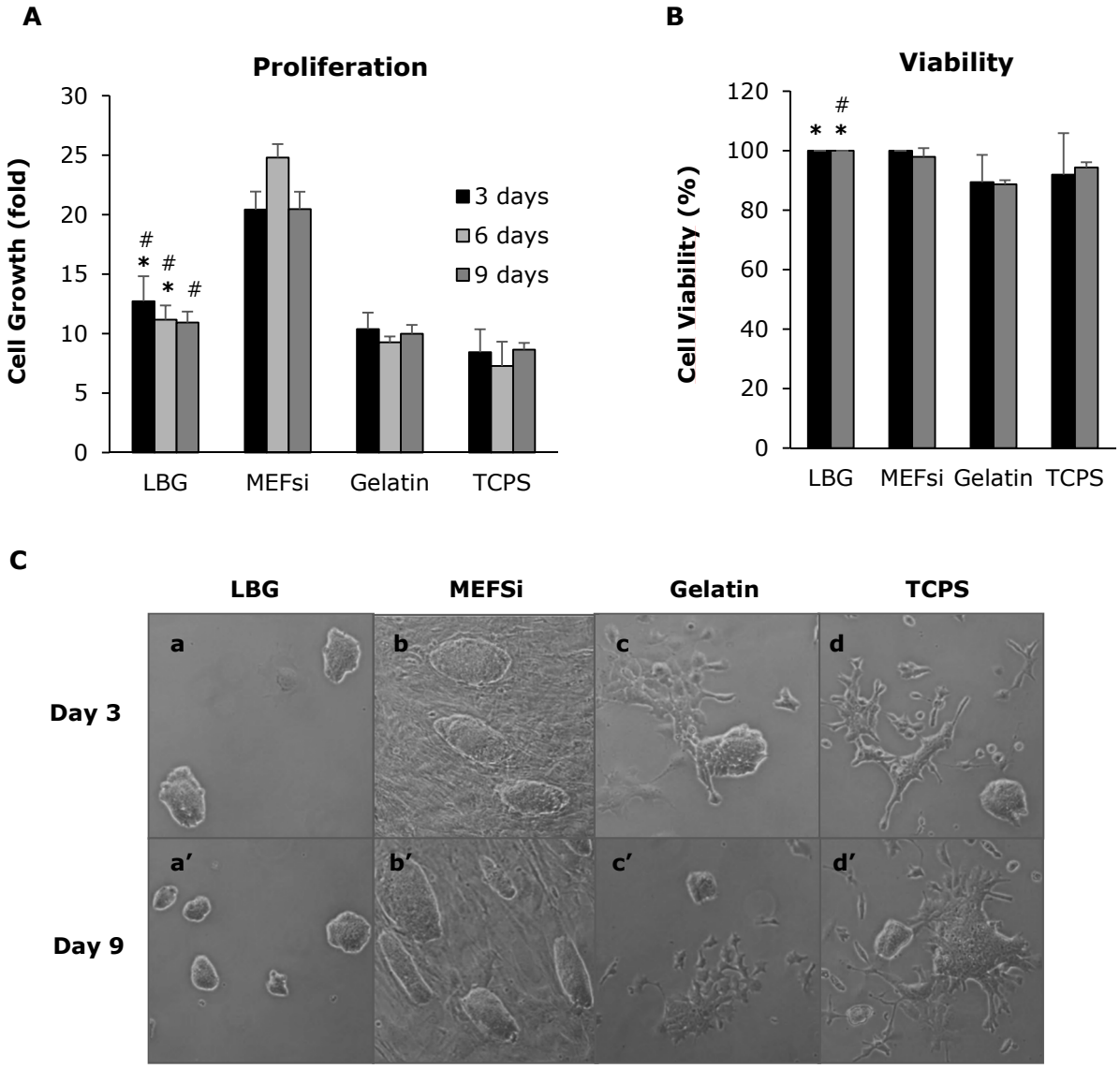

Figure 3.2: Cell morphology, proliferation and viability. A. Cell proliferation tests performed during 3, 6 and 9 days of mouse ESC culture. Cell growth (fold) is presented as the ratio between viable cells and initial cell number. **B.** Cell viability results obtained at day 3 and 9 of culture. Viability is expressed in percentage of the ratio between the number of viable cells and total number of cells. (*) significantly different cell number in LBG ($p<0.05$) compared to gelatin. (#) significantly different cell number in LBG compared to negative control value (TCPS) ($p<0.05$). Data presented as mean + SD from three independent experiments performed in technical triplicates (n=9) **C.** Representative contrast phase images of E14GFP8 mouse ESCs at day 3 (a-d) and day 9 (a'-d') of culture in a. 0.1% LBG, b. MEFsi, c. 0.1% Gelatin and d. TCPS. Magnification is 100x.

3.3.3. LBG coating supports self-renewability of ESCs

To evaluate the pluripotency when ESCs endure long-term subculture on LBG coating, undifferentiated mouse ESCs were seeded at the same density and passaged every 3 days during 30 days. Thereafter, ESCs pluripotency was determined through alkaline phosphatase (ALP) activity assay at passage 3 (day 9), and qPCR analysis to determine the expression of pluripotency markers at passage 3 and 10 (day 30). The expression of ALP, a marker of undifferentiated, pluripotent and capable of long-term self-renewal ESCs (Lanza et al., 2009; Martí et al., 2013), is commonly used to discriminate between pluripotent and differentiated cells. This can be easily assessed with a staining due to the capacity of this enzyme to change the conformation of a colorimetric reagent from a soluble to a precipitated state (Martí et al., 2013).

As depicted in Fig. 3.3 A, at passage 3 there were no significant differences in the expression of *Oct4* and *Sox2* between ESCs cultured in LBG, gelatin or MEFsi. In contrast, it is observed that the expression of *Nanog*, which encodes for a transcription factor critically involved in self-renewal of ESCs, was only similar in ESCs cultured in LBG (0.92-fold) and in MEFsi (1.01-fold), and significantly higher than ESCs cultured in gelatin (0.44-fold) or in TCPS (0.57-fold). Furthermore, ALP stainings at passage 3 revealed that ESCs cultured in gelatin or in TCPS showed more differentiated ALP-negative ESCs (white arrows) than those cultured in LBG (Fig. 3.3 C: a-d). In fact, all the colonies in LBG were, similarly to ESCs cultured in MEFsi, ALP-positive (red arrow) and exibited regular shape (Fig. 3.3 C: a, b). At passage 10, the differences in the expression of pluripotency markers among culture conditions became more evident. *Nanog* expression in ESCs cultured in LBG (0.98-fold) was comparable to ESCs cultured in MEFsi or in gelatin, 1- and 0.77-fold, respectively, and significantly higher than ESCs cultured in TCPS (0.46-fold) (Fig. 3.3 B). Furthermore, even though *Sox2* expression in ESCs cultured in LBG (1.13-fold) and in MEFsi (1-fold) was similar, *Sox2* expression in ESCs cultured in gelatin or in TCPS was significantly lower, 0.51- and 0.39-fold, respectively. (Fig. 3.3 B). The more evident differences were observed in the expression of *Oct4*, the pivotal regulator of pluripotency (Shi and Jin, 2010), in ESCs grown in LBG coating. *Oct4* expression in ESCs cultured in LBG (1.97-fold) was approximately the double of the expression in MEFsi (1-fold) or in gelatin (1-fold) or in TCPS (0.84-fold) (Fig. 3.3 B).

At passage 10, the morphology of the ESCs colonies cultured in LBG coating was comparable to the morphology of ESCs cultured in MEFsi, resembling the typical dome-shape pluripotent colonies (Fig. 3.3 C: a, b). In contrast, ESCs cultured in gelatin or in TCPS presented a fibroblast-like morphology, with colonies that clearly lost their discreet borders, suggesting ESC differentiation (Fig. 3.3 C: a'-d'). Taken together, these data suggest that, unlike ESCs cultured in gelatin coating or TCPS, ESCs cultured in LBG coating efficiently maintain their pluripotency even during long-term culture.

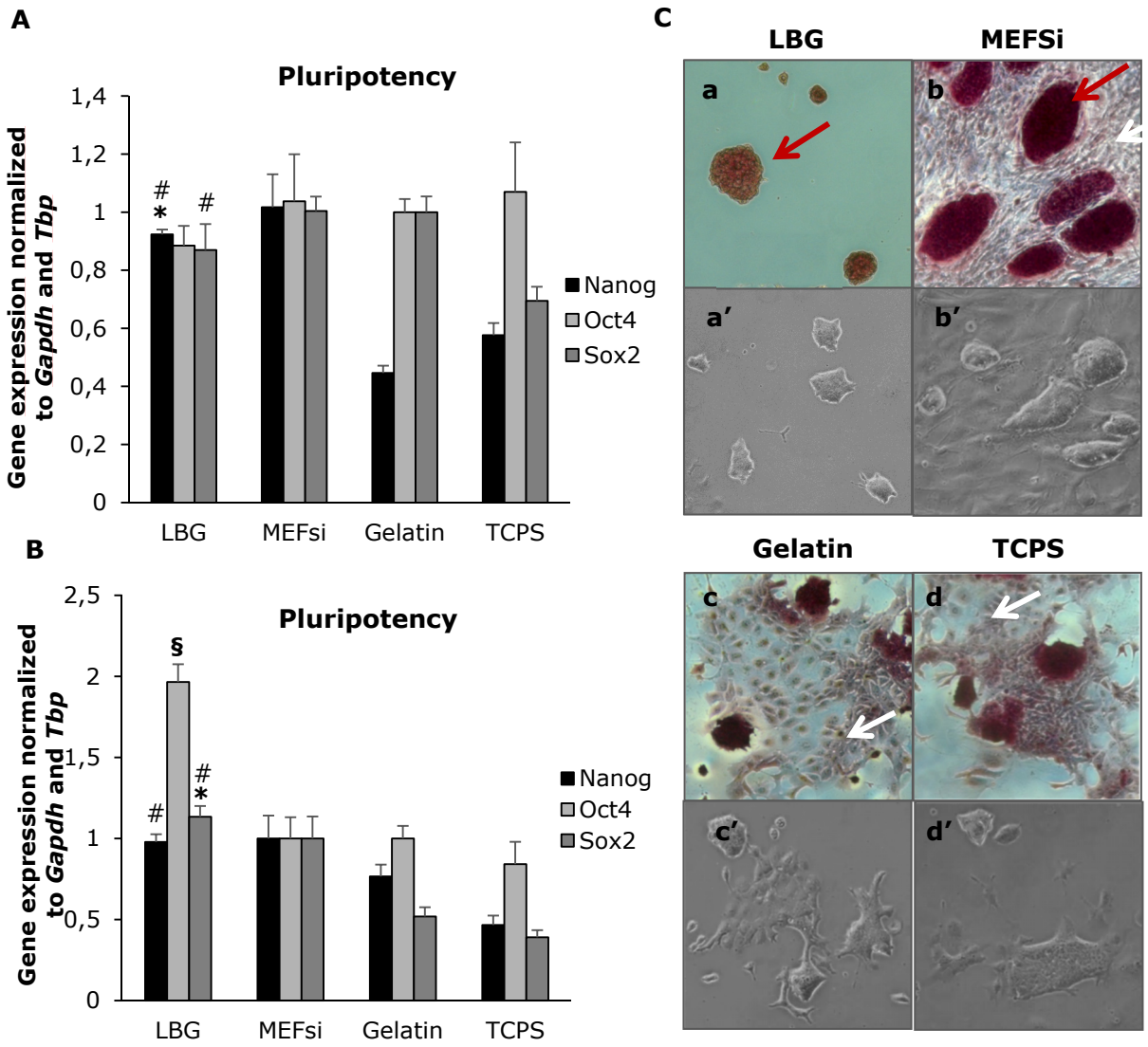

Figure 3.3: Preservation of ESCs Pluripotency. Analysis of stem cell markers expression, *Nanog*, *Oct4* and *Sox2*, at passage 3 (**A.**) and at passage 10 (**B.**) of cells cultured on LBG and in standard conditions. (*) significantly different expression levels in LBG compared to gelatin. (#) significantly expression levels in LBG compared to TCPS. (§) significantly expression levels in LBG compared to gelatin, TCPS and MEFsi. Data are presented as mean + standard error of mean, representative of two independent experiments performed in technical triplicates (n=6) **C.** Representative pictures of mouse

ESCs cultured on natural polymers at passage 3 stained for ALP assay (a-d). ESCs morphology at passage 10 (a'-d'), cultured in a. 0.1% LBG, b. MEFsi, c. 0.1% Gelatin and d. TCPS. Magnification is 100x. White arrow indicates differentiated cells and red arrows point pluripotent colonies.

3.3.4. ESCs grown on LBG coating maintain their *in vitro* tri-lineage differentiation capacity

The expression of pluripotency markers is not sufficient to determine if ESCs are pluripotent. Only the simultaneous presence of pluripotency markers and the demonstration that the ESCs are able to differentiate into cells from all three germ layers define them as truly pluripotent (Martí et al., 2013). On the other hand, it is important to evaluate this tri-lineage differentiation capacity upon long-term culture to evaluate if long exposure to the coating leads to the accumulation of cytotoxic effects and to alterations in cell behavior (Klim et al., 2010). Therefore, the differentiation capacity was assessed after long-term culture of ESCs in LBG coating or in standard conditions mentioned earlier, by *in vitro* differentiation of ESCs through embryoid body (EB) formation. Briefly, undifferentiated mouse ESCs were passaged every 3 days at the same cell density for 10 passages in LBG coating or in control conditions. At day 30, spontaneous differentiation of mouse ESCs was induced by forcing ESCs to aggregate and form three dimensional aggregates, known as EBs. ESCs were allowed to differentiate for 10 days. Subsequently, at day 10 of differentiation, the EBs were collected for RNA isolation and the differentiation evaluated by quantitative analyses of the expression of gene markers of the three germ layer. Morphological analysis indicates that the cells migrated or extend outwards from the EBs and displayed either epithelial- or mesenchymal-like morphologies. Cells were organized in multilayers and presented beating foci, thus confirming that the ESCs were differentiated (Fig. 3.4 A). In the case of ESCs cultured in LBG coating, the EBs also gave rise to more complex structures, such as fibrous and more organized structures (Fig. 3.4 A).
Quantitative analysis revealed that the ESCs cultured in LBG coating or in MEFsi presented higher expression of differentiation markers than ESCs cultured in TCPS or in gelatin. Indeed, the expression of the endoderm marker *Afp* was significantly higher in ESCs cultured in LBG than in gelatin, MEFsi or TCPS. Nonetheless, no significant differences were found in the expression of the endoderm and mesoderm marker *Gata4* among the culture conditions (Fig. 3.4 B). In addition,

the expression of the mesoderm marker *Hand1* was similar between all the culture conditions, and the expression of another mesoderm marker *αSma* was significantly higher in ESCs cultured in LBG than in gelatin, MEFsi or TCPS (Fig. 3.4 C). Regarding the differentiation in ectoderm lineages, expression of both *βIII-Tubulin* and *Nestin* in ESCs cultured in LBG was identical to ESCs cultured in MEFsi and significantly higher than ESCs cultured in gelatin or TCPS (Fig. 3.4 D). These data indicate that ESCs cultured in LBG coating retain their tri-lineage differentiation capacity even after long-term culture. In addition, increased expression of some of these marker genes in ESCs cultured in LBG when compared to ESCs cultured gelatin or TCPS suggests that ESCs cultured in LBG coating retain their pluripotency much more efficiently than ESCs cultured in gelatin or TCPS.

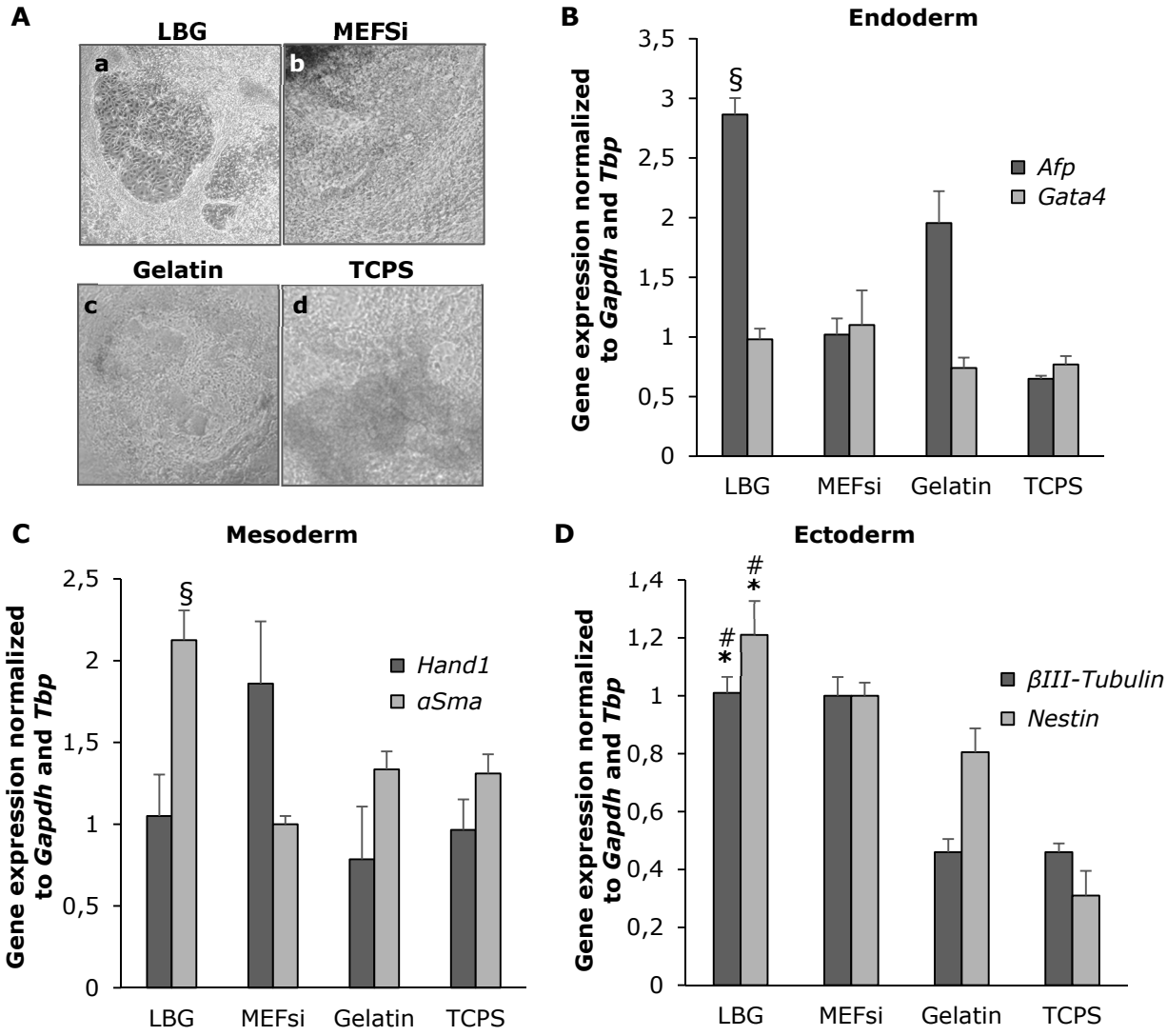

Figure 3.4: *In vitro* trilineage differentiation. ESCs cultured on LBG coating for 30 days were induce to differentiate through hanging drops method. **A.** Morphology of EBs at day 10 of differentiation. **B-D**. Levels of gene expression of germ layer markers of EBs

collected after 10 days of differentiation. **B.** *Afp* and *Gata4* are expressed in endoderm and mesoderm. **C.** *Hand1* and *αSma* mark mesoderm. **D.** *βIII-Tubulin* and *Nestin* are ectodermal marker genes. The relative gene expression was normalized to *Gapdh* and *Tbp* and to MEFsi control. (*) significantly different expression levels in LBG compared to gelatin. (#) significantly different expression levels in LBG compared to TCPS. (§) significantly different expression levels in LBG compared to gelatin, TCPS and MEFsi. Data are presented as mean + standard error of mean, representative of two independent experiments, performed in technical triplicates (n=6).

As a whole, these data indicate that natural polymer LBG coating is a suitable support to maintain pluripotent mouse ESCs in culture, even better than the animal-derived gelatin.

3.4. DISCUSSION

The polysaccharide LBG, which is commonly used due to its biocompatibility, adhesive and thickening properties, revealed to be able to form a coating with rough topography. The formation of such a ridged pattern could be attributed to the properties of the LBG solution as it is known that, when dissolved, LBG adopts a disordered fluctuating random coiled conformation (Lundin and Hermansson, 1995). Interestingly, our data suggest that when undifferentiated mouse ESCs were cultured in LBG coating and in standard culture conditions, the proliferation rates were proportional to the roughness of growth surface. Rough surfaces have higher contact area which increases the availability of integrin binding sites or cell-matrix adhesion spots, thus promoting integrin assembly into focal contacts and further cell adhesion (Park et al., 2007). Indeed, according to our data, at passage 1 the total cell number increased in cultures from TCPS to gelatin, which are smooth surfaces, followed by LBG and MEFsi that are rough surfaces. Furthermore, the same was observed at passage 2 and 3. This suggests that the rough topography of the LBG coating is able to promote ESCs growth more efficiently than the smoother surfaces provided by TCPS or gelatin coating.

On the other hand, cell viability assessment showed that 100% of ESCs cultured in LBG coating were viable, while the culture in gelatin only provided 89% viability. According to I.S. EN ISO 10993-5:2009 a material is considered cytotoxic if cell viability becomes lower than 70% (Europeam Commitee for Standardization, 2009). Even though 89% of cell viability is acceptable, the accumulation of unviable cells along the time of culture in gelatin may become problematic, especially if it leads to the activation of mechanisms that change the fate of the ESCs (Ardehali et al., 2011). In sum, these data indicate that in short-term culture LBG coating is better than that of gelatin to support ESC proliferation and viability.

A biomaterial is considered suitable for culture of pluripotent ESCs when, besides supporting growth and viability, it has the capacity to maintain the ESCs undifferentiated and their tri-lineage differentiation capacity. Pluripotent cells growing as EBs are able to differentiate spontaneously to cell types of all three germ layers (Martin and Evans, 1975; Ying et al., 2008). Our data demonstrated that EBs composed of ESCs grown in long-term culture in LBG coating gave rise to differentiated heterogeneous populations of cells exhibiting different morphologies

and tissue-like structures. Furthermore, gene expression analysis revealed the presence of derivatives of all three primary germ layers, confirming that ESCs grown in LBG coating efficiently maintain their tri-lineage differentiation capacity, even upon long-term culture. On the other hand, the colonies of ESCs grown for long-term in LBG coating, used for the spontaneous differentiation in EBs, were compact with the shape of undifferentiated pluripotent ESC colonies. According to the results of ALP staining and, unlike ESCs cultured in TCPS or gelatin coating, all the ESCs cultured in LBG coating and on top of MEFsi were ALP-positive. This indicates that while LBG coating is able to maintain the ESCs in their pluripotent state, ESCs grown gelatin and TCPS are able to differentiate, thus losing their pluripotency. In fact, there is a general agreement that topography has an important role in cell adhesion, growth and pluripotency (Blin et al., 2010; McNamara et al., 2010; Qi et al., 2013). Furthermore, gene expression analysis of pluripotency markers after 3 or 10 consecutive passages was comparable or higher in ESCs cultured in LBG than ESCs cultured in gelatin or in MEFsi. The differences in the expression of pluripotency markers were more evident at passage 10 highlighting the importance of making long-term assays for the validation of a material for ESC culture. The obtained results suggest that LBG coating is able to support pluripotent ESCs in culture and that, possibly, LBG is more suitable than gelatin as a coating for stem cell research applications. Besides from being an animal-derived material, gelatin may also, as shown here, introduce potential variability into the cell culture system. Furthermore, when the ESCs are cultured in gelatin, part of the population is differentiated during the culture, which is consistent with previous reports (Ramírez et al., 2011). Some authors are so critical of the usage of gelatin that they use it as a negative control, considering it is not the ideal substrate to maintain pluripotent ESCs (Ramírez et al., 2011; Yue et al., 2012). The alternative proposed here, the LBG coating, is even more economic than gelatin and is a vegetal-derived polymer. The combination of price, origin, topography, adhesive and gelling properties, makes LBG a perfect candidate to substitute gelatin coating. Indeed, LGB coating seems to be able to overcome one of the biggest challenges for culture defined matrices, which is long-term pluripotent undifferentiated stem cell propagation.

Chapter III. I

Supplementary Study

Comparative study of LBG from different sources

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Author´s contribution:

The majority of the experimental work was performed by A.R. Perestrelo with the exception of the coating characterization by electronic microscopy performed by A. Grenha and the purification of LBG performed by L. Braz and A.M.R. Costa

3.1.1. ABSTRACT

Biopolymers (or natural polymers) are an attractive class of biodegradable polymers since they are derived from natural sources, easily available, cheap and can be modified using suitable reagents. There are several advantages and disadvantages of using natural polymers instead of synthetic polymers, being the main advantage the high levels of biocompatibility and the principal disadvantage the possible batch-to-batch and manufacturer-to-manufacturer variation. In fact, LBG, our biomaterial of interest, is a galactomannan, obtained from endosperm of the seeds of the carob tree, in which it acts as a carbohydrate reserve during germination.

The purpose of the present chapter is to validate the use of LBG instead of gelatin for embryonic stem cell culture research, even when LBG is obtained from different sources and with different purification levels. In general, the results showed no significant differences between LBG from Roeper, Industrial Farense and purified LBG from Industrial Farense, but still similar or potentially better than gelatin. These data confirms the application potential of LBG for pluripotent ESC culture.

Keywords: batch-to-batch and manufacturer-to-manufacturer variation, biopolymers, embryonic stem cell culture, locust bean gum, gelatin substitute growth support.

3.1.2. INTRODUCTION

Featuring different physicochemical properties, galactomannans are a versatile material used for many applications: they are excellent stiffeners and stabilizers of emulsions, and the absence of toxicity allows their use in the textile, pharmaceutical, biomedical, cosmetic and food industries (Vipul D Prajapati et al., 2013).

Our galactomannan of interest, LBG, consists of galactose and mannose in the ratio 1:4 and derived from the endosperm (gum) of carob seeds (Dionísio and Grenha, 2012). Despite the advantage of deriving LBG from a natural source, plant-based materials display variations in their properties according to the type of culture, growth conditions, collecting season, method of extraction, purification process and manufacturing practices (Beneke et al., 2009). In fact, in the case of LBG the ratio of galactose to mannose is approximately 1:4, but it differs depending on the age of the plant, growth conditions of the plant during production and the method of extraction of the polysaccharide (Dionísio and Grenha, 2012). In addition, as most of the galactomannans used in pharmaceutical technology and cosmetics, which are usually unpurified gums (Üner and Altinkurt, 2004), LBG can contain impurities. These include husk, germ, residual amounts of ethanol or isopropanol and microbiological contaminations that can affect the protein and ash content, which may affect cells cultured in the presence of LBG. In terms of composition, usually the commercial samples of LBG contain approximately 5–12% moisture, 1.7–5% acid-soluble ash, 0.4–1.0% ash, and 3–7% protein (Vipul D. Prajapati et al., 2013). In our perspective, the variations of LBG concerning the batch, producer and possible impurities were sufficient to motivate an additional study to determine whether LBG from different sources and purified *versus* non purified are equally able to replace gelatin in mouse ESC culture.

3.1.3. RESULTS

In the present study we used LBG from Roeper and Industrial Farense (LBG I. F.), and purified LBG from Industrial Farense (LBG I.F.P.), to support pluripotent mouse ESCs in culture.

3.1.3.1. Characterization of LBG

3.1.3.1.1. Chemical composition

LBG is a high molecular weight branched galactomannan polysaccharide consisting more in detail of a linear chain of (1→4)-linked β-D-mannopyranosyl units with evenly spaced (1→6)-linked α-D-galactopyranosyl residues as side chains. In general, it presents a molecular weight range of 50 to 1000 kDa (Dakia et al., 2008).

LBG from Roeper has origin in Spain, Italy and Turkey, and is commercialized as a white to yellowish-white colored powder containing galactomannan (75% minimum), water (14% maximum), protein (7% maximum) and ash (1.2% maximum). The LBG that was kindly given by Industrial Farense, was provided without quality or analysis datasheet. The latter LBG was purified by heating followed by precipitation, filtration and drying. It was assumed that samples of clarified or purified LBG should contain approximately 3–10% moisture, 0.1–3% acid-soluble matter, 0.1–1% ash, and 0.1–0.7% protein (Dakia et al., 2008).

3.1.3.1.2. Topographic characterization of LBG coating

We started by characterizing the coatings used for ESC culture by FE-SEM. To do that, LBG and gelatin aqueous solutions were incubated overnight in TCPS coverslips. The next day, the excess of solution that was not adsorbed to TCPS surface was removed and samples were processed for FE-SEM.

FE-SEM micrographs revealed that the LBG coating from Roeper acquires a relatively organized striated topography (Fig. 3.5 A). The coating disposition acquired for LBG from Industrial Farense (LBG I.F.) was also rough, but presented a somewhat different pattern when compared to LBG from Roeper (Fig. 3.5 B, C). Both purified and non-purified coatings generated using LBG from Industrial Farense, form a rough and porous coating (Fig. 3.5 B, C). Therefore, no apparent

differences were found between purified and non-purified LBG from the same supplier (Fig. 3.5 B, C). In contrast, the gelatin coating and negative control TCPS exhibited an almost smooth surface (Fig. 3.5 D, E).

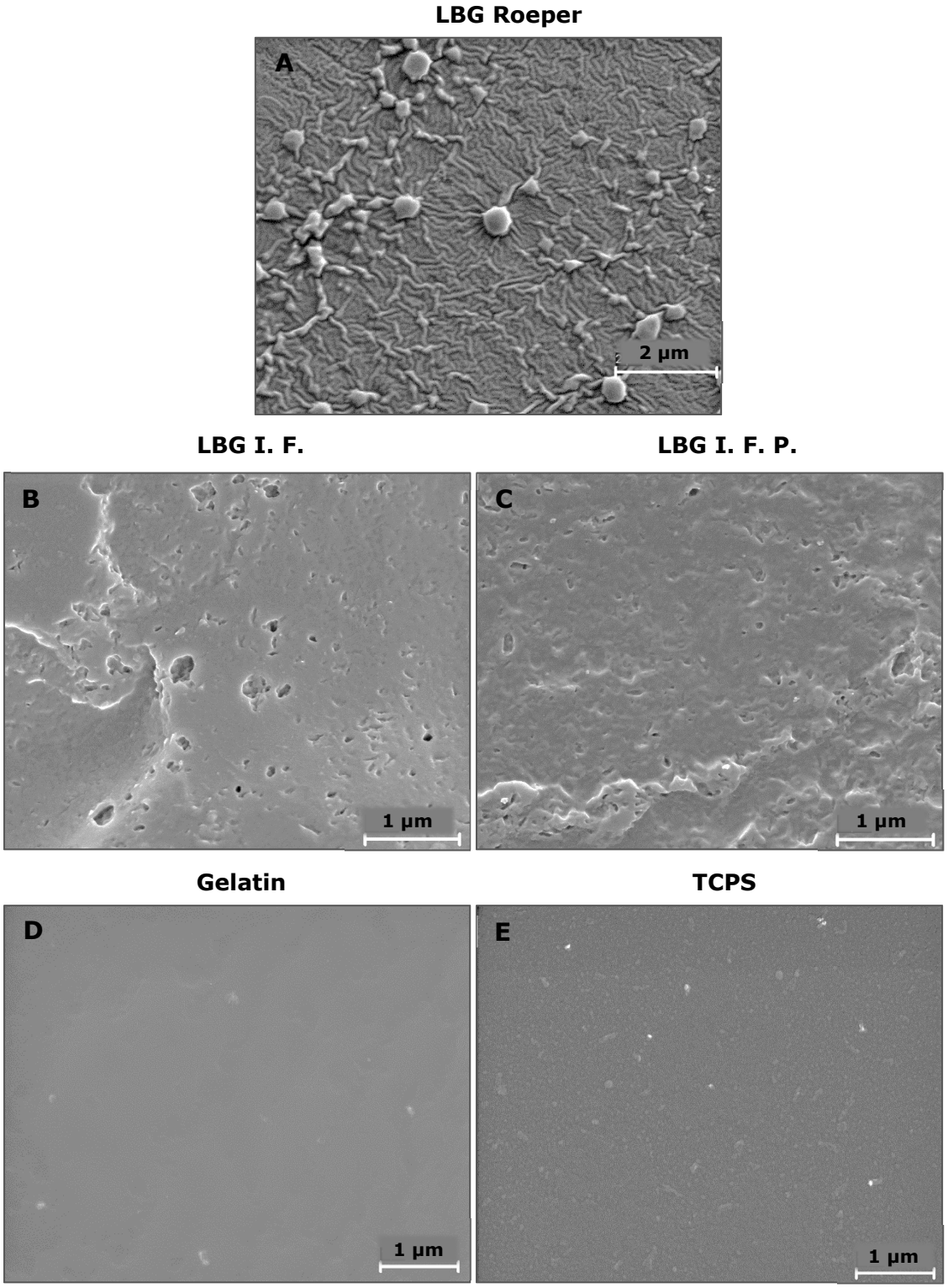

Figure 3.5: FE-SEM photographs of coatings prepared for ESC culture. A. 0.1% of LBG from Roeper. **B.** 0.1% of LBG from Industrial Farense. **C.** 0.1% of purified LBG from Industrial Farense. **D.** 0.1% of gelatin **E.** TCPS. LBG forms a rough film.

3.1.3.2. Cell Proliferation

In order to evaluate the ability of LBG to support cell adhesion and growth, undifferentiated mouse ESCs were cultured in 0.1% of non-purified LBG from Roeper and from Industrial Farense, 0.1% of purified LBG from Industrial Farense, 0.1% of gelatin and in negative control TCPS for 3 passages (9 days). According to the analysis of cell morphology at passage 1 and 3, colonies of ESCs cultured in LBG displayed a round, regular and dome-shape characteristic of pluripotent colonies (Fig. 3.6 A-C, A'-C'). In contrast, the majority of ESCs cultured in gelatin or TCPS showed cytoplasmic extensions suggesting spontaneous differentiation (Fig. 3.6 C: D, D', E, E'). In ESC culture, a heterogeneous cell population or changes in cell morphology can be a first indication that culture is entering in differentiation (Roccio et al., 2013). Furthermore, at the morphological level, there were no evident differences between ESCs cultured in LBG from Roeper or from Industrial Farense, purified or non-purified. This indicates that LBG seems to be able to support pluripotent ESC colonies independently of the supplier.

Analysis of the proliferation at day 3 showed that LBG from Roper was able to promote better ESCs proliferation than gelatin (Fig. 3.7 A). At day 6 and 9, however no significant differences were found in cell growth between ESCs cultured in LBG coatings or in gelatin. In addition, at days 3, 6 and 9, ESCs cultured in commercial LBG from Roeper proliferated significantly more than ESCs cultured in commercial LBG from Industrial Farense (Fig. 3.7 A). Furthermore, no significant differences were observed in proliferation of ESCs cultured in purified versus non-purified LBG from Industrial Farense (Fig. 3.7 A). This suggests that the purification step does not favor the proliferation of ESCs in LBG.

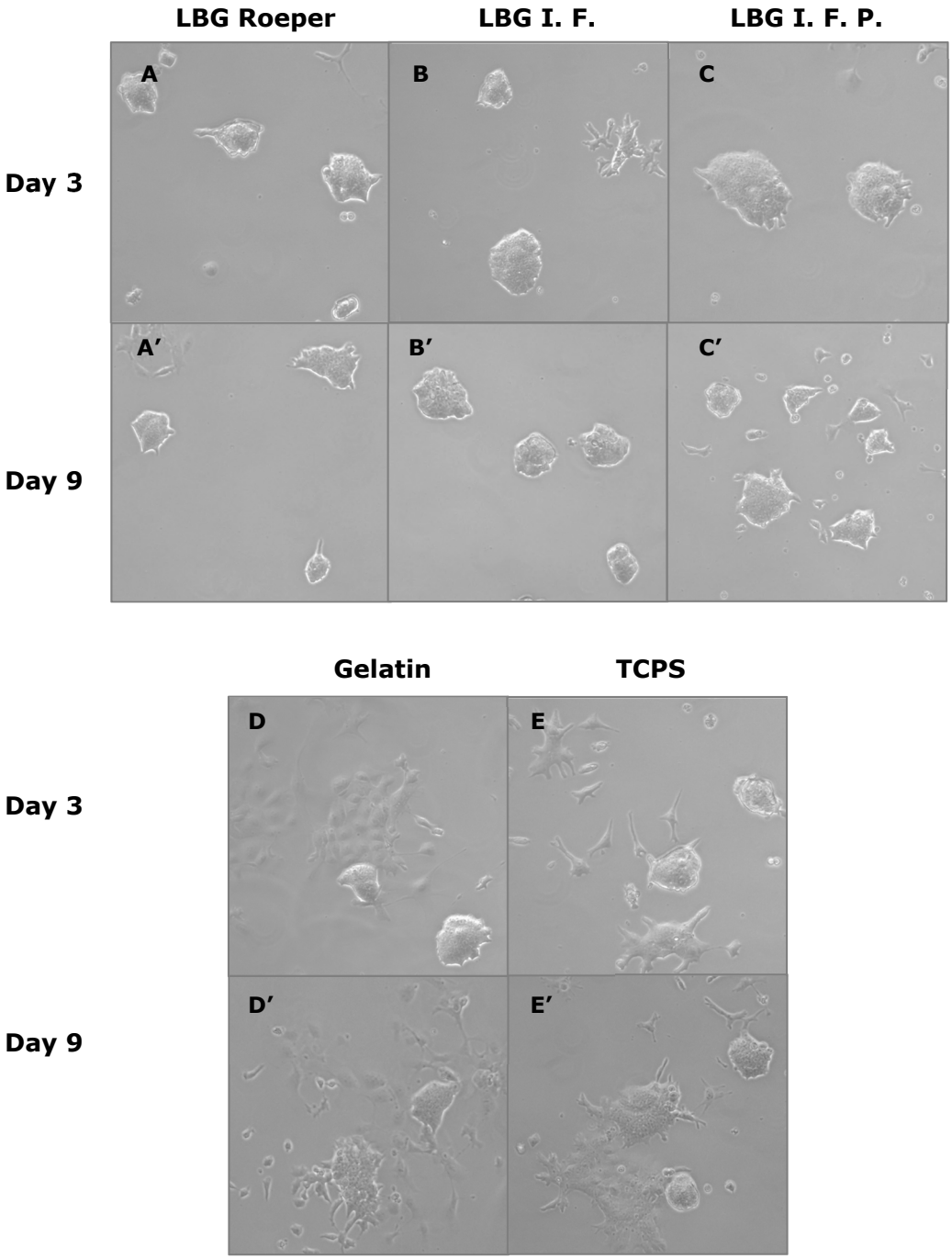

Figure 3.6: Cell Morphology. Representative contrast phase images of E14GFP8 mouse ESCs at day 3 (A-E) and day 9 (A'-E') of culture in **A.** 0.1% of LBG from Roeper. **B.** 0.1% of LBG from Industrial Farense. **C.** 0.1% of purified LBG from Industrial Farense. **D.** 0.1% of gelatin **E.** TCPS. Magnification is 100x.

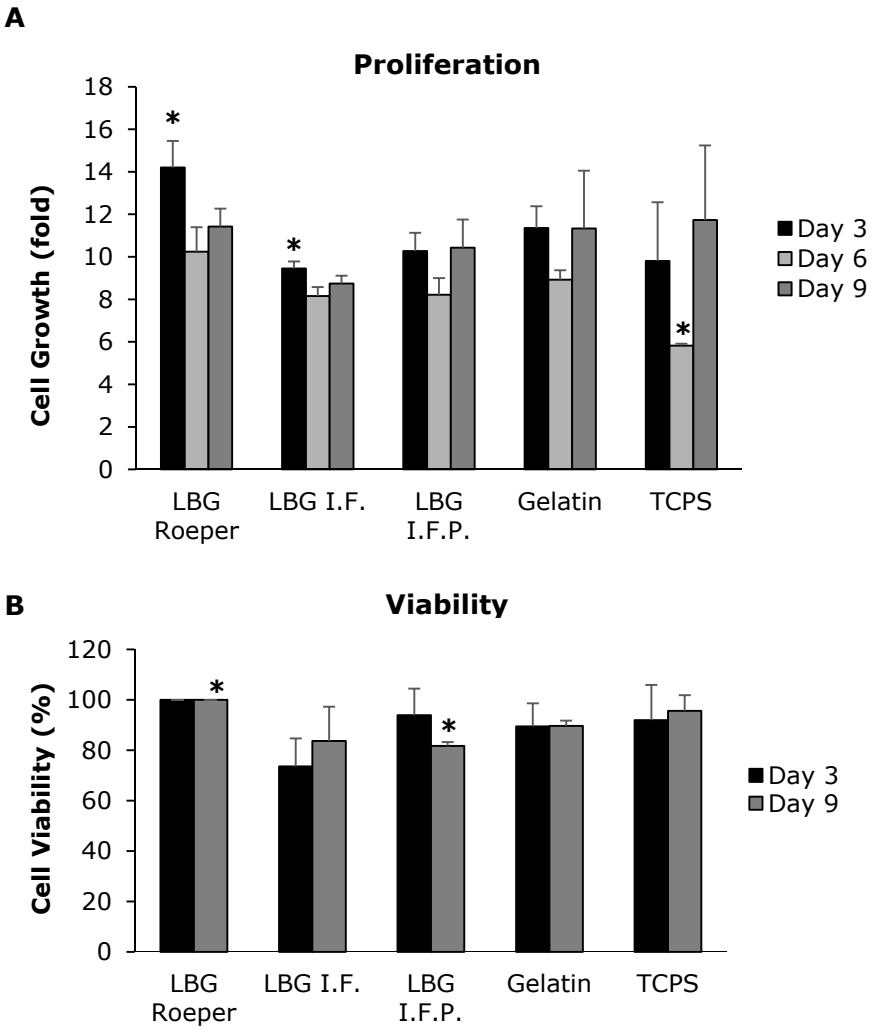

Figure 3.7: **Cell proliferation and viability assays**. **A.** ESCs were cultured for 9 days. Cell growth (fold) is presented as the ratio between cells and initial cell number. **B.** Cell viability results obtained at day 3 and 9 of culture were determined by trypan blue incubation. Cell viability is represented as percentage of viable cells to the total number of cells. (*) significantly different compared to gelatin ($p<0.05$). Data presented as mean + SD from one independent experiment performed in technical triplicates (n=3).

In addition to cell proliferation, cell viability was also tested by incubating the ESCs with trypan blue, at day 3 and day 9 of culture, and counting the number of death versus viable cells. According to our data, no significant differences in the viability of ESCs cultured in LBG coatings or in gelatin at day 3, but at day 9 ESCs cultured in LBG from Roeper seemed to present better viability than ESCs cultured in gelatin (Fig. 3.7 B). At day 3, the percentage of viable ESCs cultured in LBG from Roeper was significantly higher than in ESCs cultured in non-purified LBG from Industrial Farense (Fig. 3.7 B). At day 9, however the viability was similar among all the ESCs cultured in the different types of LBG coatings. Furthermore, at day 3 and 9,

there were no significant differences in cell viability between ESCs cultured in purified and non-purified LBG from Industrial Farense, indicating that the purification procedure did not influence the ability of LBG to support ESC viability (Fig. 3.7 B). Even though LBG from Roper seemed to support the viability of ESCs more efficiently than LBG from Industrial Farense, all LBG coatings proved to be suitable to replace gelatin, as ESC proliferation and viability in LBG coatings was equivalent or higher than ESCs grown in gelatin.

3.1.3.3. Analysis of stem cell markers

As previously mentioned, long-term support of pluripotent ESCs in culture is an important issue to validate the use of a material for ESC culture. With this in mind, we examined whether LBG coating could support mouse E14GFP8 ESCs self-renewal for 3 and 10 passages. Pluripotency was assessed through the analysis of alkaline phosphatase activity and *Nanog*, *Oct4* and *Sox2* expression at passage 3. According to our data, there were no significant differences in the expression of *Nanog, Oct4*, *Sox2* between ESCs cultured in LBG from Roeper or in gelatin (Fig. 3.8 A). Furthermore, there were no significant differences in the expression of pluripotency markers between LBG from Roeper and non-purified LBG from Industrial Farense (Fig. 3.8 A). Nonetheless, the expression of *Nanog* and *Sox2* in ESCs cultured in purified LBG from Industrial Farense was significantly higher than in ESCs cultured in gelatin (Fig. 3.8 A). These two genes were also significantly higher in purified LBG when compared to non-purified LBG from Industrial Farense and, hence, the purification of LBG seems to be beneficial for the maintenance of ESC pluripotency (Fig. 3.8 A). In addition, all the colonies grown in LBG from Roeper, purified and non-purified LBG from Industrial Farense were ALP-positive and exibited regular shape typical of plutipotent colonies (Fig. 3.8 B: a-c). On the other hand, analysis of the activity of ALP at passage 3 revealed that ESCs cultured in gelatin or in TCPS presented more differentiated ALP-negative ESCs than ESCs cultured in LBG (Fig. 3.8 B).

At passage 10, even though the expression of pluripotency markers of ESCs cultured in LBG coatings was, in some cases, equivalent to ESCs cultured in gelatin (Fig. 3.9 A), the ESCs cultured in LBG from Roeper, purified and non-purified LBG from Industrial Farense coatings grew as compact colonies characteristic of

undifferentiated pluripotent stem cells (Fig. 3.9 B: a-c). In contrast, ESCs cultured in gelatin or in TCPS presented some cells with a fibroblast-like morphology, with colonies that clearly lost their well-defined borders, suggesting ESC differentiation (Fig. 3.9 B: d, e). Taken together, these data suggest that ESCs cultured in LBG coatings from different producers, purified or non-purified are, at least, equally efficient as gelatin in maintaining pluripotency upon long-term culture.

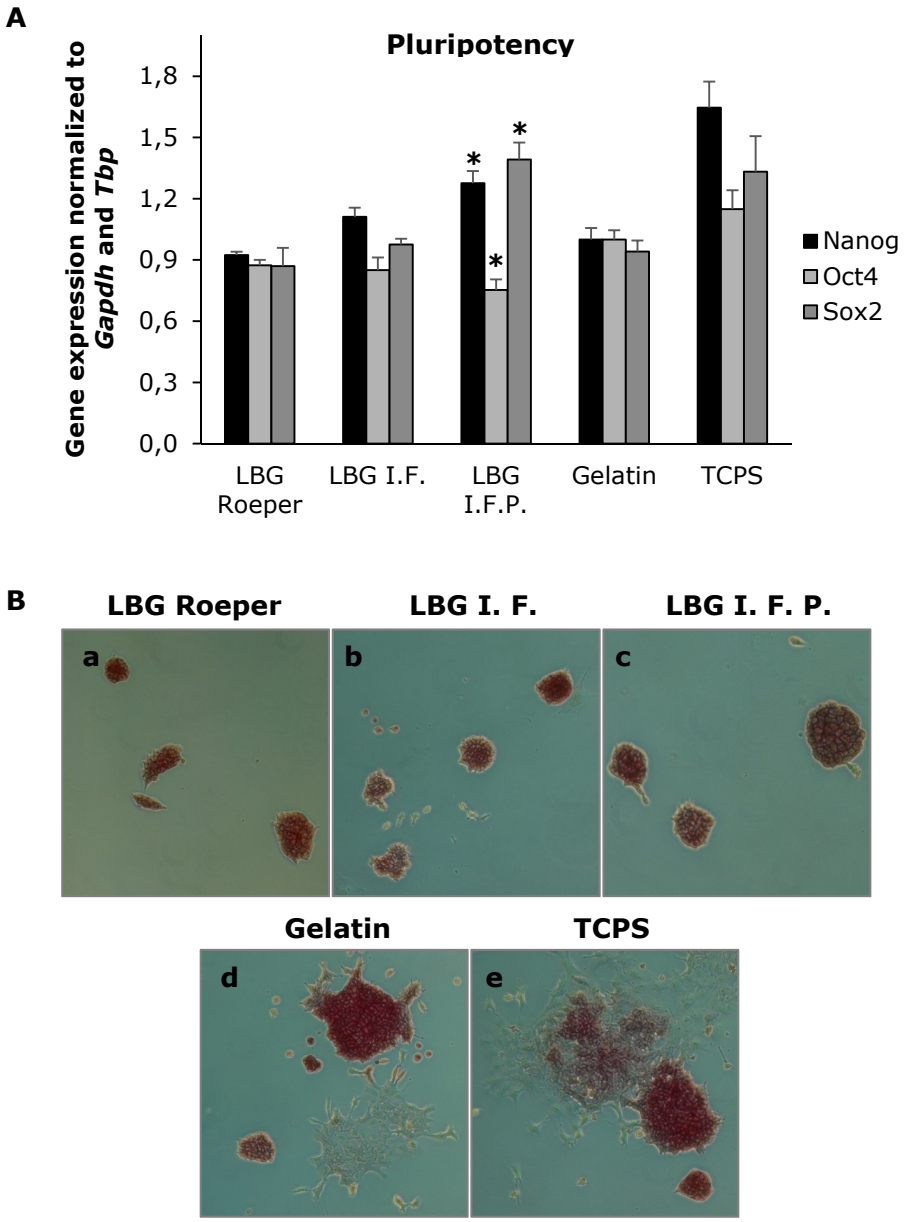

Figure 3.8: Pluripotency evaluation at passage 3. A. Analysis of expression of *Nanog*, *Oct4* and *Sox2* pluripotency markers and representative pictures of mouse ESCs stained for ALP activity (**B.**) of ESCs cultured in a. 0.1% of LBG from Roeper, b. 0.1% of LBG from Industrial Farense, c. 0.1% of purified LBG from Industrial Farense, d. 0.1% of gelatin, e. TCPS for 9 days. Magnification is 100x. (*) significantly different in LBG compared to gelatin ($p<0.05$). Data presented as mean + SD from one independent experiment performed in technical triplicates (n=3).

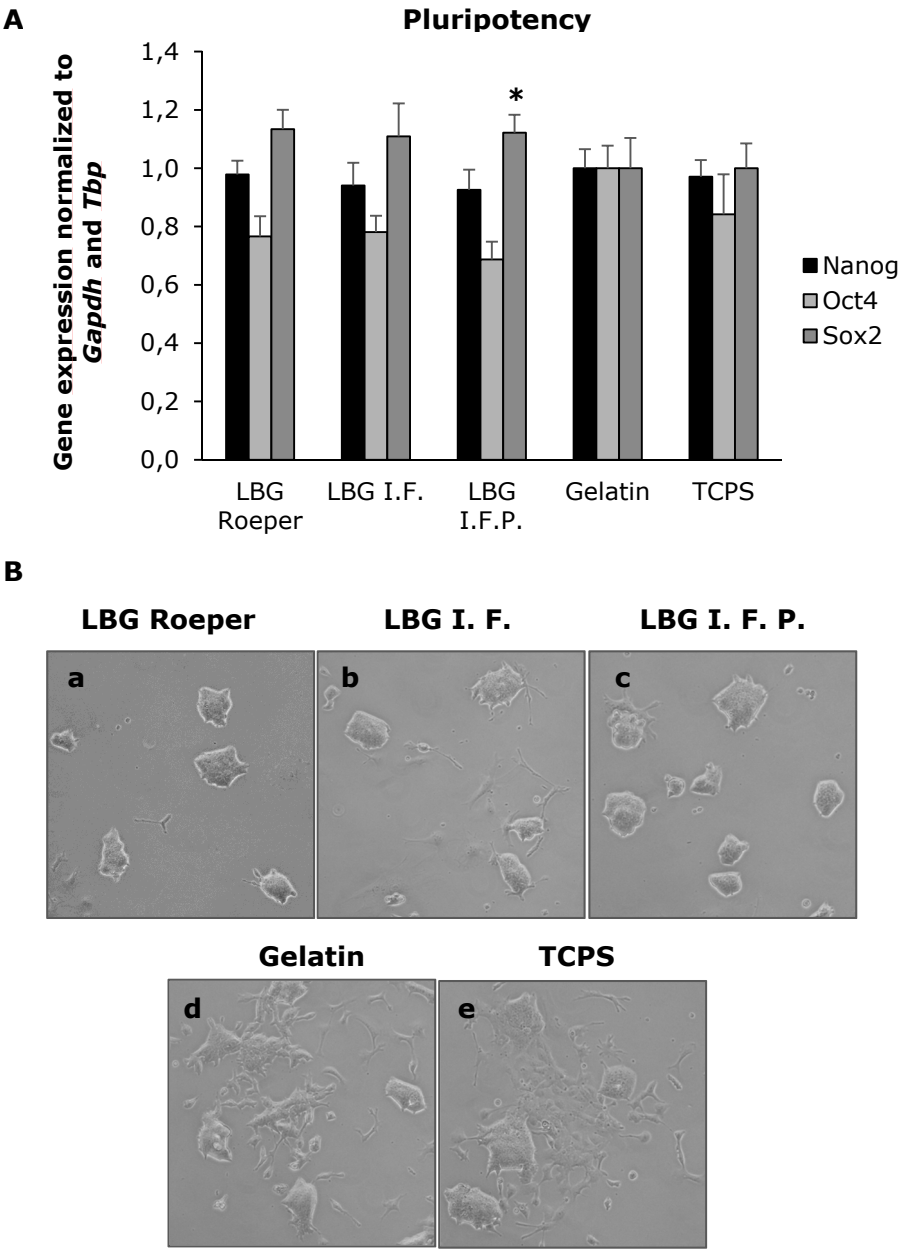

Figure 3.9: Pluripotency and morphology of ESCs at passage 10. A. Expression of *Nanog*, *Oct4* and *Sox2* pluripotency markers and representative phase contrast pictures of ESCs (**B.**) cultured in a. 0.1% of LBG from Roeper, b. 0.1% of LBG from Industrial Farense, c. 0.1% of purified LBG from Industrial Farense, d. 0.1% of gelatin, e. TCPS for 30 days. Magnification is 100x. (*) significantly different compared to gelatin ($p<0.05$). Data presented as mean + SD from one experiment performed in technical triplicates (n=3).

3.1.3.4. *In vitro* differentiation of ESCs

As mentioned earlier, pluripotent cells have the ability to differentiate into cell types of all organs and tissues of a living organism, so it is fundamental that ESCs cultured *in vitro* retain this feature. To study early stages of differentiation, ESCs are normally differentiated into three-dimensional structures called embryoid bodies (EBs). The potential of differentiation of ESCs cultured on the different LBG coating was tested by differentiating ESCs in EBs after enduring 10 passages in LBG, gelatin or TCPS. At day 5 of differentiation EBs were plated onto an adherent surface. ESCs were differentiated for 10 days. At the end of differentiation, it was possible to observe elongated cell projections emanating out from EBs. In addition, all EBs differentiated into heterogeneous populations of cells displaying morphological evidences of endothelial cells, fibroblasts and other cell types, and were organized resembling tissue-like structures (Fig. 3.10 A). This suggests that cells cultured for a long term in LBG retain their tri-lineage differentiation capacity (Fig. 3.10 A). Indeed, the differentiation was confirmed by quantitative analysis of markers of ectoderm (*βIII-Tubulin* and *Sox1*), endoderm (*Afp, Gata4*) and mesoderm (*Gata4*, *Hand1*, and *αSma*). According to qPCR data, ESCs cultured in LBG coatings obtained higher or comparable expression of markers of all three primary germ layers than ESCs cultured in TCPS or in gelatin. As expected, the lowest expression of differentiation markers was obtained for ESCs cultured for long-term in TCPS, indicating that the ESCs cultured in these conditions lost at least in part the capacity to differentiate in all three primary germ layers. The endoderm and mesoderm markers *Afp* and *Gata4* were similarly expressed in ESCs cultured in LBG from Roeper, in LBG from Industrial Farense, purified or non-purified, or in gelatin (Fig. 3.10 B). In addition, the expression of the mesoderm marker *Hand1* was similar between all the culture conditions, but the expression of another mesoderm marker, *αSma*, was significantly higher in ESCs cultured in LBG from Roeper and purified LBG from Industrial Farense LBG than in gelatin (Fig. 3.10 C). The expression of *Hand1* and *αSma* in ESCs cultured in LBG from Roeper and LBG from Industrial Farense were equivalent (Fig. 3.10 B). Regarding the differentiation into ectodermal lineages, expression of both *βIII-Tubulin* and *Nestin* in ESCs cultured in LBG from Roeper was identical to ESCs cultured in LBG from Industrial Farense, purified or non-purified, but significantly higher than ESCs cultured in gelatin (Fig. 3.10 D).

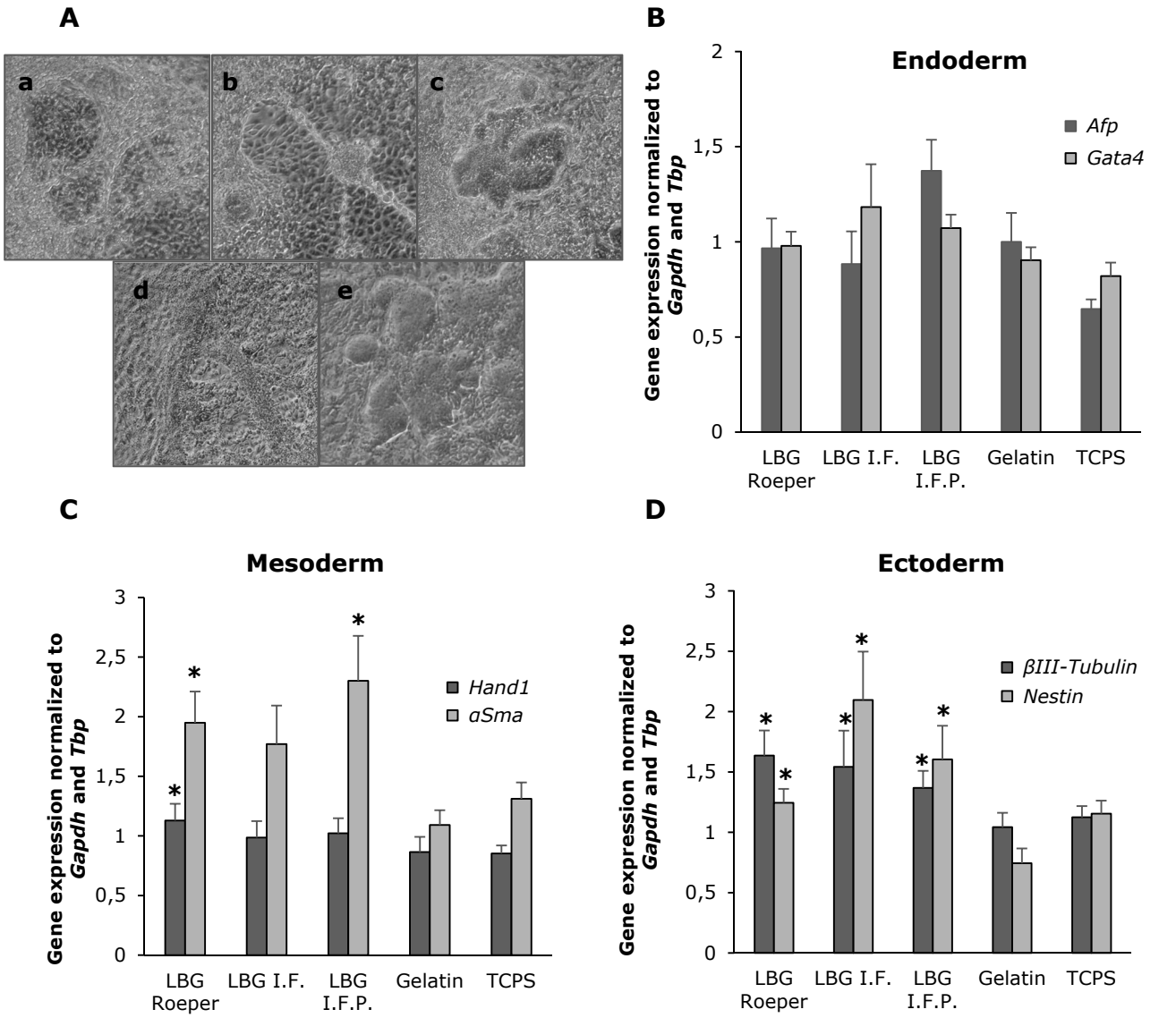

Figure 3.10. In vitro tri-lineage differentiation. A. Morphology of EBs at day 10 of differentiation. **B-D.** Levels of gene expression of germ layer markers of EBs collected after 10 days of differentiation. **B.** *Afp* and *Gata4* are expressed in endoderm and mesoderm. **C.** *Hand1* and *αSma* mark mesoderm. **D.** *βIII-Tubulin* and *Nestin* are ectodermal marker genes. The relative gene expression was normalized to *Gapdh* and *Tbp* and to Gelatin control. (*) significantly different expression levels compared to gelatin. Data are presented as mean + standard error of mean, representative of two independent experiments, performed in triplicate (n=6).

In global, these data shows that ESCs cultured in LBG coatings retain their tri-lineage differentiation capacity even after long-term culture. Furthermore, increased expression of some of the genes from the different germ layers in ESCs cultured in LBG indicates that they retain the pluripotency much more efficiently than ESCs cultured in gelatin or TCPS.

3.1.4. DISCUSSION

LBG or E410 is one of the three major galactomannans of commercial importance in food and non-food industries, but its application in research is almost unexplored. In the previous chapter, we demonstrated the ability of LBG to support mouse ESC culture. The possibility remained, however, that the properties of this plant-based material could be affected by the geographic location of the source of the plants, collecting season or manufacturer. Therefore, the aim of the present chapter was to validate the generic use of LBG in ESC culture.

According to our data, the topography of LBG coatings from Roeper and from Industrial Farense was somewhat different. Nevertheless both LBG coatings gave rise to a slightly rough surface, contrasting to the smooth gelatin coating. The topography of purified and non-purified LBG from Industrial Farense was similar, suggesting that the purification process does not affect the ability of LBG to give rise to slightly rough and porous films. ESCs are anchorage-dependent cells and normally function better if they are grown on a permeable or porous surface (Ryan, 2008). Indeed, it is known that ESCs encounter and respond to topography *in vivo* at length scales ranging from the nano- to microscale (Dolatshahi-Pirouz et al., 2011; Hatano et al., 2013; Leclerc et al., 2013). In addition, there is a general agreement that topography has an important role in cell adhesion, growth and pluripotency (Blin et al., 2010; McNamara et al., 2010; Qi et al., 2013). Therefore, the topography acquired by LBG coating probably brings an asset to the ESC *in vitro* cell culture systems making it resembling closer the *in vivo* situation. In general, according to our results, when ESCs were cultured in LBG coatings for a short-term culture, no significant differences were found in cell growth between ESCs cultured in LBG coatings or in gelatin, and also between ESCs cultured in purified or non-purified LBG. This suggests that all LBG coatings were suitable to grow ESCs, independently of the producer and additional purification step. Analysis of the tri-lineage differentiation capacity by quantifying the expression of pluripotency markers showed that ESCs cultured in purified or non-purified LBG coatings from different producers, were able to maintain their pluripotency upon long-term culture. LBG coatings allowed maintenance of pluripotency similarly, if not even more efficiently than ESCs cultured in gelatin. Accordingly and as presented in the previous chapter, in contrast to ESCs grown in gelatin, all ESCs grown in LBG for 3 or 10 passages were ALP positive and organized in compact

colonies with well-defined borders. This is consistent with the ESCs cultured for a long-term in LBG coatings retaining much more efficiently their tri-lineage differentiation capacity than ESCs cultured in gelatin.

Natural gums, such as LBG, are natural polymers commercially available, which mainly consist of carbohydrates sometimes with small amounts of proteins and minerals. Therefore, depending on the application, a purification step could be required to purify natural products from water solutions. For example, in the formulation of nanoparticles for drug-delivery, the impurities may alter the physico-chemical and release characteristics of nanoparticle system, so the purification of the natural polymers prior to nanoparticles formulation is highly recommended (Dalwadi et al., 2005). In addition, sometimes the purification is required to ensure the purity of the protein or polysaccharide before implantation to avoid activating an immune response (Willerth et al., 2008). In the present comparative study, one of the main aims was to test whether the ash and protein content affects the pluripotency of ESCs in culture or if the ESCs respond mainly to galactomannan, the major component of LBG. The purified LBG has higher galactomannans content and no longer contains the cell wall structures or materials (Kawamura, 2008). According to ESC proliferation, viability, pluripotency and differentiation data, ESC response to purified or non-purified LBG was almost equivalent, with the exception of the pluripotency maintenance at passage 3, in which the purification of LBG revealed to be beneficial. Taking together, the fact that the results using the purified gum match those obtained with the commercial one, indicates that protein and ash impurities did not have any deleterious effect on the ESCs. Furthermore, since ESC response among the different LBG coatings was equivalent the observed results may be attributed to the galactomannan.

Chapter IV

Novel triblock copolymer nanofiber system as an alternative support for embryonic stem cells growth and pluripotency

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Author´s contribution:

The majority of the experimental work was performed by A.R. Perestrelo with the exception of the design of chemical synthesis of nanofibers and their characterization by TEM performed by Fouzi Mouffouk and the chemical synthesis of poly (ethyleneglycol-β-trimethylsilyl methacrylate-β-methacrylic acid) and further production of nanofibers performed by Ana Maria da Rosa Costa.

4.1. ABSTRACT

Conventionally, embryonic stem cells (ESCs) are cultured on gelatin or over a mitotically inactivated monolayer of mouse embryonic fibroblasts (MEFsi). Considering the lack of versatile, non-animal-derived and inexpensive materials for that purpose, we aimed to find a biomaterial able to support ESC growth in a pluripotent state that avoids the need for laborious and time-consuming MEFsi culture in parallel with mouse ESC (mESC) culture. Undifferentiated mESCs were cultured in a new nanofiber material designed for ESC culture, which is based on the self-assembly of a triblock copolymer, poly(ethyleneglycol-β-trimethylsilyl methacrylate-β-methacrylic acid), conjugated with the peptide glycine–arginine–glycine–aspartate–serine, to evaluate its potential application in ESC research.

The morphology, proliferation, viability, pluripotency and differentiation potential of ESCs were assessed. Compared to conventional stem cell culture methodologies, the nanofibers promoted a higher increase in ESCs number, enhanced pluripotency and were able to support differentiation after long-term culture.

This newly developed synthetic system allows the elimination of animal-derived matrices and provides an economic method of ESC culture, made of a complex network of nanofibers in a scale similar to native extracellular matrices, where the functional properties of the cells can be observed and manipulated.

Keywords: embryonic stem cells, embryonic stem cell culture, gelatin substitute, growth support, pluripotency, polymeric nanofibers

4.2. INTRODUCTION

Stem cell research has grown from unexplored to becoming an important field in biomedical sciences today. ESCs have theoretically two unique abilities, an unlimited self-renewal capacity and multilineage differentiation potential (pluripotency; Evans and Kaufman 1981). Indeed, a deeper understanding of the basic biology of stem cells holds the key to unlock new hopes to various so-far incurable human diseases (Silva et al., 2012).

Even though the first mouse ESC lines were derived three decades ago (Evans and Kaufman 1981) and standard protocols for ESC derivation and maintenance are widely used today, the technical difficulties of these protocols still pose a challenge for many investigators attempting to produce pluripotent ESCs at high quality levels. The gold standard supportive material for *in vitro* mESC culture is MEFsi, which allows mESCs to continue proliferating without differentiating (E. Michalska, 2007). Besides the immunity problems and batch-to-batch variation, the use of MEFsi as a support for ESC culture is a laborious and time-consuming process. MEFs are primary cells and stop dividing after a couple of passages and hence they need to be isolated freshly from time to time, and one week is required to reach the correct confluency to perform ESC culture (E. Michalska, 2007). Concerning that, many efforts are being made to avoid the use of MEFs without losing ESC pluripotency through replacing the supportive MEFsi by synthetic systems. The major challenge is to find a cheap, defined, user-friendly and feeder-free condition that properly mimics the ESC niche, in order to obtain high quality undifferentiated ESCs. Many efforts have been made to closely mimic the real microenvironment of cells. So far, a wide range of approaches have been explored, including the use of the new 2i defined medium (Ying et al., 2008), coating with proteins (Heng et al., 2012) and peptides (Klim et al., 2010), carbon nanotubes (Lizundia et al., 2012), hydrogels (Geckil et al., 2010), a diversity of natural and synthetic scaffolds from different sources (Li et al., 2010) and nanofibers (nfs; Nur-E-Kamal et al., 2006). Nanofibers have exciting geometry properties that have drawn much attention recently, particularly in the field of ESCs and tissue engineering. Special properties of nanofibers, such as the ability of mimicking the arrangement of fibers and fibrils of the ECM makes them suitable for a wide range of biomedical applications that are improved when combined with ESCs (Kanani and Bahrami, 2010).

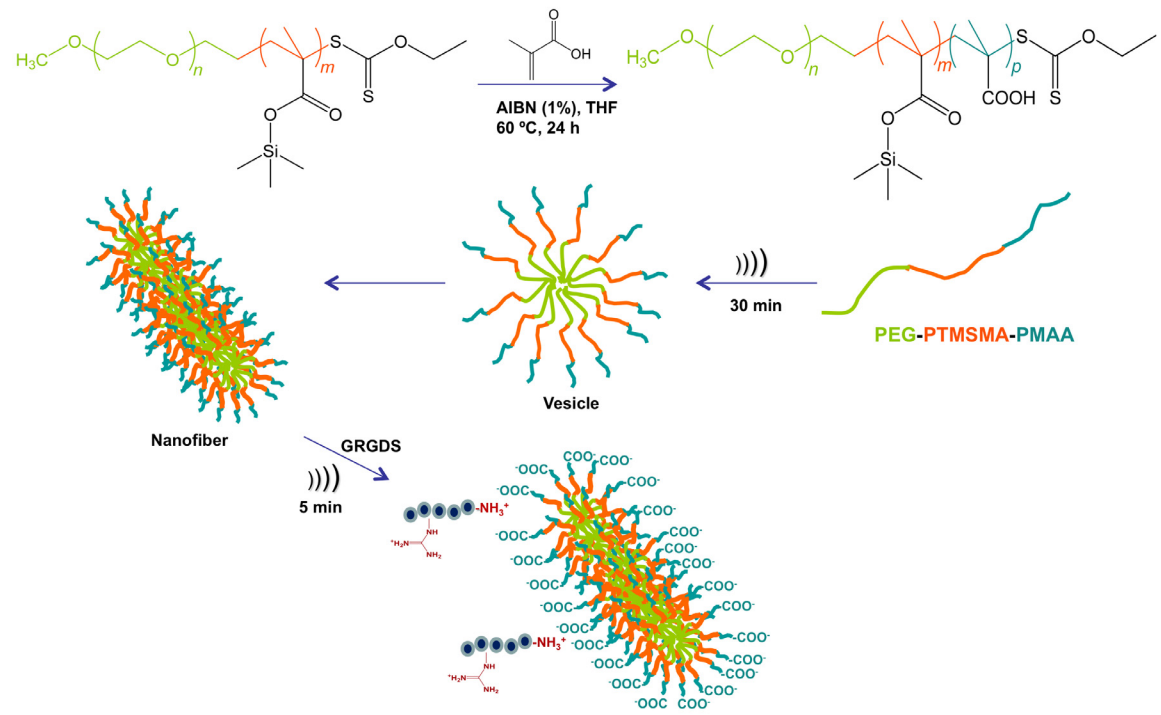
Here, we report the synthesis of poly(ethyleneglycol-*β*-trimethylsilyl methacrylate-*β*-methacrylic acid)-Glycine-Arginine-Glycine-Aspartate-Serine (PEG-PTMSMA-PMAA-GRGDS) - based nanofibers and the capability of this new artificial nanofiber network to support mouse ESC culture.

4.3. RESULTS

4.3.1. Synthesis and characterization of nanofibers

The mechanism of PEG-PTMSMA-PMAA-GRGDS nanofiber formation involves three phases: first, the self-assembly of the amphiphilic copolymer into vesicles; second, conversion of polymeric vesicles into nanofibers via a stacking process; and third, incorporation of GRGDS into the nanofibers surface by electrostatic interaction (Fig. 4.1 A).

A



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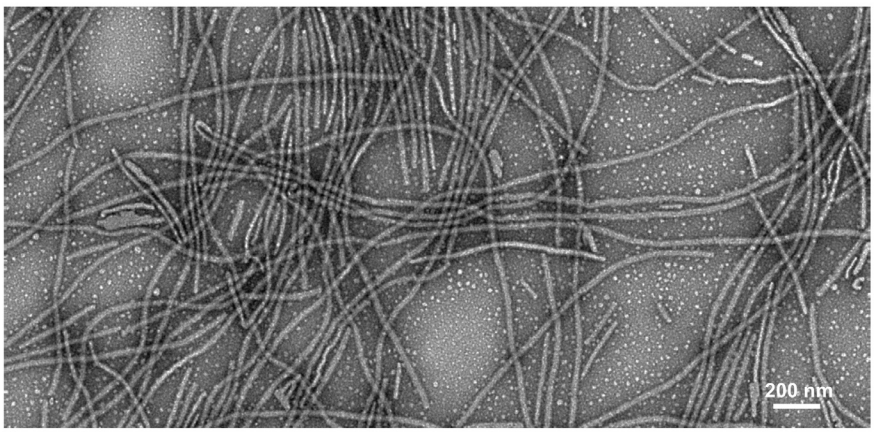


Figure 4.1: Synthesis and characterization of nanofibers. A. Synthetic approach to PEG–PTMSMA–PMAA and nanofibers formation: self-assembly of the amphiphilic co-polymer into vesicles, conversion into nanofibers via stacking, and GRGDS incorporation.

B. Cryo-TEM images of polymeric nanofibers by negatively staining with 1% uranyl acetate. These nanofibers are predisposed to form a mesh.

PEG-PTMSMA-PMAA was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization (Chiefari et al., 1998) from the diblock copolymer α-(O-ethylxanthate)-ω-methyl PEG-PTMSMA as macro-chain transfer agent (macro-CTA) and methacrylic acid (MAA). A monomer:macro-CTA ratio of 106:1 and AIBN (1 mol% of the monomer), as radical initiator were used (Fig. 4.1 A). The polymer was characterized by ^{1}H NMR spectroscopy triple-detection GPC, FTIR, and MALDI-TOF. A DP_n value of 94 was inferred from the NMR peak area at 3.64-3.72 ppm (CH_2 in PEG) and those at 1.09-1.19 ppm (CH_3 in TMSMA and MAA) and 0.06-0.10 ppm [$(CH_3)_3Si$ in TMSMA]. GPC analysis reveals a monomodal molecular weight distribution. The presence of TMSMA units was patent on both the FTIR and NMR spectra as evidenced by the bands at 1255, 1181 and 846 cm^{-1} {δ and ν[$Si(CH_3)_3$], respectively} and the singlets at 0.06 and 0.10 ppm [$(CH_3)_3Si$]. On the MALDI-TOF spectrum, interpeak distances corresponding to the masses of the three repeating units (44 for EG, 86 for MAA, and 158 for TMSMA) were observed.

Self-assembly of the polymer into nanofibers was achieved by means of its solubilization in an aqueous medium through sonication. The synthetic nanofibers were observed by Cryo-TEM and found to be 1-2 μm long and approximately 30 nm diameter (Fig. 4.1 B).

4.3.2. Cell adhesion

Analysis of the expression of *β1-Integrin* revealed that culture of undifferentiated ESCs in 10 and 100 μg/ml of nanofibers for 13 days results in a significantly higher expression of *β1-Integrin* (1.32- and 1.55-fold, respectively) when compared to gelatin and MEFsi (1.10 and 1, respectively) (Fig. 4.2 A). Furthermore, ESCs cultured in 100 μg/ml of nanofibers presented significantly higher expression levels of Collagen type I alpha 1 (*Col1a1*; 0.12-fold) than standard polystyrene culture plates and gelatin (0.08- and 0.09-fold, respectively) (Fig. 4.2 B). Furthermore, previous experiments already revealed the importance of GRGDS bioconjugation with PEG-PTMSMA-PMAA nanofibers in ESCs adhesion and growth (Fig. 4.3). These

data suggest that nanofibers bioconjugated with GRGDS are able to improve cell adhesion by inducing the expression of adhesion molecules that will allow the reorganization of the microenvironment and incorporation in a fiber-based matrix.

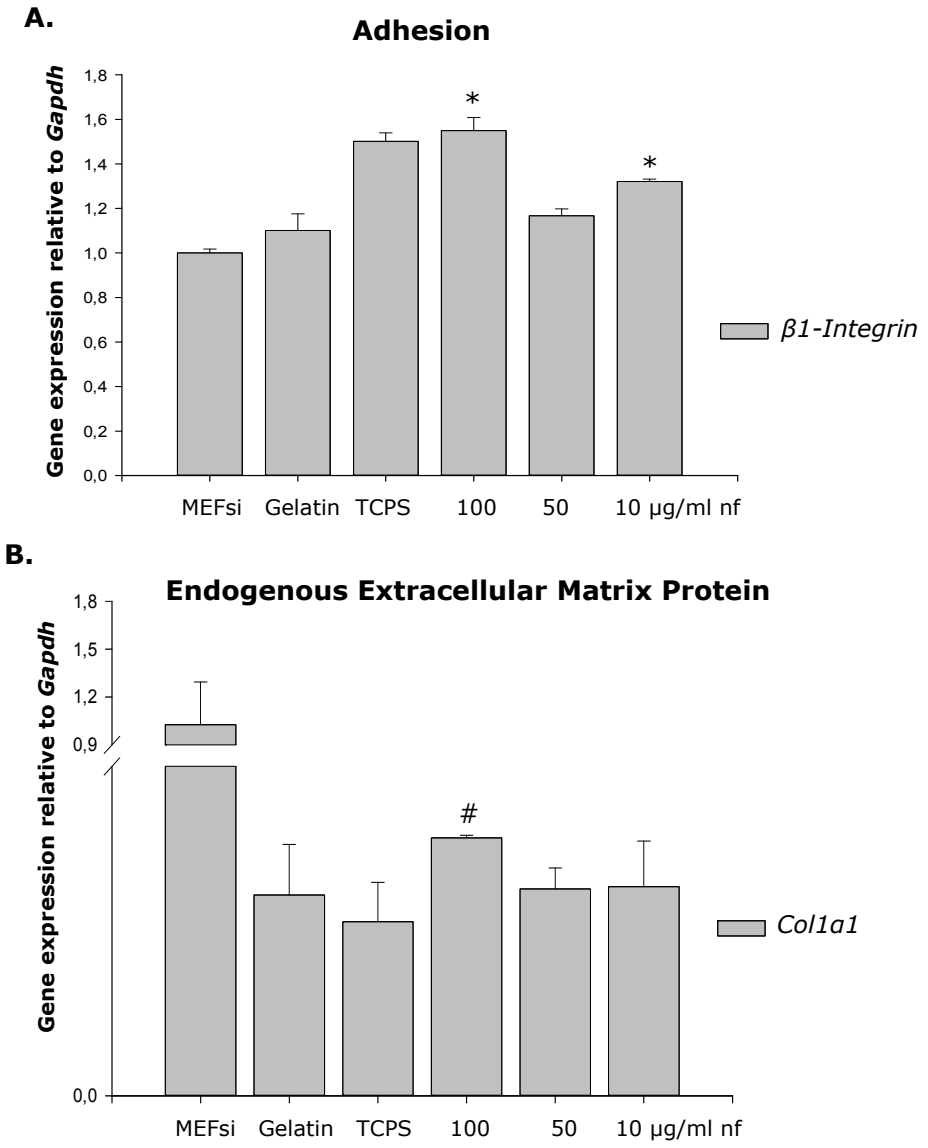


Figure 4.2: Cell Adhesion. Expression of endogenous ECM proteins and adhesion proteins in ESCs grown for four subcultures on PEG-PTMSMA-PMAA-GRGDS nanofibers and under standard conditions. **A**. Adhesion marker, levels of β1-integrin gene expression; (*) significantly different expression levels between nanofibers, MEFsi and gelatin ($p<0.05$). **B.** Endogenous ECM protein marker, levels of Col1α1 gene expression; (#) significantly different expression levels between nanofibers and gelatin and tissue culture polystyrene plates (TCPS) ($p<0.05$). The relative expression was normalized to *Gapdh* and to MEFsi control.

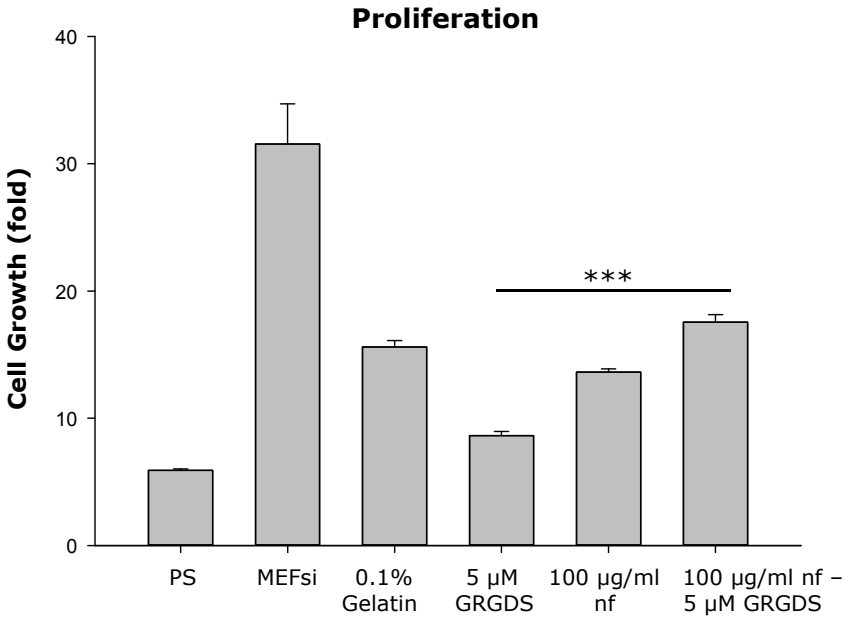


Figure 4.3: Cell proliferation test performed at day 3 of ESC culture in untreated polystyrene (PS), MEFsi, 0.1% of gelatin, 5 µM of GRGDS, PEG-PTMSMA-PMAA nanofibers and PEG-PTMSMA-PMAA nanofibers bioconjugated with 5 µM GRGDS. (***) statistically differences among the group ($p<0.001$). Data presented as mean + SD for n=6.

4.3.3. Cell Proliferation

As a first approach to evaluating the capacity of the nanofibers to sustain pluripotent ESCs cultures, we tested whether the nanofibers were able to support mESCs adhesion and growth. Undifferentiated E14GFP8 ESCs were cultured for 3 and 5 days on different concentrations of PEG-PTMSMA-PMAA-GRGDS nanofibers and compared to cultures in MEFsi, gelatin and TCPS used as control conditions. The morphology, viability and the proliferation of the cells were assessed.

Morphological analysis of mESCs cultured for 5 days on nanofibers (Fig. 4.4 A', B', C') showed that colonies were tightly-packed, dome-shaped and presented clear and defined borders, similar to the colonies obtained when using MEFsi as substrate (Fig. 4.4 D'). Conversely, ESCs cultured in gelatin and in TCPS plates lost the capacity of colony formation, presented an irregular shape and acquired undefined borders, a typical characteristic of loss of pluripotency (Fig. 4.4 E, E', F, F').

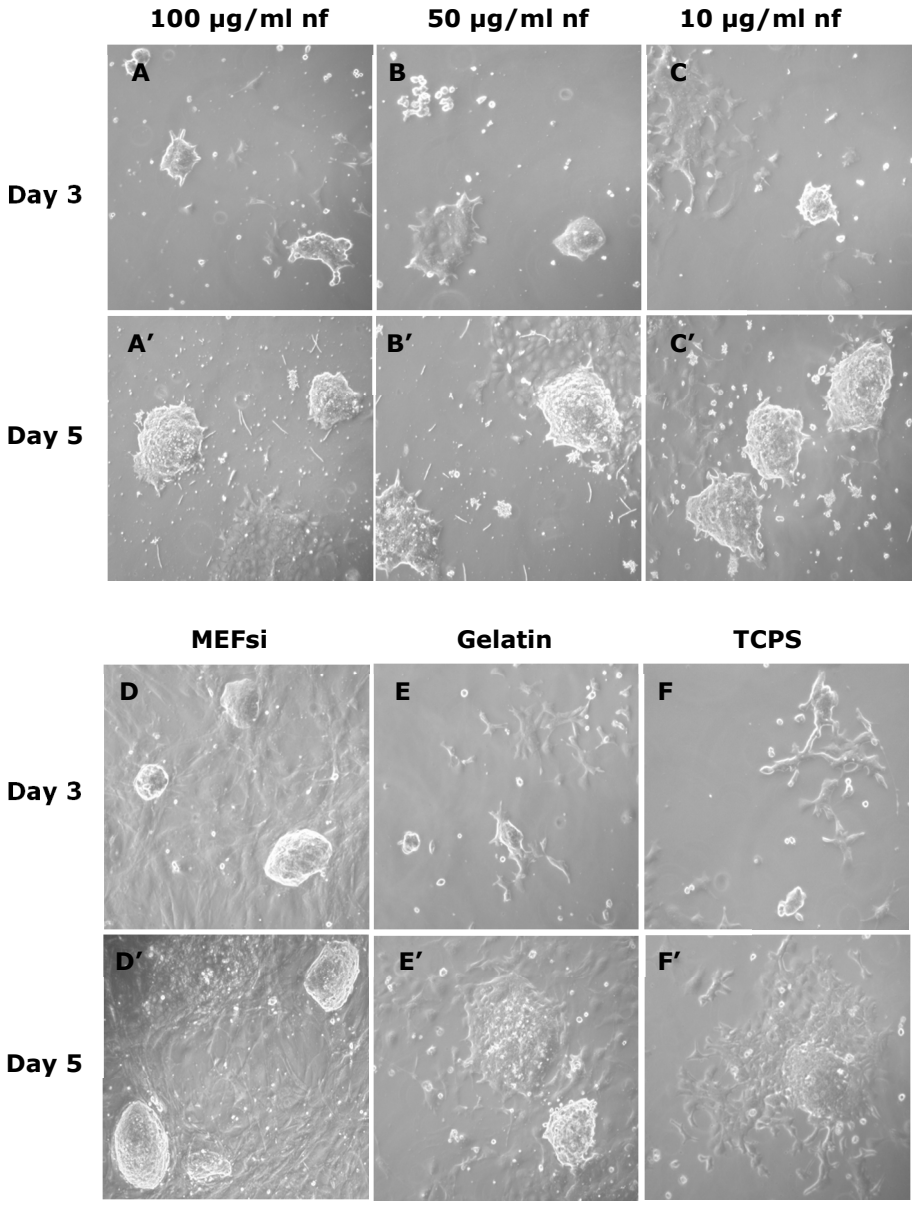


Figure 4.4: Cell morphology. Representative images of E14GFP8 embryonic stem cells during 3 (A-F) and 5 (A'-F') days in culture. The wells were covered by: **A.** 100 µg/ml nf, **B.** 50 µg/ml nf, **C.** 10 µg/ml nf. **D.** MEFsi **E.** 0.1% Gelatin, **F.** TCPS. Magnification is 100x.

After 3 days in culture, the cell growth in 100 and 50 µg/ml of nanofibers was 16.04- and 15.27-fold respectively, which was significantly higher than the 10.62-fold observed in TCPS plates. In addition, no significant difference was observed in proliferation between 100, 50, 10 µg/ml of nanofibers, gelatin and MEFsi at day 3 of culture (Fig. 4.5 A). On the other hand, ESC proliferation in nanofibers for 5 days was higher than not only the cultures in TCPS but also cultures in gelatin. Concurrently, 50 µg/ml of nanofibers promoted a 27-fold increase in cell number, which is significantly higher than that observed in gelatin (17.63-fold) or TCPS

(14.53-fold) (Fig. 4.5 A). Therefore, the results obtained for the proliferation of ESCs in PEG-PTMSMA-PMAA-GRGDS nanofibers in a short-term culture were similar or, in some cases, even better than those in gelatin. This suggests that the nanofibers may be used to replace gelatin in supporting ESC growth.

The viability ratio of ESCs was approximately 1 for all the tested conditions indicating that viability maintenance was independent from the culture conditions and the time of culture (Fig. 4.5 B).

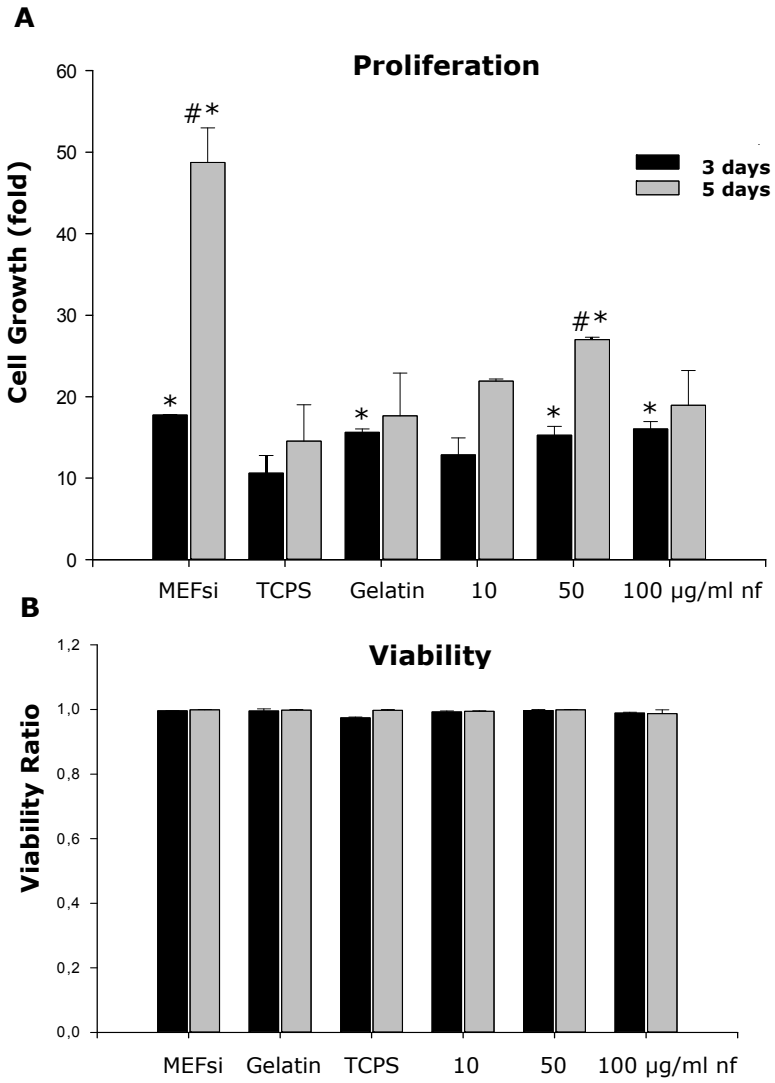


Figure 4.5: Cell proliferation. A. Cell proliferation and viability tests (**B.**) performed during 3, 5 of ESC culture. Cell growth (fold) is presented as the ratio between viable cells and initial cell number. Viability ratio is the ratio between the number of viable cells and total number of cells. (*) Significantly different cell number in nanofibers compared with negative control value (TCPS) ($p<0.05$). (#) Significantly different cell number in nanofibers compared with gelatin. Data presented as mean + SD for n=6**.**

4.3.4. Maintenance of Pluripotency

The pluripotent status of stem cells was detected by ALP test performed in ESCs cultured in 100, 50 and 10 μg/ml PEG-PTMSMA-PMAA-GRGDS nanofibers and under control conditions. Undifferentiated pluripotent stem cells presented elevated levels of ALP, therefore ALP staining was used to distinguish between pluripotent and differentiated cells (Tsuji et al., 2008). ESCs were passaged every 3 days at the same density and the ALP test was performed at passage 1 (day 3) and at passage 5 (day 15). ALP staining revealed that, contrarely to ESCs cultured in gelatin (Fig. 4.6 E, E') or TCPS (Fig. 4.6 F, F'), ALP activity is present in all cells cultured in nanofibers independently of the time of culture (3 or 15 days) (Fig. 4.6 A, A', B, B', C, C'). Indeed, ESCs cultured on nanofibers (Fig. 4.6, A, A', B, B', C, C') resemble more the colonies that form when ESCs are cultured on a monolayer of fibroblasts where all cells are ALP-positive and the colonies are dome-shaped (Fig. 4.6 D, D'). These results suggest that nanofibers may be used as an alternative to conventional gelatin to support the growth of undifferentiated ESC cultures.

Quantitative RT-PCR for stem cell markers was performed using total RNA isolated from ESCs at passage 4. After 13 days, ESCs cultured in 10 and 100 μg/ml nanofibers presented *Oct4* expression levels of 1.37- and 1.24- fold, which were significantly higher than ESCs cultured in MEFsi (one-fold), gelatin (1.09-fold) or TCPS (0.99-fold) (Fig. 4.7). As *Oct4* is one of the major regulators of ESC "stemness" (Shi and Jin, 2010), these results suggest that the nanofibers not only support but also stimulate further ESC "stemness". Furthermore, ESCs cultured in 10 μg/ml nanofibers also presented *Nanog* expression level similar to that of ESCs cultured in MEFsi (Fig. 4.7). Moreover, the expression levels of *Sox2* in ESCs cultured in 10 μg/ml nanofibers (0.72-fold) were higher than ESCs cultured in gelatin (0.65-fold) or TCPS (0.59-fold) (Fig. 4.7).

Taken together, these data suggest that mESCs cultures in the nanofibers remain self-renewable.

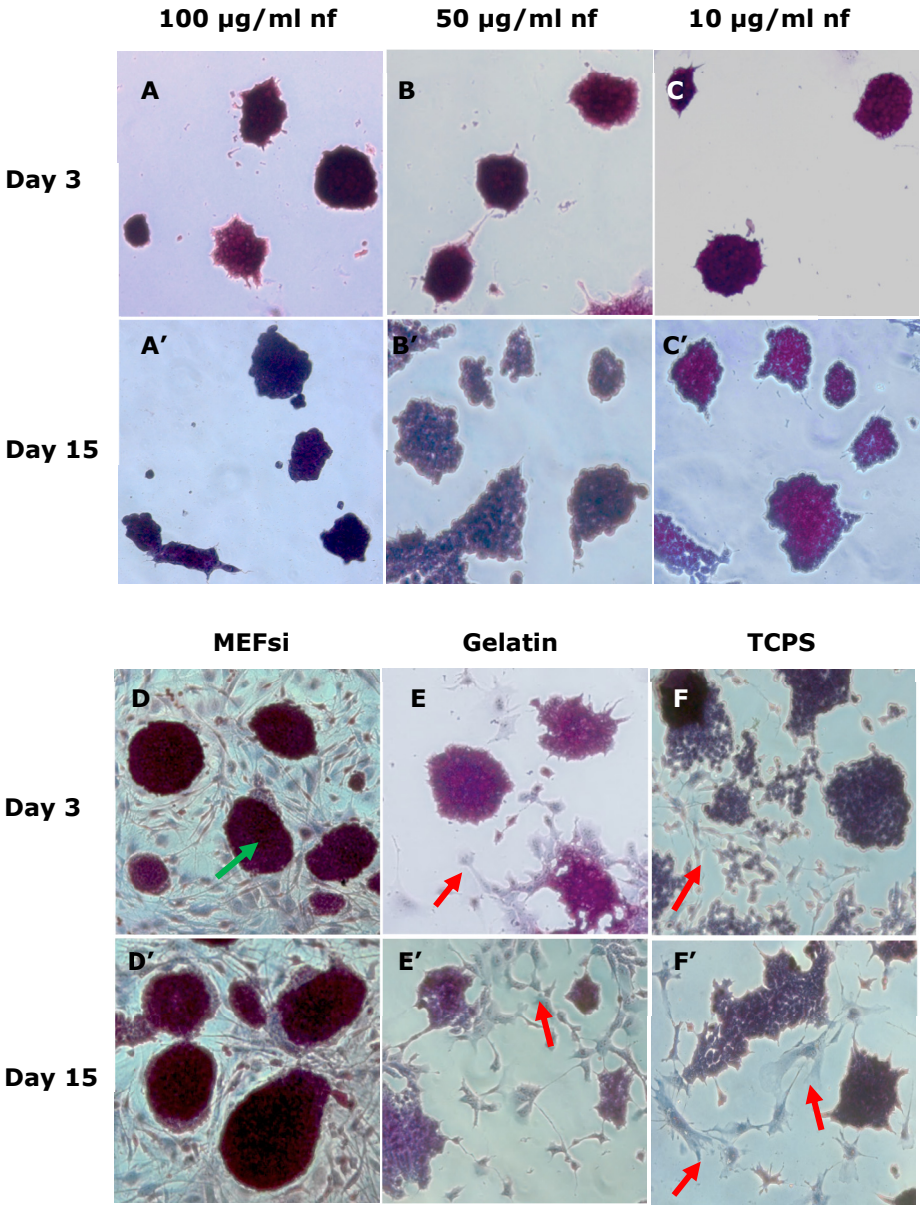


Figure 4.6: Maintenance of Pluripotency. Representative images of ESCs stained for ALP after 3 (A-F) and 15 days (A'-F') of culture. The wells were covered by: **A.** 100 µg/ml nf, **B.** 50 µg/ml nf, **C.** 10 µg/ml nf **D.** MEFsi, **E.** 0.1% Gelatin, **F.** TCPS. Magnification is 100x. The red arrows indicate the differentiated cells and the green arrow indicates the pluripotent colonies with a regular shape.

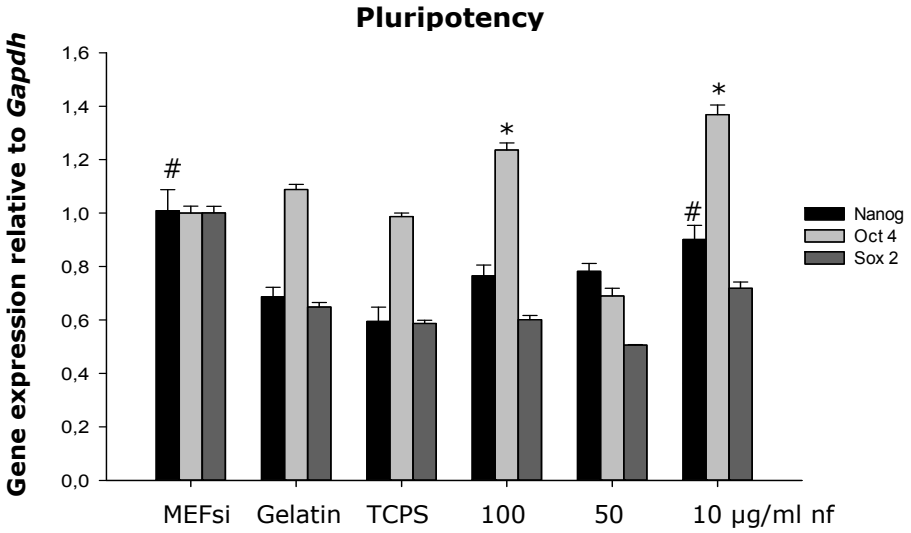


Figure 4.7: Maintenance of Pluripotency. Expression of the pluripotency markers *Nanog*, *Oct4* and *Sox2* in cells cultured on PEG-PTMSMA-PMAA-GRGDS nanofibers, and under standard conditions, for four subcultures. (*) significantly different expression levels in nanofibers compared with gelatin, MEFsi and TCPS ($p<0.05$); (#) significantly different expression levels compared with TCPS ($p<0.05$).

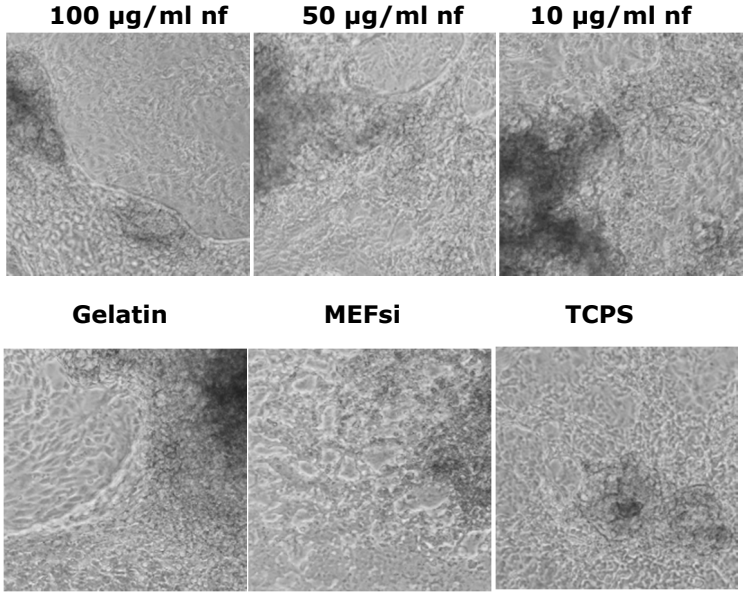
4.3.5. Tri-lineage differentiation

Determination of long-term effects of nanofibers is important in screenning nanomaterials for their potential benefits or pathogenic properties. Therefore, we tested whether the nanofibers could support ESC differentiation potential over multiple passages.

ESCs were subcultured in nanofibers and under standard conditions for 10 passages, then induced to form EBs by the hanging drops method and differentiated for 10 days. At day 10 of differentiation, some fibroblast-like cells had migrated out to form a halo around the EBs and the cultures become dense, confluent and multilayered (Fig. 4.8 A). Different structures with diverse types of organization and arrangement had formed (e.g. beating foci) suggesting that cells cultured for a long term in nanofibers retain their tri-lineage differentiation capacity (Fig. 4.8 A). Indeed, these results were confirmed by qRT for markers of ectoderm (*βIII-Tubulin* and *Sox1*), mesoderm (*Hand1* and *αSma*) and endoderm (*Afp* and *Gata4*) (Fig. 4.8 B). According to our qRT data, ESCs cultured in 50 and

10 µg/ml nanofibers for 30 days were able to give rise to the three germ layers more efficiently than the MEFsi and gelatin (Fig. 4.8 B).

A



B

EBs	ΔΔ Cq	100	50	10 µg/ml nf	MEFsi	Gelatin	TCPS
Ectoderm	*βIII-Tubulin*	1.13	1.47	1.54	1.39	1.33	1.88
	Sox 1	0.22	0.40	0.65	1.02	0.58	0.61
Mesoderm	*Hand 1*	0.53	0.65	1.05	0.98	1.53	1.79
	αSma	0.97	1.32	0.50	1.01	0.59	0.73
Endoderm	*Afp*	0.46	0.89	0.42	1.01	0.63	1,76
	Gata 4	0.06	0.44	0.47	1.14	0.47	0.34

ΔΔ Cq:
≥ MEFsi
≥ Gelatin

Figure 4.8: Tri-lineage Differentiation. Differentiation potential of ESCs cultured on nanofibers for 30 days. **A.** Morphology of EBs at day 10 of differentiation. **B.** Levels of gene expression of germ layer markers in EBs collected at day 10 of differentiation, *βIII-Tubulin* and *Sox1* for ectoderm, *Hand1* and *αSma* for mesoderm and *Afp* and *Gata4* for endoderm and mesoderm. The relative gene expression was normalized to *Gapdh* and *Tbp* and to MEFsi control. Data are from two biological and two technical replicates, performed in triplicate (n=8).

4.4. DISCUSSION

The PEG-PTMSMA-PMAA-GRGDS nanofibers were designed taking advantage of the ideal and diverse properties of each block. PEG is a flexible, water-soluble well-established biodegradable polymer with many applications from industrial manufacturing to medicine (Duncan, 2003). On the other hand, a combinatorial library of biomaterials formed from different acrylate and methacrylate monomers has proved to be useful for identifying environments suitable for ESCs differentiation (Anderson et al., 2004). Furthermore, methacrylates have been used in biomedical applications for many years and have been shown to promote adhesion and proliferation of endothelial cells when tethered to adhesive proteins (Fussell and Cooper, 2004). Polymers containing methacrylic acid and trimethylsilyl methacrylate have also been described to improve adhesion between the resist film and the wafer surface, as well as dry etch resistance in photolithographic applications (Mormann and Ferbitz, 2002). RGD is a well-known cell attachment peptide (Bellis, 2011). Taking this into account, our nanofibers have many features that may promote stem cell adhesion and control of cell proliferation. In addition to these potential benefits, they represent a good substitute for the existing systems, due to the advantages of being cheap and easy to produce, presenting chemical and physical properties tailored via molecular synthesis.

We successfully synthetized PEG-PTMSMA-PMAA by RAFT polymerization and the polymer self-assembly into nanofibers also succeeded. In the self-assembly process, it is expected that the neutral hydrophilic segment of the copolymer (PEG) becomes majorly oriented to the core of the particles, and the negatively charged one (PMAA) to their surfaces, in order to minimize charge repulsion. Attachment of GRGDS to the fibers surface would occur by interaction between the positively charged terminal amino group of the former and the negatively charged carboxylate groups of the latter, leaving the guanidium group of the arginine residue available for cell adhesion. After polymerization, nanofiber assembly and bioconjugation with GRGDS, our purpose was to test the authentic effect of the nanofibers culture system, not only in supporting high-quality mouse embryonic stem cells growth but also in keeping cells undifferentiated and pluripotent.

Cell-biomaterial interaction mechanisms are poorly understood and many efforts have been made to clarify this matter. Nonetheless, several studies have demonstrated that cells do not interact with surfaces directly, but via deposition of adhesive proteins secreted by themselves and adsorbed on the adhesive surface, forming and remodeling their own ECM (Vladkova, 2010). Therefore, an increased expression of ECM and adhesion proteins, such as integrins, is evidence that surrounding microenvironment fosters cell adhesion and interaction with the support system. Our hypothesis was that the functionalization of the nanofibers with GRGDS may promote the expression of integrins, like e.g. *β1-Integrin*. Indeed, culture of ESCs in 100 µg/ml of nanofibers lead to an increase in *β1-Integrin* expression and *Col1α1* suggesting that integrins mediate the interaction between cells and the surrounding engineered bioadhesive motifs (GRGDS) of the nanofiber matrix. This indicates that the nanofibers play an important role in the regulation of synthesis, secretion and deposit of endogenous ECM proteins. This newly developed synthetic system allows the establishment of a more realistic and controlled microenvironment for mESC culture, by providing a complex network of nanofibers, gaps and pores through which oxygen and nutrients can be delivered and metabolites can be filtered away in a scale similar to the native ECM, where the functional and biological properties of mESCs could be understand more precisely and manipulated.

The first approach to evaluate the capacity of the nanofibers to sustain ESCs culture was to test whether the nanofibers were able to support ESCs' growth for 3 and 5 days. According to cell adhesion and proliferation results, the cell proliferation in 100 µg/ml nanofibers for a short-term culture was revealed to be higher than or similar to that in gelatin or TCPS. Besides the similarity of proliferation results between gelatin and in nanofibers, ESCs cultured in gelatin were found to be less pluripotent than in nanofibers, which could be explained by a selective process in which nanofibers potentiate the growth of pluripotent cells. Indeed, according to our morphological analyses of the ESCs cultures at different time points of culture, the ability of nanofibers to support growth of undifferentiated ESCs seemed to be better than gelatin as, in the latter, ESCs were rearranged in irregular colonies resembling a spontaneous differentiation process. Nevertheless, despite the translational effects that cell morphology has on different cell functions, a study pointed out that the association of the undifferentiated state of the ESCs with their pluripotency might not necessarily be related to a specific

cellular morphology (Tsuji et al., 2008). The regulation of pluripotency in ESCs is provided by a complex network of transcription factors, cell-ECM interactions, cell-cell contacts and niche-support cells. Due to the complexity of pluripotency maintenance mechanism, we performed an extensive assay using nanofibers and standard culture conditions to more accurately verify the effect of the nanofibers on ESCs pluripotency. The maintenance of pluripotency was verified through ALP activity test, analysis of the quantitative expression of pluripotency marker genes (*Nanog*, *Oct4* and *Sox2*) and, lastly, the differentiation potential of ESCs after long-term culture in nanofibers. Results of ALP staining showed that, independently of the time of culture, ALP activity was higher in ESCs cultured in the nanofibers than those in gelatin, in which there seemed to be many more differentiated cells. In addition, ESCs long-term cultured in 10 μg/ml nanofibers exhibited *Nanog* and *Oct4* expression levels similar to or higher than those in ESCs cultured in MEFsi, emphasizing that nanofibers not only support but also promote ESCs to retain their "stemness".

The differentiation potential of ESCs was tested after 30 days of culture in nanofibers via the hanging drop method. EBs formation stimulates disordered and heterogeneous patterns of differentiated ESCs into three germ lineages. Consequently, in some cases there is a preponderance of specific germ layer-derived cells, whereas only a small fraction of cells differentiate into the other lineages (P. T. W. Kim et al., 2010). Therefore, to clarify the differentiation status of EBs, the global gene expression profile of ESCs population differentiated in nanofibers was quantitatively analyzed by qPCR (Koike et al., 2007; Sakai et al., 2011).

The *in vitro* differentiation assay showed that ESCs cultured in 50 and 10 μg/ml nanofibers for 10 passages were able to preserve their tri-lineage differentiation capacity, which validates the authenticity of ESCs cultured in nanofibers. Interestingly, the higher levels of pluripotency markers and differentiation were observed in ESC cultures in 10 and 100 μg/ml nanofibers as well as the higher levels of *β1-Integrin* and *Col1α1* expression, suggesting that nanotopography may play a role in regulation of cell attachment, spreading, proliferation and, most importantly, in regulation of self-renewal of undifferentiated ESCs. This phenomenon of influence of nanometric-scale surface topography and roughness of biomaterials in cell fate was also observed in other studies (Park et al., 2007). In summary, this newly developed synthetic system brings an alternative substrate

for mESC culture with the advantage of being inexpensive and easy to produce, and researchers can really control their chemical and physical properties via molecular synthesis. The major advantage of this system is that mESCs cultured on PEG-PTMSMA-PMAA-GRGDS nanofibers maintain their pluripotent state and present increased expression of *Col1α1* and *β1-Integrin*, which may help establishing a microenvironment that supports ESC attachment, proliferation, and pluripotency.

Chapter V

Characterization of *Ccbe1* mutant MEFs

Ccbe1 and its effect in proliferation and migration

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Manuscript in preparation

Author´s contribution:

The majority of the experimental work was performed by A.R. Perestrelo with the exception of the maintenance of the *C57Bl6 Ccbe1* mutant mice lineage in animal house by PNG Pereira and the production and purification of mouse CCBE1 protein by J. M. Inácio.

5.1. ABSTRACT

Ccbe1 or collagen and calcium-binding EGF domains 1, is involved in lymphangiogenesis, cardiogenesis and carcinogenesis. However, its function and molecular action in biological events is still unknown. Indeed, little is known about the Ccbe1 gene and protein, except that the amino acid sequence of the CCBE1 protein predicts the presence of a signal peptide and collagen and calcium binding EGF domains. As these domains are also found in some of the ECM proteins, CCBE1 might be an ECM protein and the loss of its expression may result in changes in cellular characteristics, such as adhesion and motility.

Experimental data from literature and from experimental work conducted in our lab revealed that, even though *Ccbe1*$^{-/-}$ mice die prenatally, *Ccbe1* is highly expressed in MEFs. Therefore the aim of this work was to evaluate the effect of *Ccbe1* in cell adhesion, proliferation and migration using MEFs as a model.

Here, we show that the proliferation of *Ccbe1*$^{-/-}$ mouse embryonic fibroblasts ceases prematurely. The *Ccbe1*$^{-/-}$ MEFs presented senescent cellular morphology, a reduction in growth fraction, and increased expression of the cell proliferation inhibitor *Bax*. Wound healing migration assay using different protein ECM as a coating revealed that Ccbe1$^{-/-}$ MEFs presented high motility and that the migration is substrate-dependent. The culture of *Ccbe1*$^{-/-}$ MEFs culture on top of a CCBE1 coating rescued the defective proliferation and viability of *Ccbe1*$^{-/-}$ fibroblasts, and the highly migratory behavior was also reduced.

Even though the underlying mechanism of action of *Ccbe1* is still unknown and complex, the data presented here suggests that *Ccbe1* coordinates cell adhesion, migration and proliferation, thus playing a key role in the ECM remodeling.

Keywords: *Ccbe1*, ECM, migration, MEFs, survival

5.2. INTRODUCTION

Collagen and calcium-binding EGF domain 1 (*Ccbe1*) was identified during a gene expression profiling of chick heart/hemangioblast precursors using Affymetrix® GeneChip Arrays, in our laboratory (Bento et al., 2011). Data from our laboratory indicates that *Ccbe1* might be responsible for alterations in cell migration during chick heart development. As described in detail, in the General Introduction – *Ccbe1 State of the art*, the absence or presence of *Ccbe1* is responsible for several phenotypes in vertebrates. In ovary cancer cells and breast cancer, absence of *CCBE1* has been shown to favor the migration of the cells (Barton et al., 2010; Yamamoto and Yamamoto, 2007). In mice, administration of CCBE1 facilitates VEGF-C-induced lymphangiogenesis in corneal micropocket assays in mice (Bos et al., 2011). Furthermore, Ccbe1 is required for proper lymphangioblast migration and lymphatic vessels development in zebrafish (Hogan et al., 2009). Moreover, fetal liver erythroid precursors of *Ccbe1*$^{-/-}$ mice exhibit reduced proliferation and increased apoptosis (Zou et al., 2013).

Many efforts have been made, but the function or molecular action of CCBE1 in biological events is poorly understood. Knockout (KO) mice are important animal models that represents a powerful tool to study the role of genes which have been sequenced but whose functions have not been determined. The existence of a *Ccbe1*$^{-/-}$ mice makes possible to take full advantage of the potential of mice and their MEFs to study the effect of *Ccbe1* in embryonic development events, such as cell proliferation, migration and cell-ECM interactions. Furthermore, analysis of the Affymetrix array data generated in Greber *et al* revealed that Ccbe1 is highly expressed in MEFs (Greber et al., 2007). Moreover, as *Ccbe1*$^{-/-}$ mice died prenatally at about E16.5 (Zou et al., 2013) makes possible to isolate the embryonic fibroblasts between E13.5 and E14.5, for further studies.

Ccbe1 has been shown to interact with extracellular matrix (ECM) proteins vitronectin, collagen type I and V, indicating that Ccbe1 may be as well an ECM component (Bos et al., 2011). Besides conferring support, interaction of ECM components with membrane receptors integrins is known to regulate several cellular processes, such as proliferation, survival, cellular shape and migration. For that reason, the main aim of this work was to characterize *Ccbe1*$^{-/-}$ MEFs and attempt to understand how Ccbe1 affects viability, proliferation and migration processes.

5.3. RESULTS

5.3.1. Genomic organization of mouse Ccbe1 gene

Collagen and calcium-binding EGF-like domain 1 gene (GeneBank reference sequence number NM_178793.4) has 11 exons, 1631 bps and is localized in the *Mus musculus* chromosome 18, oriented in the reverse strand at the genomic region between 66,060,967 and 66,291,838 K (Fig. 5.1 A).

CCBE1 protein is a 408 aminoacid (aa) secreted protein with a predicted molecular weight of 44.4 kDa. According to bioinformatic analyses performed using InterPro and Vega Genome Browser, CCBE1 contains a predicted signal peptide with a cleavage site (1-37 aa), a EGF domain (93-134 aa), a calcium-binding EGF-like domain (135-176 aa) presenting an aspartate/asparagine hydroxylation site (151-162 aa) and two collagen-like domains (248-335 aa; Fig. 5.1 B). Also, CCBE1 has a conserved RGD domain (177-179 aa; Fig. 5.1). Mouse CCBE1 protein is conserved across vertebrates. Mouse CCBE1 amino acid sequence is 90% identical to the human and chimpanzee and 66% identical to zebrafish (Fig. 5.2).

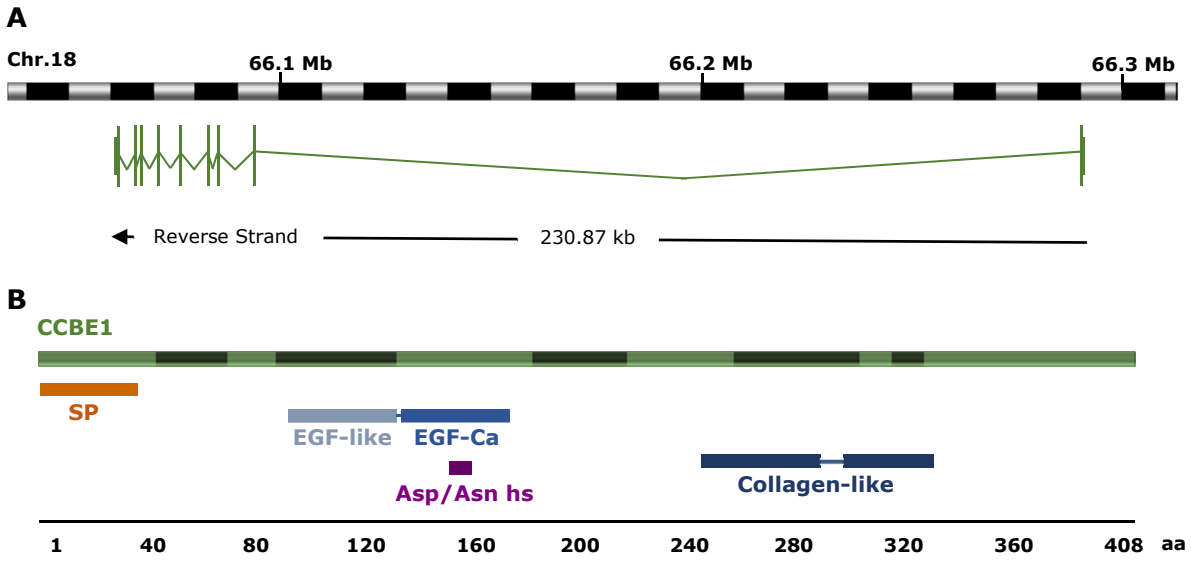

Figure 5.1: Schematic representation of the predicted Ccbe1 gene and protein. A. The Ccbe1 gene (NM_178793.4) is located in the chromosome 18 of mouse genome and orientated in the reverse strand. The vertical lines represent the 11 exons and the lines the introns. The resulting transcript has 1631 bps length**.** Transcript analysis conducted on-line http://www.ensembl.org, in November 2013. **B.** Predicted functional domains of CCBE1. Exons are represented in alternated light and dark green segments. CCBE1 is composed of a SP – signal peptide, an EGF – epidermal growth factor-like domain, an EGF-Ca – calcium-binding EGF-like domain, an Asp/Asn hs – aspartic acid and asparagine hydroxylation site and *two* collagen-like domains. Protein sequence analyses was conducted on-line using InterPro (http://www.ebi.ac.uk/interpro/) and Vega Genome Browser (http://vega.sanger.ac.uk/index.html), in November 2013.

A

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Mus musculus     1 MVPPPlPSRGGAAKRQLGKSLGPL--LLLLALGHTWTYREEPEDRDREVCSENKITTTKYPCLKSSGELTTCFRKKCCKG 78
Homo sapiens     1 MVPPP-PSRGGAARGQLGRSLGPL--LLLLALGHTWTYREEPEDGDREICSESKIATTKYPCLKSSGELTTCYRKKCCKG 77
Pan troglodytes  1 MVPPP-PSRGGAARGQLGRSLGPL--LLLLALGHTWTYREEPEDGDREICSESKIATTKYPCLKSSGELTTCYRKKCCKG 77
Gallus gallus    1 M-------------GRPARSLAPLrlLLLVALGHAWTYREEPQDSDREVCSENKIATTRYPCLKPTGELTTCFRKKCCKG 67
X.tropicalis     1 MW------ERGARRGCL--CLGCL--LLLLSLGLAWTYREELE-TDREICSESKITTTKYPCVKPTGELATCFRKKCCKG 69
Danio rerio      1 MIYPG-------RGASL--SVAVA--LVLFSSGAPWTFREEKEDVDREVCSESKIATTKYPCVKSTGEVTTCYRKKCCEG 69

Mus musculus    79 YKFVLGQCIPEDYDICAQAPCEQQCTDNFGRVLCTCYPGYRYDRERHQKRERPYCLDIDECATSNTTLCAHICINTMGSY 158
Homo sapiens    78 YKFVLGQCIPEDYDVCAEAPCEQQCTDNFGRVLCTCYPGYRYDRERHRKREKPYCLDIDECASSNGTLCAHICINTLGSY 157
P.troglodytes   78 YKFVLGQCIPEDYDVCAEAPCEQQCTDNFGRVLCTCYPGYRYDRERHRKREKPYCLDIDECASSNGTLCAHICINTLGSY 157
Gallus gallus   68 YKFVLGQCIPEDYDVCAEAPCEQQCTDNFGRVLCTCYPGYRYDRERHRNREKPYCLDIDECAASNGTLCSHICINTVGSY 147
X.tropicalis    70 YKFVLGQCIPEDYDVCSEAPCEQQCTDNFGRVLCTCYPGYLYDRERHRNREKPYCLDIDECASKNETVCSHICINTPGSY 149
Danio rerio     70 FKFVLGQCIPEDYDVCAGAPCEQQCTDHFGRVVCTCYDGYRYDRERHRNREKPYCLDIDECANNNETVCSQMCVNTPGSY 149

Mus musculus   159 HCECREGYILEDDGRTCTRGDKYPNDTGHEeKSENEVKAGTCCATCKEFSQMKQTVLQLKQKMALLPNNAAELGKYVNGD 238
Homo sapiens   158 RCECREGYIREDDGKTCTRGDKYPNDTGHE-KSENMVKAGTCCATCKEFYQMKQTVLQLKQKIALLPNNAADLGKYITGD 236
P.troglodytes  158 RCECREGYIQEDDGKTCTRGDKYPNDTGHE-KSENVVKAGTCCATCKEFYQMKQTVLQLKQKIALLPNNAADLGKYITGD 236
G.gallus       148 RCECHEGYTRGEDGRTCTKGDKGMCPGLSE-KSENVAKPGTCCASCKEFHQIKQTVLQLKQKVSLLPNNAADLSKQITGE 226
X.tropicalis   150 RCECPEGYSLEEDGKTCTKGSQGD----FE-KSNNVMKAGVCSETCKDFHQIKQTVLQLKQKLAFLPNSVSESSKHITAE 224
Danio rerio    150 RCDCHSGFYLEDDGKTCTKGERAP---LFE-KSDNVMKEGTCSATCEDFHQMKMTVLQLKQKMSLLSSN-TEINKQMTNE 224

Mus musculus   239 KVLASN-AYLPGPPGLPGGQGPPGSPGPKGSPGFPGMPGPPGQPGPRGSMGPMGPSPDLSHIKQGRRGPVGPPGAPGRHG 317
Homo sapiens   237 KVLASN-TYLPGPPGLPGGQGPPGSPGPKGSPGFPGMPGPPGQPGPRGSMGPMGPSPDLSHIKQGRRGPVGPPGAPGRDG 315
P.troglodytes  237 KVLASN-TYLPGPPGLPGGQGPPGSPGPKGSPGFPGMPGPPGQPGPRGSMGPMGPSPDLSHIKQGRRGPVGPPGAPGRDG 315
G.gallus       227 KVLASN-AYIPGPPGQPGQGPPGAPGPKGSQGVPGSPGPPGQPGPRGSMGPMGPSPDISHIKQGRRGPVGPPGAPGRDG 305
X.tropicalis   225 KVLAST-TYVQGPPGLPGAQGPPGLPGPKGSAGQSGIPGPPGPPGPRGFMGPVGPSPEISQLKQGRRGPVGPPGAPGKDG 303
Danio rerio    225 KMMMTTnSFLPGPPGPPG---PAGTPGAKGSSGSPGQMGPPGLPGPRGDMGPIGPSPDLSHIKQGRRGPVGPPGAPGRDG 301

Mus musculus   318 SKGERGAPGPPGSPGPPGSFDFLLLVLADIRNDIAELQEKVFGHRTHSSAEDFP-LPQEFSSYPETLDFGSGDDYSRRTE 396
Homo sapiens   316 SKGERGAPGPRGSPGPPGSFDFLLLMLADIRNDITELQEKVFGHRTHSSAEEFP-LPQEFPSYPEAMDLGSGDDHPRRTE 394
P.troglodytes  316 SKGERGAPGPRGSPGPPGSFDFLLLMLADIRNDITELQEKVFGHRTHSSAEEFP-LPQEFPSYPEAMDLGSGDDHPRRTE 394
G.gallus       306 SKGERGAPGPKGIPGPPGSFDFLLLMMADIRNDIAELQERVFGRRTHSSTEEFP-LPQEFTNYHDTVDFGSGEDYKPRAA 384
X.tropicalis   304 TKGDRGAPGPRGPPGPPGPSGFDFLLLMMADIRNDIAELQDKVFGRRTHSSAEEFP-LPHEFTNHHESVDLGSGEDYKHRTV 382
Danio rerio    302 MKGERGFPGPSGPPGPPGSFDFLLLMMADIRNDIAELQSKVFSRPLHSSFEDFPsAPDSWRDTPENLDFGSGEDYKSQSP 381

Mus musculus   397 ARDPEAPRNFYP    408
Homo sapiens   395 TRDLRAPRDFYP    406
P.troglodytes  395 TRDLRAPRDFYP    406
G.gallus       385 PRDSRIQKAAHP    396
X.tropicalis   383 SKNLRTDKNSRH    394
Danio rerio    382 PKSSRKRKLPRN[8] 401
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B

Specie	Ccbe1 percent homology to *Mus musculus*
Homo sapiens	90
Pan troglodytes	90
Gallus gallus	79
Xenopus tropicalis	72
Danio rerio	66

Figure 5.2: Predicted Ccbe1 homology among vertebrate species. **A.** Multiple protein sequence alignment was computed using *Cobalt Constraint-based Multiple Protein Alignment Tool* to obtain conserved domains and local sequences similarities. **B.** Homology analysis was further obtained from the *NCBI Blast Local Alignment Search Tool* database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). GenBank Accession Number of sequences used for alignment were: *Mus musculus* - NP_848908.1, *Homo sapiens* - NP_597716.1, *Pan troglodytes* - XP_512156.3, *Gallus gallus* - XP_001233358.1, *Xenopus tropicalis* - XP_002936721.2 and *Danio rerio* - NP_001157395.1. Red corresponds to highly conserved

residues in the species listed, while black corresponds to moderately conserved residues, and blue corresponds to less conserved residues. Delimited zone corresponds to RGD domain. Alignment and analysis were conducted on-line http://www.ncbi.nlm.nih.gov/tools/cobalt, in September 2013

5.3.2. Ccbe1-/- fibroblasts exhibited premature proliferation arrest

The effect of *Ccbe1* in the autonomous proliferative capacity of MEFs was firstly explored by analyzing the survival curves of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs in culture. Therefore, taking advantage of the conventional *Ccbe1* mutant mice already existent in our animal facility, *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs were isolated separately from littermate embryos at approximately E13.5 days of gestation. The majority of the *Ccbe1*$^{-/-}$ embryos appeared edematous, which is consistent with defective lymphatic vasculature in these mutants (Bos et al., 2011). *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ primary mouse embryonic fibroblasts were plated at a density of 15 000 cells/cm^2 in a 6-well plate, in technical triplicates, and subcultured or passaged every 3 days at the same cell density, in parallel and under identical cell culture conditions.

Analyses of the cumulative population doubling (CDP) of isolated MEFs showed a decrease or delay in proliferation of *Ccbe1*$^{-/-}$ MEFs when compared to the wild-type. Several independent assays revealed that *Ccbe1*$^{-/-}$ MEFs proliferated normally in the first two passages but slowed dramatically by passage 3 and then arrested permanently (Fig. 5.3 A). Indeed, from passage three forward, *Ccbe1*$^{-/-}$ MEFs cumulative doubling population (CPD) is approximately half (CPD=2.95) of the *Ccbe1*$^{+/+}$ MEFs (CPD=4.48). In addition, morphological analysis showed that cultures of either genotype had indistinguishable spindle-shaped cells at initial plating (Fig. 5.3 B: a, d), but after two passages *Ccbe1*$^{-/-}$ fibroblast cultures started to accumulate cells that had a flattened appearance and cytoplasmic enlargement (Fig. 5.3 B: e), which is reminiscent of senescent cells. Furthermore, at passage six *Ccbe1*$^{-/-}$ fibroblasts resemble senescent cells and also presented binuclei, in contrast to *Ccbe1*$^{+/+}$ fibroblasts that exhibited a spindle-shape even after 21 days of culture (Fig. 5.3 B: f). Together, these data suggest that *Ccbe1* is involved in proliferation.

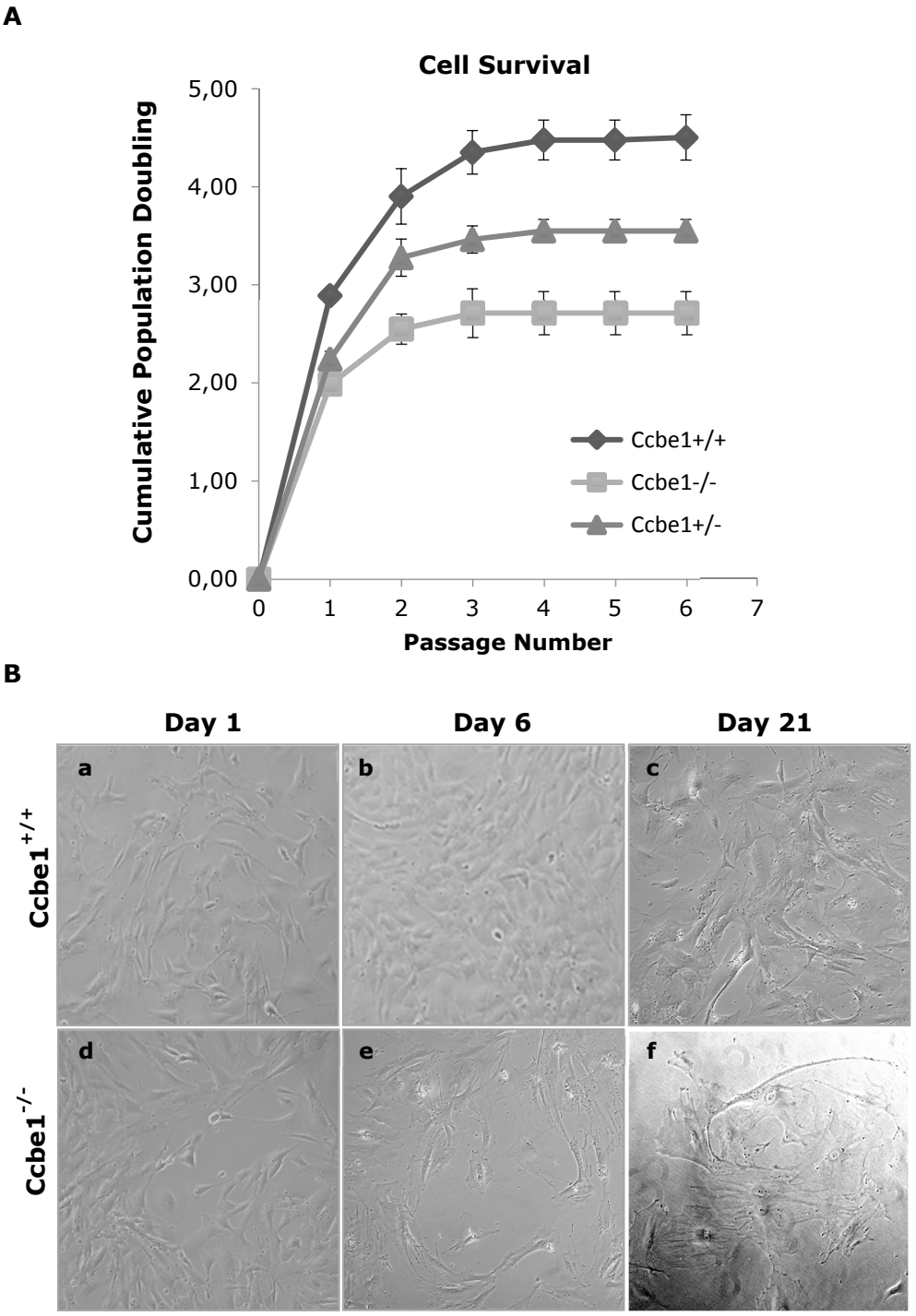
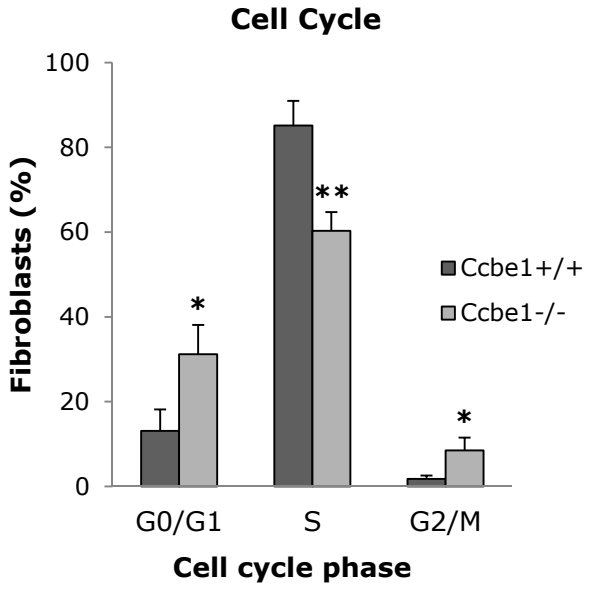


Figure 5.3: Proliferation properties and morphology of *Ccbe1*$^{-/-}$ MEFs. A. Growth curve of primary mouse embryonic fibroblasts from Ccbe1$^{+/+}$, Ccbe1$^{+/-}$ and Ccbe1$^{-/-}$ embryos. Data are representative of eight independent experiments, performed in technical triplicates, using three littermates at approximately E13.5. Population doubling (PD) per passage was calculated as *log(nf/n0)/log2*, where *n0* is the initial and *nf* the final number of cells at each passage. Cumulative population doubling (CPD) at each passage was calculated by adding population doubling per passage. Data presented as mean ± SD. **B.** Representative contrast phase images of MEFs at day 1, day 6 (passage 2) and day 21 (passage 6). Magnification is 100x (a, b, d, e) and 200x (c, f).

The BrdU labeling assay started with the seeding of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 2, in technical triplicates, in a 6-well plate. Cells were cultured for 24h, incubated with BrdU at the last 14h of culture and supplemented with 7-aminoactinomycin D (7-AAD) immediately before flow cytometry acquisition. Flow cytometry analysis showed that the percentage of *Ccbe1*$^{-/-}$ MEFs in phase G0/G1 (31.20%) was approximately the double of Ccbe1$^{+/+}$ MEFs (13.10%) (Fig. 5.4 A) suggesting that *Ccbe1*$^{-/-}$ fibroblasts may leave cell cycle and stop dividing, and/or the G1 phase in KO MEFs takes longer than WT MEFs (Fig. 5.4 A). The percentage of *Ccbe1*$^{-/-}$ proliferating MEFs (cells in S phase) is approximately 25% less than *Ccbe1*$^{+/+}$ MEFs (Fig. 5.4 B), which is consistent with the growth curves presented above (Fig. 5.3 A). Furthermore, *Ccbe1* mutant MEFs presented high percentage of cells in G2/M phase (8.50%) than *Ccbe1*$^{+/+}$ (1.77%; Fig. 5.4 A). The results from the analysis of survival growth curves and cell cycle indicate that the lack of *Ccbe1* in MEFs promote decrease of proliferating cells (S phase) and G0/G1 or G2/M phase arrest leading to cell quiescence.

A



B

%	WT	KO	Δ (KO-WT)
G0/G1	13,10	31,20	+18,10
S	85,13	60,30	-24,83
G2/M	1,77	8,50	+6,73

Figure 5.4: Cell cycle. A. Cell cycle phases (Go/G1, S and G2/M) of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 2. Representative results of two independent experiments performed in technical triplicates. **B.** Ratio of the difference in cell cycle phases population between *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs, in percentage. (*; **) indicates significantly different percentage ($p<0.05$; $p<0.01$) compared to *Ccbe1*$^{+/+}$ MEFs.

To evaluate if the proliferation is arrested in *Ccbe1*$^{-/-}$ fibroblasts, the proliferative properties of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ were also analyzed by a Cell Proliferation Dye eFluor® 670 assay. Cell Proliferation Dye eFluor® 670 (eBioscience) is a red fluorescent dye that can be used to monitor individual cell divisions. This fluorescent dye binds to proteins containing primary amines and as cells divide, the dye is distributed equally between daughter cells, which can be measured as successive halving of the fluorescence intensity of the dye. Therefore, eFluor® 670-labeled *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ fibroblasts were seeded and the tracking of cell division was performed at day 2, 4 and 7 of culture by flow cytometry. In order to analyze the loss of fluorescence intensity with more accuracy, the resultant charts were divided in 4 levels of fluorescence in which quadrant (Q) 1 corresponds to the maximum and 4 to the lower level of fluorescence intensity. Analysis of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs histograms showed that at day 0 all the cells were eFluor® 670-labeled and the intensity of fluorescence decreased along the time of culture (Fig. 5.5 A), as expected. At day 2, the percentage of undivided cells, i.e. the cells that remained with the initial intensity of fluorescence (Q1), was higher in *Ccbe1*$^{-/-}$ (29.01%) than in *Ccbe1*$^{+/+}$ (16.70%) fibroblasts population (Fig. 5.5 B). A similar cell behavior was observed at day 4, where the undivided cells (Q1+Q2) in *Ccbe1*$^{-/-}$ fibroblast population was approximately a half (53.22%) of the *Ccbe1*$^{+/+}$ fibroblast population (35.55%, Fig. 5.5 B). Subsequently, at day 7, while *Ccbe1*$^{+/+}$ histogram revealed a pronounced shift to the left, *Ccbe1*$^{-/-}$ presented a peak approximately in the middle of the fluorescence intensity axis (Fig. 5.5 A) indicating that Ccbe1 mutant MEFs divided less. Taking together, these results indicate that *Ccbe1*$^{-/-}$ MEFs proliferated less than *Ccbe1*$^{+/+}$ MEFs. In addition, these results indicate that the proliferation of *Ccbe1*$^{-/-}$ MEFs is not arrested, but instead, severely delayed.

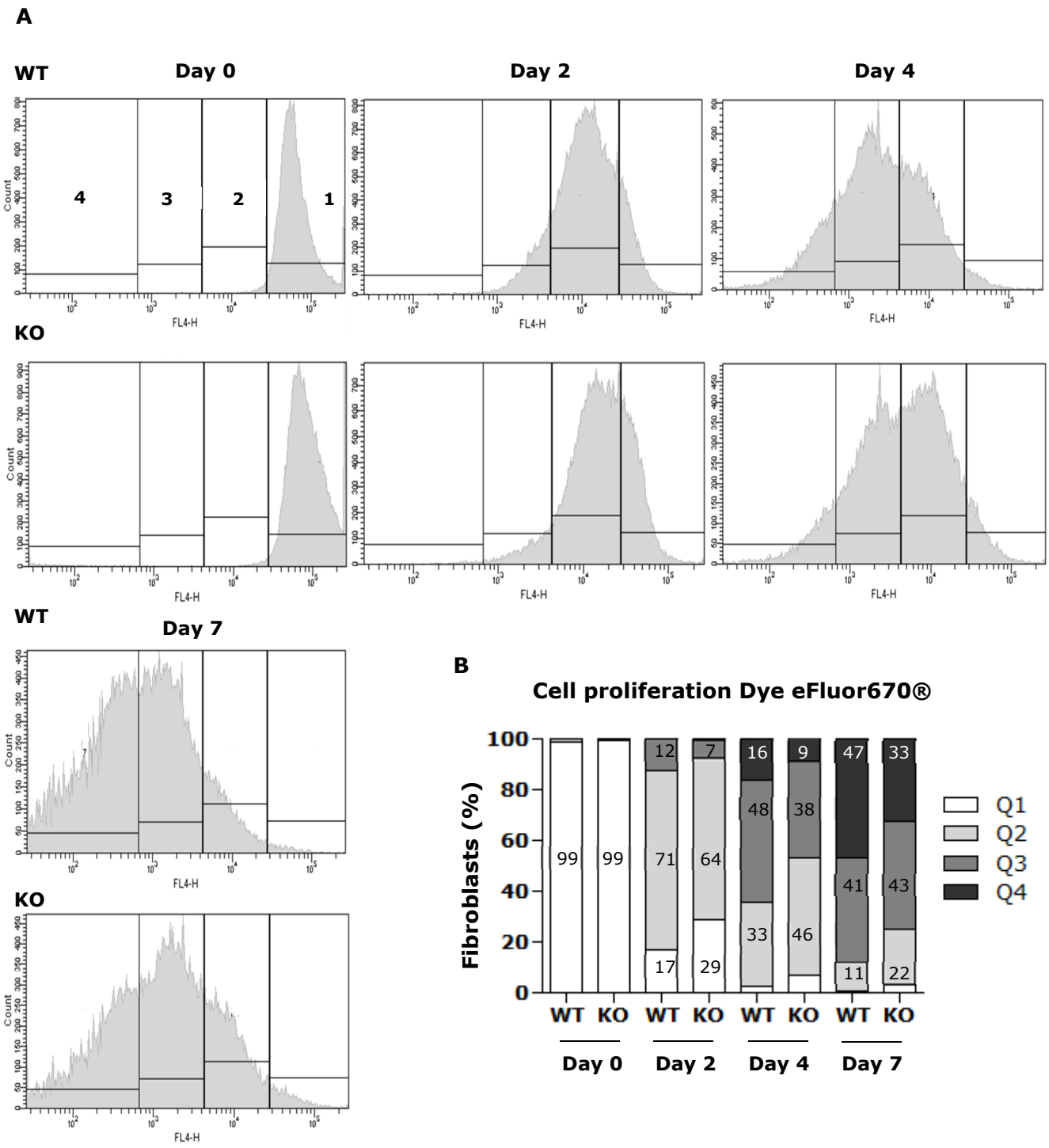


Figure 5.5: Cell division tracking assay. A. Histograms of fluorescence decay during 7 days of culture. The undivided cells maintain their fluorescence intensity. In divided cells the fluorescence intensity decrease. Y axis – Cell number, X axis - Fluorescence **B.** Percentage of fibroblasts per quadrant of fluorescence intensity (Q). Data are resultant of one experiment performed in technical triplicates.

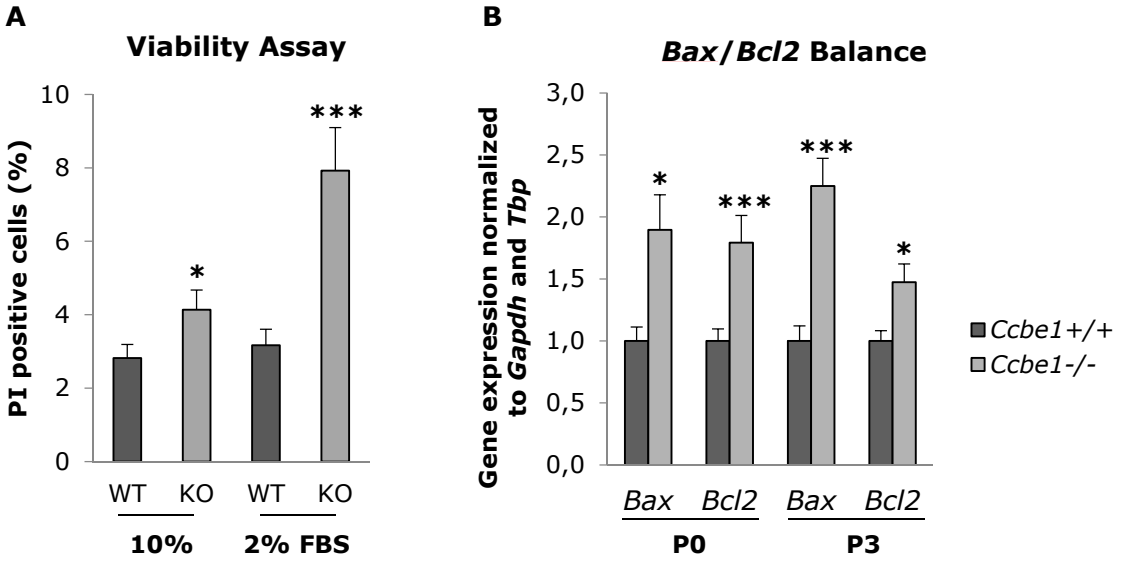
5.3.3. *Ccbe1*$^{-/-}$ MEFs are susceptible to apoptosis

The analysis of cell proliferation and cell cycle results was complemented with an apoptosis assay to determine whether *Ccbe1* has a role in cell viability. The apoptosis or viability assay was performed using propidium iodide (PI) which is a membrane-impermeant dye generally excluded from viable cells and, therefore, it can be used to differentiate necrotic or apoptotic from viable cells and consequently to evaluate cell viability. In short, viable *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 2 were cultured for 44h in growth medium (10% FBS) and in starvation medium (2% FBS) to induce cell stress, allowing a more clear analysis of cell viability. Then, cells were collected and incubated with PI immediately before FACS acquisition.

According to our data, the percentage of apoptotic (PI positive) *Ccbe1*$^{+/+}$ MEFs was unaffected by starvation conditions (Fig. 5.6 A). However, the percentage of apoptotic *Ccbe1*$^{-/-}$ MEFs was higher (4.14%) than *Ccbe1*$^{+/+}$ MEFs (2.45%) in standard conditions and the double in starvation conditions (7.93; Fig. 5.6 A). Therefore, the differences in apoptosis susceptibility of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs become more evident in starvation conditions. In fact, the percentage of apoptotic fibroblasts in standard conditions was 47% higher in *Ccbe1*$^{-/-}$ than in *Ccbe1*$^{+/+}$ population, while in starvation conditions the percentage of *Ccbe1*$^{-/-}$ PI positive MEFs increased 150% in comparison to *Ccbe1*$^{+/+}$ MEFs (Fig. 5.6 A). The viability assay revealed that *Ccbe1*$^{-/-}$ MEFs seem to be more susceptible to apoptosis suggesting that *Ccbe1* may be important to maintain cell viability, especially in stress conditions.

In order to confirm that the absence of *Ccbe1* increases the susceptibility to apoptosis, the mRNA levels of *Bax* (an apoptosis promoter) and *Bcl2* (an apoptosis inhibitor) were quantified. The ratio of *Bax* to *Bcl2* acts as a cell autonomous rheostat and is considered to be a marker of a cell's susceptibility to apoptotic stimuli (Yin et al., 1994). Following a signal for programmed cell death, cells die if *Bax* is in excess, but live if *Bcl2* predominates. The evaluation of *Bax* and *Bcl2* expression levels was performed using mRNA from *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 0 and 3. At passage 0, the *Bax*/*Bcl2* ratio was approximately 1 for *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs, suggesting an equilibrium between both apoptosis promoter and inhibitor factors (Fig. 5.6 B, C). Nonetheless, when *Ccbe1*$^{-/-}$ MEFS endured three passages, the expression levels of *Bax* increased and the expression

levels of *Bcl2* decreased, resulting in an increase of approximately 50% of *Bax*/*Bcl2* ratio (Fig. 5.6 B, C). This is consistent with the decreased cell viability of *Ccbe1* mutant MEFs. Normally, an increase of *Bax*/*Bcl2* ratio leads to the activation of the caspases, especially Caspase3, which are known to act downstream of *Bax/Bcl2* control and play a key role in the execution of apoptosis (Salakou et al., 2007).



C

	P0		P3	
	Ccbe1$^{+/+}$	*Ccbe1*$^{-/-}$	*Ccbe1*$^{+/+}$	*Ccbe1*$^{-/-}$
Bax/Bcl2	1.00	1.06	1.00	1.53

Figure 5.6: Viability and apoptosis study. A. Evaluation of viability of Ccbe1$^{+/+}$ and Ccbe1$^{-/-}$ fibroblasts after 44h under standard (10% FBS) or starvation (2% FBS) conditions. Data are expressed as mean+SD, from two experiments performed in technical triplicates. **B.** *Bax* and *Bcl2* expression levels of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ fibroblasts at passage 0 and passage 3. Data are expressed as mean+SEM, from three biological samples in technical triplicates. **C**. *Bax*/*Bcl2* ratio of mRNA levels. (*, ***) indicates significantly different ($p<0.05$; $p<0.001$) compared to *Ccbe1*$^{+/+}$ MEFs.

Therefore, *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 3 were seeded in coverslips for 24h. Then, the activity of Caspase3 was analyzed by immunocytochemistry using antibodies against cleaved-Caspase3. Preliminary results (n=1) showed that approximately 18% of *Ccbe1*$^{-/-}$ MEFs exhibited active (cleaved) Caspase3, whereas Caspase3 seemed to be inactive in *Ccbe1*$^{+/+}$ MEFs (Fig. 5.7), which is consistent with the results obtained for *Bax/Bcl2* ratios. Altogether, these results

indicate that *Ccbe1*$^{-/-}$ MEFs seem to be less viable and more susceptible to apoptotic stimuli.

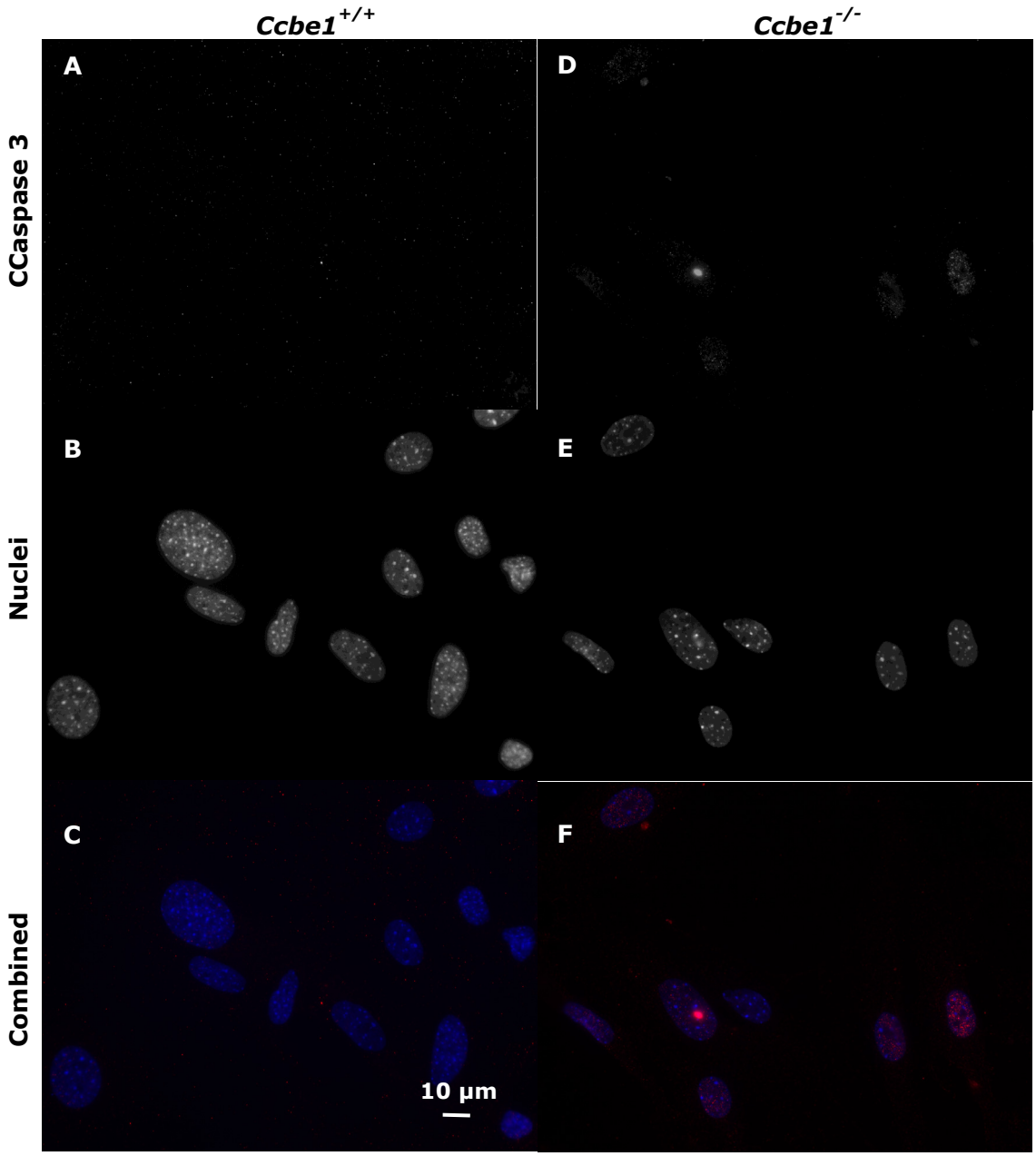


Figure 5.7: Activated caspase3 expression. Representative pictures of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs at passage 3 labeled with cleaved caspase3 antibody. Red – CCBE1. Blue (Dapi) – Nuclei.

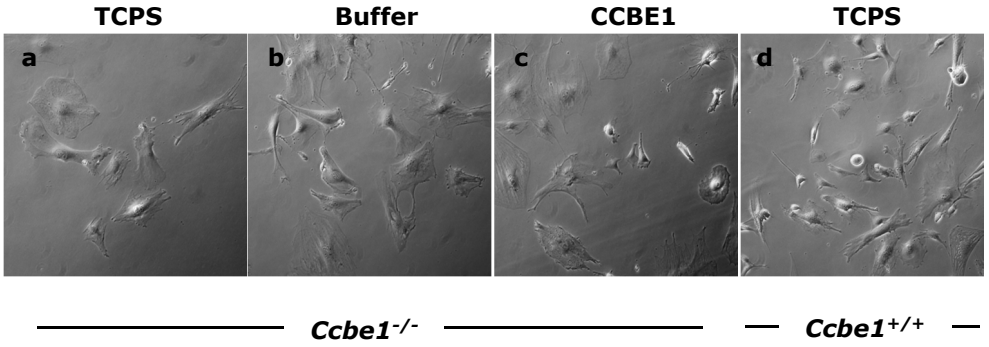
5.3.4. Rescue of *Ccbe1*$^{+/+}$ proliferative phenotype

A rescue assay was performed to verify whether the addition of CCBE1 contributes to cell proliferation and prevention of apoptosis. Therefore, 10 µg/ml of mouse CCBE1 protein solution and the buffer where CCBE1 was diluted (25 mM Tris, 0.15 M NaCl pH 7.4, 2 mM $CaCl_2$, 10% Glycerol) as control were incubated o.n. at 4ºC in the culture plates to cover entire growth area. Then, the excess of the solution was removed and approximately 22 000 MEFs were seeded per well of a 6-well plate. Culture of *Ccbe1*$^{-/-}$ and *Ccbe1*$^{+/+}$ fibroblasts in TCPS was used as negative control. The culture was monitored by microscopic observation. Cell culture was stopped and cells were counted approximately after 48h, when cell density started to reach confluency. According to our data, *Ccbe1*$^{-/-}$ fibroblasts cultured in TCPS or in dialysis buffer presented similar values of cell growth (Fig. 5.8 A). In contrast, *Ccbe1*$^{-/-}$ fibroblasts cultured in CCBE1 coating exhibited a significant increase in growth, corresponding to a gain of 63% of the cell growth obtained for *Ccbe1*$^{-/-}$ fibroblasts cultured in TCPS (Fig. 5.8 A). In addition, *Ccbe1*$^{-/-}$ fibroblasts cultured in CCBE1 coating appeared to have a small size and spindle-shape morphology, similar to fibroblasts found in *Ccbe1*$^{+/+}$ MEFs culture and in contrast to the flattened appearance with extensive lamellipodia of *Ccbe1*$^{-/-}$ MEFs cultured in TCPS (Fig. 5.8 B). Furthermore, preliminary results (n=1) revealed that the viability of *Ccbe1*$^{-/-}$ MEFs in starvation conditions can be improved as well through the use of a CCBE1 coating (Fig. 5.8 B). Even though *Ccbe1*$^{-/-}$ MEFs cultured in CCBE1 did not achieve the levels of proliferation and viability of *Ccbe1*$^{+/+}$ MEFs, rescuing CCBE1 loss-of-function by providing CCBE1 protein as a coating improves proliferation and prevent apoptosis of *Ccbe1*$^{-/-}$ MEFs.

A

	Ccbe1$^{-/-}$			*Ccbe1*$^{+/+}$
	TCPS	**Buffer**	**CCBE1**	**TCPS**
Cell number	8626	8104	14214**	22694
Cell growth (fold)	0,38	0,36	0,63	1,01
Normalization to TCPS	0	-0,07	0,63	1,60

B



C

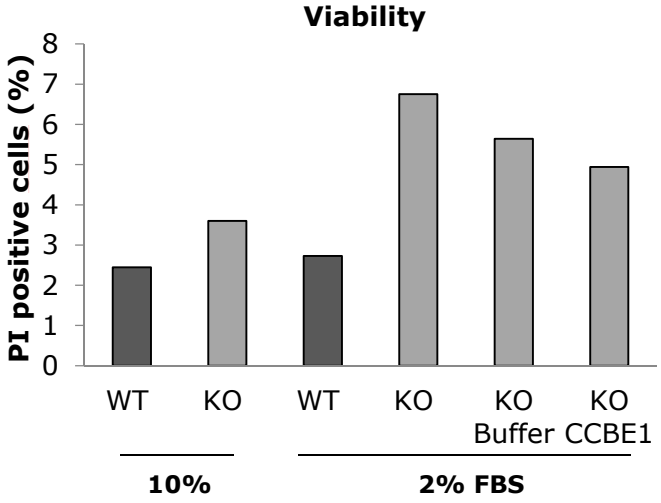
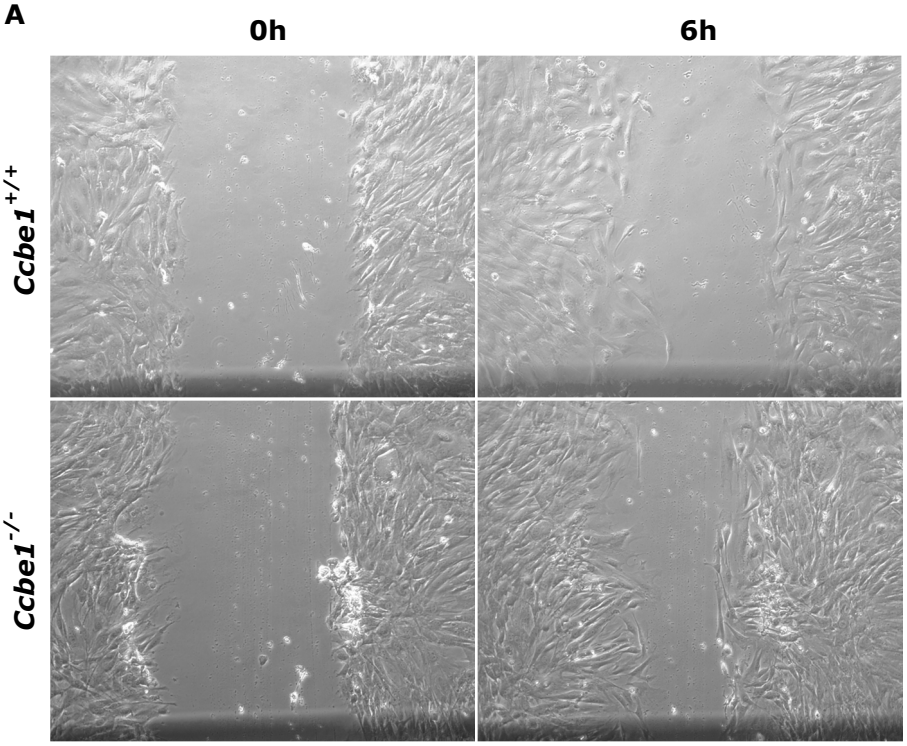


Figure 5.8: Rescue of proliferative phenotype of *Ccbe1*$^{+/+}$ MEFs. A. Proliferation of *Ccbe1*$^{-/-}$ MEFs in TCPS, CCBE1 and buffer coatings and *Ccbe1*$^{+/+}$ MEFs in TCPS, for 48h. Data are representative of two independent experiments. ** $p<0.01$ relative to Ccbe1$^{-/-}$ MEFs cultured in TCPS. **B.** Morphology of *Ccbe1*$^{-/-}$ and *Ccbe1*$^{+/+}$ MEFs after 48h of culture. Magnification is 100x. **C.** Evaluation of viability of *Ccbe1*$^{-/-}$ fibroblasts cultured in TCPS, buffer and CCBE1 coatings and *Ccbe1*$^{+/+}$ fibroblasts after 44h under standard (10% FBS) or starvation (2% FBS) conditions. Data from one sample per condition.

5.3.4. *Ccbe1*$^{-/-}$ MEFs presented high motility

Data from our lab showed that absence of *Ccbe1* leads to alterations in cell migration events during chick heart development. Furthermore, absence of *Ccbe1* in ovary cancer cells has been shown to favor the migration of the cells (Barton et al., 2010). Moreover, in mice, *Ccbe1* seems to be required for proper lymphatic cell migration (Bos et al., 2011). In order to evaluate if migration is affected as well in *Ccbe1*$^{-/-}$ MEFs and to determine the possible mechanism whereby Ccbe1 may modulate cell migration, we performed comparative analysis of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs in wound healing assays. In one of the first approaches to characterize the migration of *Ccbe1*$^{-/-}$ MEFs, 500 000 of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ inactivated MEFs were seeded in 6-well plate pre-coated with gelatin, and in the following day cell migration was evaluated by a wound-induced migration assay: the basic steps involved creation of a "wound"/scratch in the MEFs monolayers with the help of a pipette tip, capture of micrographs at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. Since *Ccbe1* affects proliferation, MEFs were mitotically inactivated to isolate the effect of the migration in wound closure. Observation of micrographs at 0 and 6h, showed that *Ccbe1*$^{-/-}$ MEFs are able to close the gap faster than *Ccbe1*$^{+/+}$ MEFs (Fig. 5.9 A). The delay was confirmed by the calculations of migration rate where *Ccbe1*$^{-/-}$ fibroblasts were 11.26 % faster than *Ccbe1*$^{+/+}$ fibroblasts (Fig. 5.9 B).

A



B

	v (µm/h)
***Ccbe1*$^{+/+}$**	39.64 ± 1.45
***Ccbe1*$^{-/-}$**	44.10 ± 1.77
% KO/WT	+11.26

Figure 5.9: Motility of Ccbe1$^{-/-}$ and Ccbe1$^{+/+}$ MEFs. A. Representative pictures of wound-induced migration assay. Magnification is 100x. **B.** Quantitative analysis of the migration rate of the cells into the denuded area over 6h. Data are representative of 3 independent experiments and presented as mean±SD**.**

5.3.5. Migration of Ccbe1$^{-/-}$ MEFs is substrate dependent

Ccbe1 has been shown to interact with ECM proteins vitronectin, collagen type I and V, indicating that Ccbe1 may be as well an ECM component (Bos et al., 2011). Besides conferring support, interaction of ECM components with membrane receptors integrins is known to regulate several cellular processes, such as proliferation, survival, cellular shape and migration (Kim et al., 2011). Whether Ccbe1 is a signaling molecule or simply an ECM component remains unclear. The *in vitro* scratch assay is particularly suitable for studies on the effects of cell–matrix

and cell–cell interactions on cell migration, mimic cell migration during wound healing *in vivo*. So, in order to start to understand the mechanism whereby Ccbe1 may regulate cell migration, were performed wound healing assays in the presence of several of these ECM components, namely vitronectin and fibronectin. Therefore, 0.1% of gelatin, 10 µg/ml of fibronectin and vitronectin were incubated o.n. at 4ºC in a 6-well plated and then mitotically inactivated Ccbe1$^{-/-}$ and Ccbe1$^{+/+}$ MEFs were seeded at same density, in technical triplicates. As *Ccbe1* is involved in proliferation process MEFs were mitotically inactivated prior to the migration assay to avoid the interference of proliferation events. In the next day, all the cells were adhered forming a monolayer and three gaps or scratches per well were done using a blue pipette tip. In general, *Ccbe1*$^{-/-}$ MEFs demonstrated to be faster than *Ccbe1*$^{+/+}$ MEFs, independently of the coating (Table 5.1). Nevertheless, the difference of migration rate between *Ccbe1* KO and WT MEFs was matrix dependent and more evident for migration in vitronectin (28%) (Table 5.1). Migration was favored in vitronectin, a substrate that possibly interacts with CCBE1. These results suggest that probably the interaction of CCBE1 with vitronectin block migration and, in absence of CCBE1, fibroblasts are able to migrate easier.

Table 5.1: Main results of migration in gelatin, fibronectin and vitronectin, over 6h. *Ccbe1*$^{-/-}$*/Ccbe1*$^{+/+}$ (%) = (v (*Ccbe1*$^{-/-}$*/Ccbe1*$^{+/+}$)*100) – 100. Data are representative of 2 independent experiments as mean±SD

	***Ccbe1*$^{+/+}$ v (µm/h)**	***Ccbe1*$^{-/-}$ v (µm/h)**	***Ccbe1*$^{-/-}$*/Ccbe1*$^{+/+}$ (%)**
Gelatin	41,40 ± 3,39	45,72 ± 4,01	10,46
Fibronectin	51,30 ± 2,30	60,05 ± 2,50	17,06
Vitronectin	48,55 ± 2,69	62,09 ± 1,49	27,90

Migrating cells make new adhesions under the leading edge, have stable adhesions under the cell body and break adhesions at the trailing edge (Moissoglu and Schwartz, 2006). Furthermore, adhesion is mediated primarily by integrins; transmembrane receptors, each containing an α and a β subunit, that bind ECM

proteins outside the cell and link to cytoskeletal proteins and signaling pathways inside the cell (Moissoglu and Schwartz, 2006). Moreover, the differences in adhesion stability during migration give rise to gradients in multiple signaling pathways that promote or maintain the directionality of cell movement (Moissoglu and Schwartz, 2006). Taking this into account, we performed a wound-healing assay including antibodies (ab) to block specific subtypes of integrins. More specifically, scratches were performed and *Ccbe1*$^{-/-}$ MEFs were incubated with growth medium supplemented with antibodies against β1-integrin (usually part of collagen receptor; Santa Cruz; 1:100) and β4-integrin (usually part of laminin receptor; Abcam; 1:100) (Srichai and Zent, 2010). In addition, *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs incubated with growth medium were used as control. A rescue condition was also tested by incubating *Ccbe1*$^{-/-}$ MEFs with growth medium supplemented with 10 μg/ml of CCBE1 protein. *Ccbe1*$^{-/-}$ MEFs migrated 8.17% faster than *Ccbe1*$^{+/+}$ MEFs, as expected (Table 5.2). The incubation of *Ccbe1*$^{-/-}$ MEFs with antibodies against both β1 and β4-integrins induced a decrease of migration rate, however the blocking of migration was not selective and cells responded similarly to the blocking of interaction via collagen or via laminin-dependent pathway (Table 5.2). So this experiment was not sufficiently elucidative about the specific β-integrin signaling pathway where Ccbe1 is involved. On the other hand, the supplementation of *Ccbe1*$^{-/-}$ MEFs with CCBE1 protein rescued the migration phenotype of *Ccbe1*$^{+/+}$ MEFs. *Ccbe1*$^{-/-}$ MEFs supplemented with CCBE1 migrated 16.15% slower than *Ccbe1*$^{-/-}$ MEFs in growth medium and 9.30% slower than *Ccbe1*$^{+/+}$ MEFs (Table 5.2).

Table 5.2: Main results of migration in gelatin over 7h. Growth medium was supplemented with antibodies against β1 and β4 integrin and with CCBE1, in gelatin coating. *Ccbe1/Ccbe1*$^{-/-}$ *control* (%) = (v (*Ccbe1/Ccbe1*$^{-/-}$ *control)**100) – 100, *Ccbe1*$^{-/-}$*/Ccbe1*$^{+/+}$ (%) = (v (*Ccbe1*$^{-/-}$*/Ccbe1*$^{+/+}$)*100) – 100. Data are from one experiment performed in technical duplicates and presented as mean±SD.

		v (μm/h)	***Ccbe1/Ccbe1*$^{-/-}$ control (%)**	***Ccbe1*$^{-/-}$*/Ccbe1*$^{+/+}$ (%)**
***Ccbe1*$^{+/+}$**	control	36,84 ± 0,86	-7,55	-
***Ccbe1*$^{-/-}$**	control	39,85 ± 0,92	-	8,17
	β1-integrin ab	35,59 ± 0,89	-10,69	-3,40
	β4-integrin ab	34,37 ± 1,12	-13,75	-6,71
	CCBE1	33,41 ± 0,02	-16,15	-9,30

Collectively, these experiments confirmed that the absence of *Ccbe1* in MEFs favors migration and the addition of CCBE1 blocks migration, *in vitro*. The migration rate of MEFs was modulated by both ECM and β-integrin signaling pathways.

5.3.6. Disruption in Ccbe1 gene changes cell adhesion

The differences in migration rate between *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs might be a consequence of alterations in cell adhesion and spreading. To test this, 100 000 *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs were seeded sparsely and cell adhesion was evaluated at 0, 40, 80 and 240 min by microscopy and photography. At last time point, the non-adherent cells were washed and the adherent cells were collected and counted. After 80 and 240 min of culture, we observed more adherent *Ccbe1*$^{+/+}$ than *Ccbe1*$^{-/-}$ MEFs (Fig. 5.10 A). This result was confirmed by counting the adherent cells, where from the 100 000 seeded cells only 43 000 Ccbe1$^{-/-}$ MEFs had adhered in contrast to 79 000 adherent *Ccbe1*$^{+/+}$ MEFs. Next, to test the adhesive capacity of *Ccbe1*$^{-/-}$ MEFs, 130 000 *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs were plated and cultured for 48h to reach confluency. Then, plates were washed and the adhesive capacity was determined by incubating the cells with 0.01% of trypsin for 0, 1, 5 and 8 min and monitored by microscopy and photography (Fig. 5.10 B). *Ccbe1*$^{+/+}$ fibroblast culture presented adherent and not completely individualized cells even after 8 min of trypsin incubation. In contrast, all the Ccbe1$^{-/-}$ MEFs were detached from the substrate and individualized after 5 min of trypsin incubation (Fig. 5.10 B). These data suggest that absence of *Ccbe1* weakens the adhesive ability of cells, which may be related to the enhanced motility of Ccbe1$^{-/-}$ MEFs.
Localized degradation of the ECM is necessary for cells to migrate and involves many proteolytic enzymes (Lu et al., 2011). Most of these enzymes are either serine proteases or matrix metalloproteinases (MMPs; Ennis and Matrisian, 1994). To understand if any of these enzymes was upregulated in *Ccbe1* mutants MEFs, we analyzed the profile of urokinase-type plasminogen activator (*uPa*) and *Mmp2* expression in *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs. *uPA* is a serine protease that can initiate proteolytic cascades, resulting in remodeling of extracellular matrix and basement membrane, allowing cells to move across and through these barriers (Pepper and Vassalli, 1987).

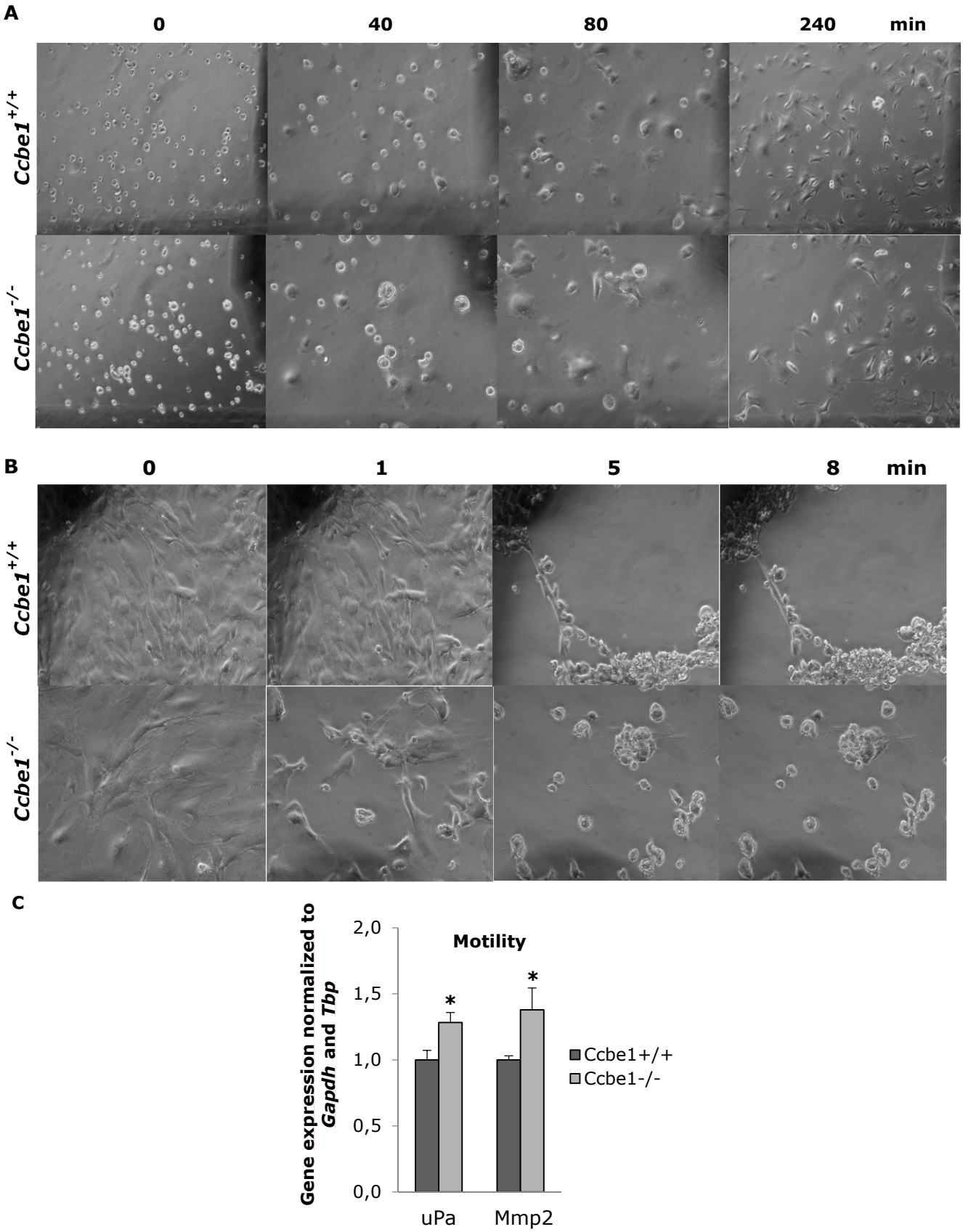


Figure 5.10: Adhesive properties of *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs. A. Evaluation of cell adhesion during 4h**. B.** Confluent monolayers of MEFs were incubated with 0.01% of trypsin and cell detachment was monitored during 8 min. All these pictures are representative of two independent experiments. **C.** Expression levels of *uPa* and *Mmp2* from two biological replicates, presented as mean + standard error of mean.

On the other hand, MMPs are a family of matrix-degrading enzymes, which include collagenases, stromelysins and gelatinases. MMP2 is a gelatinase which degrades collagen type IV, the major structural component of basement membranes and the first barrier for migrating cells (Monaco et al., 2006).
According to our qRT analysis, *Ccbe1*$^{-/-}$ fibroblasts expressed higher levels of *uPA* and of *Mmp2* comparatively to *Ccbe1*$^{+/+}$ fibroblasts (Fig. 5.10 C), which is consistent with the higher migratory behavior of *Ccbe1* mutant MEFs. In sum, these data indicate that absence of *Ccbe1* in MEFs leads to increased migration, suggesting that *Ccbe1* interferes with cell migration in wild-type cells.

5.3.7. *Ccbe1*$^{-/-}$ MEFs do not support cardiac ESC differentiation

According to the data presented above, *Ccbe1* seems to act as ECM modulator. In the absence of *Ccbe1* in MEFs, the expression of *Mmp2* increased, which probably leads to changes in ECM structure making it more suitable for cell motility. On the other hand, the developmental fate of differentiating ESCs depends on the complex combination of growth factors, signaling molecules, and ECM proteins constituting the developmental niche in which the cells exist (Czyz and Wobus, 2001; Oyamada et al., 1996). In addition, it is known that ESC differentiation require specific signals dependent on cell-matrix and cell-cell interactions *via* gap-junctions (Oyamada et al., 1996). Therefore, in agreement with the scope of the present thesis, we co-cultured ESCs during differentiation with *Ccbe1*$^{-/-}$ MEFsi as their niche to evaluate whether there would be an effect of cardiac differentiation. This was determined by evaluating the expression of cardiac-specific markers upon ESC differentiation. To do so, undifferentiated ESCs were resuspended in differentiation medium and hanging drops were plated onto the base of a bacteriological Petri dish to form EBs. At day 5 of differentiation, EBs were collected and seeded in gelatin, wild-type and mutant *Ccbe1* MEFs. Subsequently, at day 10 of differentiation, cellular aggregates were collected out from the plates and RNA isolated for further analyses of cardiac differentiation markers. During EB differentiation, there is a characteristic pattern of the expression of cardiac-specific genes. Accordingly, ESC differentiation to cardiac fate starts with mesoderm differentiation followed by cardiac mesoderm specification in which cell transcription factors, such as *Mesp1* and *2*, are involved. At the terminal

differentiation stage, maturing cardiomyocytes can be identified by the expression of cardiac structural proteins such as α-actinin, α-myosin heavy chain (*αMhc*), or the cardiac isoform of Troponin-T (*cTnT*) (Rajala et al., 2011). Therefore, in this preliminary study, we analyzed the expression of early cardiac progenitor markers (*Isl1* and *Nkx2.5)* and terminally differentiated cardiomyocyte markers (*αMhc* and *cTnT*). The expression of these markers were also analyzed in *Ccbe1*$^{-/-}$ and *Ccbe1*$^{+/+}$ MEFs to establish baseline expression in the MEFs and were used as negative controls.

According to our data, with the exception of *cTnT,* the expression of the early and terminal cardiac differentiation markers in EBs seeded in *Ccbe1*$^{+/+}$ MEFsi was identical to EBs differentiated in gelatin (Fig. 5.11). In contrast, the expression of the early and terminal cardiac differentiation markers in EBs seeded in *Ccbe1*$^{-/-}$ MEFsi was significantly lower than in EBs seeded in gelatin or in *Ccbe1*$^{+/+}$ MEFsi (Fig. 5.11). These results, even if only preliminary, suggest that the presence of *Ccbe1*$^{-/-}$ MEFsi hampers the differentiation of ESCs to cardiac lineages.

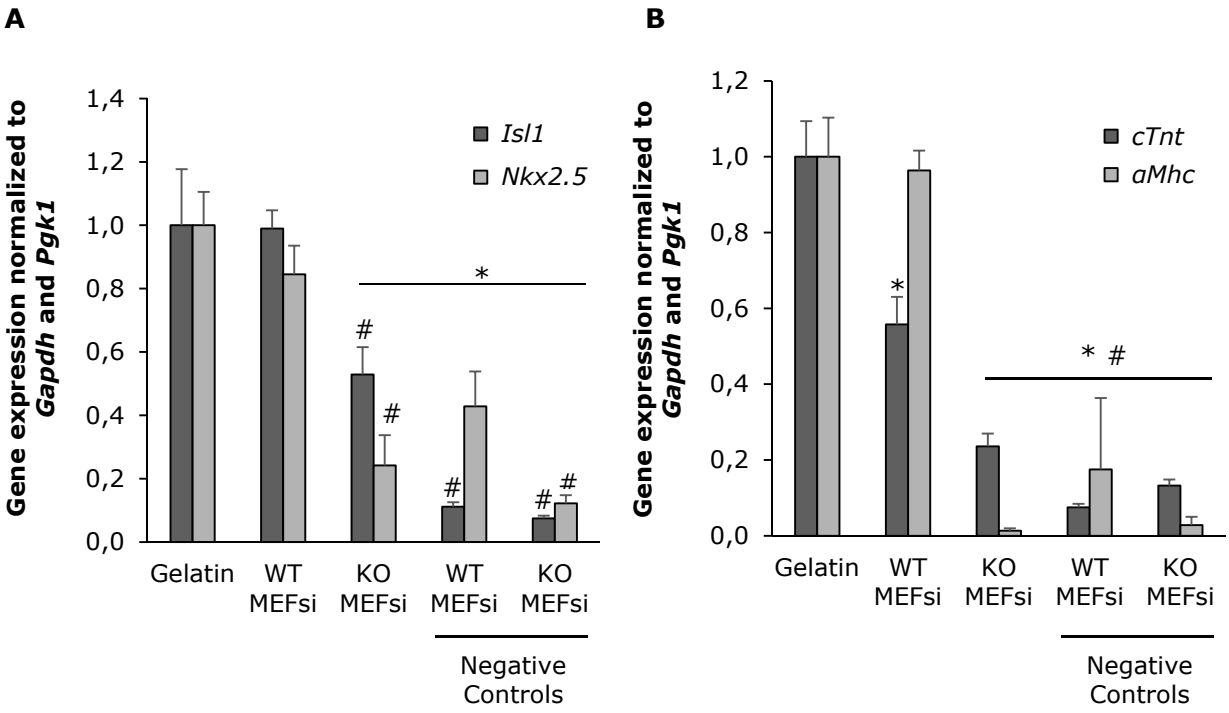


Figure 5.11: Cardiac ESC differentiation. Cardiac differentiation potential of EBs seeded in gelatin, *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFsi. Levels of gene expression of early (**A.**) and terminal (**B.**) cardiac markers, in EBs collected at day 10 of differentiation. The relative gene expression was normalized to *Gapdh* and *Pgk1* and to gelatin control. (*) significantly different expression levels compared to EBs in gelatin. (#) significantly different expression levels compared to EBs in *Ccbe1*$^{+/+}$ MEFsi. Data are from one biological replicate, performed in technical triplicates.

5.4. DISCUSSION

Several independent experiments revealed *Ccbe1*$^{-/-}$ MEFs proliferated normally until passage 2 but then proliferation decreased a half, probably due an increase of approximately 50% of *Bax*/*Bcl2* ratio. The decreased CPD *Ccbe1*$^{-/-}$ MEFs is consistent with the reduced cell proliferation exhibited by mouse *Ccbe1*$^{-/-}$ fetal livers in Zou's work (Zou et al., 2013). In addition, the analysis of cell cycle propose that the absence of *Ccbe1* in culture of MEFs promote an increase of cells in G0/G1 or G2/M phase and, consequently, a decrease of cells in proliferating phase (S). Cell cycle delay in G0/G1 or G2/M phase indicates that G0/G1 phase of *Ccbe1*$^{-/-}$ fibroblasts is longer or they tend to left cell cycle and stop dividing or to enter in quiescence. A delay in proliferation was also found by tracking cell division through Cell Proliferation Dye eFluor® 670 assay. Furthermore, viability assays revealed that *Ccbe1*$^{-/-}$ MEFs seemed to be more susceptible to apoptosis suggesting that *Ccbe1* is important to prevent cell apoptosis or protect cell viability under challenging conditions or apoptotic stimuli. Moreover, the addition of CCBE1 to the culture system reversed morphological changes in *Ccbe1*$^{-/-}$ MEFs, improved proliferation and prevented apoptosis of *Ccbe1*$^{-/-}$ MEFs.

Ccbe1 is thought to function in extracellular matrix remodeling and migration. In the present work, the possible use of *Ccbe1*$^{-/-}$ MEFs matrix to support ESC differentiation and the effect of *Ccbe1* in ECM structure was evaluated by differentiating ESCs in *Ccbe1*$^{-/-}$ MEFs and under control conditions. According to our data, the *Ccbe1*$^{-/-}$ MEFs matrix did not able support ESC differentiation. Taking into account that the cell-cell and cell-ECM interactions within the 3D structure of the EBs are important for differentiation (Bratt-Leal et al., 2009; White et al., 2013), these results are consistent with the hypothesis that Ccbe1 acts as modulator of ECM by changing ECM structural integrity. Furthermore, according to our data, absence of *Ccbe1* in MEFs favors migration and the addition of CCBE1 blocks migration. In literature, namely in a study of the migration of carcinoma cells using the transwell system coated with collagen I, the knockdown of *Ccbe1* expression increased cell migration and overexpression *Ccbe1* decreased cell migration to collagen I (Barton et al., 2010). In addition, two important factors in cell motility and in invasion, namely *uP*a and *Mmp2* (Mi et al., 2006) presented increased expression in absence of *Ccbe1* in MEFs. *uPa* either acts directly on ECM proteins or converts inert plasminogen into widely acting plasmin or activate pro

MMPs to active MMPs. Therefore, the high expression of *uPa* and *Mmp2* *Ccbe1*$^{-/-}$ MEFs suggests that cells are less adherent and more motile, which could leads to the rise of migration rates. Furthermore, *Ccbe1*$^{-/-}$ MEFs seemed to exhibited weak adhesive bonds and, morphologically displayed extensive lamellipodia, that might be also correlated with and enhanced motility of *Ccbe1*$^{-/-}$ MEFs. The wound-healing assay performed using antibodies against β-integrins showed that migration events are negatively regulated by the blocking of β-integrins, as they act as primary migration mediators (Barczyk et al., 2010). However, the blocking was not sufficiently preferential, so that experiment was not elucidative of the possible mechanism whereby Ccbe1 may modulate cell migration. Nonetheless, we decided to perform a migration assay using different matrices that may interact with CCBE1. As expected, the migration of *Ccbe1*$^{+/+}$ MEFs was favored in fibronectin, because fibronectin is the proper ECM substrate to study migration of fibroblasts (Liang et al., 2007). Furthermore, *in vivo*, fibronectin provides a crucial substrate for many forms of fibroblast migration, such as in embryonic migratory pathways and in the provisional matrix of healing wounds (Clark et al., 2003; Knox et al., 1986; Yamada, 2000). Besides acting as a substrate, fibronectin also has certain proteolytic fragments that can promote chemotactic migration (Clark et al., 1988). The differences of migration rate between *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs was matrix dependent and more evident for migration in vitronectin. Migration was favored in vitronectin, a substrate that possibly interacts with CCBE1. These results suggest that a possible complex vitronectin-CCBE1 could block migration and, in absence of CCBE1, fibroblasts are "released" and migrate easier.

The function of *Ccbe1* in migration is however still contradictory and poorly understood. On the one hand, Bos *et al* reported that Ccbe1 deficiency results in failure of specified LECs to migrate, but also observed that LEC numbers are reduced in mutants and resulting in a primary defect before the migration and further formation of lymph sacs (Bos et al. 2011). Hägerling *et al* work's also confirmed that CCBE1 deficiency results in a failure of nascent LECs to leave the veins, formation of aberrant venous sprouts and a lack of developing lymphatic vessels (Hägerling et al., 2013). On the other hand, CCBE1 has been reported to be highly down-regulated in primary breast carcinomas as compared with matched normal breast tissue (Yamamoto and Yamamoto, 2007) and modulated cell migration and survival in human ovarian cancer cells (Barton et al., 2010). In the study reported by Barton *et al*, CCBE1 overexpression in breast cancer cells

inhibited cell migration and, in opposite, loss-of-function of CCBE1 in ovarian cancer context conferred migratory advantage to cancer cells (Barton et al., 2010). In the present manuscript, we were able to study the role of *Ccbe1* in cell migration outside the context of cancer. According to our *in vitro* model using *Ccbe1*$^{+/+}$ versus *Ccbe1*$^{-/-}$ MEFs, *Ccbe1* revealed to be an important migration blocker.

Chapter VI

General Discussion

GENERAL DISCUSSION

The first years of cell research were essentially devoted to the maintenance of pieces of tissue in natural media such as plasma clots. Even though the methods and results were remarkable, animal cell culture only became a practical science with development of cell lines, cloning of cells, development of both monolayer and suspension culture methods by Eagle, and the replacement of totally natural media by the complex but defined media (Freshney, 2006). Over the last decades, embryonic stem cells have gathered a lot of attention owing to their inherent self-renewal and pluripotent capacities. Basic and clinical research carried out during the last few years on embryonic, fetal, amniotic, umbilical cord blood, and adult stem cells have constituted a revolution in regenerative medicine and cancer therapies by providing the possibility of generating multiple therapeutically useful cell types (Mimeault et al., 2007). In order to meet specific needs of stem cell-based therapies, development of bioprocessing strategies for propagation of pluripotent stem cells involves the development of medium formulations and biomaterials as substrates. Although modern tissue culture methods are widely used every day at the laboratory level, currently many stem cell culture propagation and differentiation systems incorporate animal-derived components for promoting self-renewal and differentiation (Greenlee et al., 2005; Li et al., 2009; McElroy and Pera, 2008; Zhou et al., 2013). Mouse embryonic stem cells were one of the first ESC types derived from the inner cell mass of pre-implantation blastocysts, and cultured in direct co-culture conditions on MEFsi feeder layers (Martin and Evans, 1975; Martin, 1981). For mouse ESCs, fibroblast feeder layers are often used at some phase during the culture protocol. This methodology was primarily adopted as it provides the cells with the appropriate conditions conducive to increase of plating efficiency, facilitates both the survival and the growth of stem cells, helps maintaining self-renewal rather than differentiation (Hu et al., 2012; Khademhosseini et al., 2006a). On the other hand, from a cell-based regenerative therapy perspective, the use of MEFsi for the propagation of ESCs is seen as a potential obstacle by many researchers. Even though the risk of transmitting murine viruses seems low (Amit et al., 2005), the potential immune rejection of xeno-proteins in hESCs, as demonstrated by Martin *et al* (Martin et al., 2005), shows the need for a complete xeno-free cell culture system in order to achieve the full clinical potential of ESCs. In addition, the use of feeder layers is labor

intensive and could compromise experimental data that are sometimes difficult to validate. Numerous protocols for culturing embryonic stem cells were tested, but the trend now is towards feeder-free cultures. In alternative to the use of MEFsi as feeder layer for ESC culture, several authors have explored the role of natural biopolymers, such as complex protein gels like gelatin and Matrigel™. However, gelatin is thermally denatured collagen derived from animal skin and bones and thus not xeno-free (Gorgieva and Kokol, 2011), and Matrigel is derived from the basement membrane of Enelberth-Holm-Swarm mouse sarcoma and, hence, still animal-derived (Hughes et al., 2010). Furthermore, several authors alert to the fact that when ESCs are cultured in gelatin, even when supplemented with LIF, part of the population is differentiated during the culture, indicating that the maintenance of pluripotency is compromised (Ramírez et al., 2011; Yue et al., 2012). From a biomaterials perspective, the generation of an animal- and cell-free biomimetic microenvironment that provides the appropriate physical and chemical cues for stem cell self-renewal or differentiation into specialized cell types would be ideal.

The advances of biomaterials as a scientific field dates back to approximately 50 years. Biomaterials have been investigated extensively as substrates for cell propagation, scaffolds for various organs and as delivery vehicles for drugs, growth factors and cells in many regenerative biomedical paradigms (Abraham et al., 2009). One of the potential benefits of using biomaterials for pluripotent stem cell propagation is the elimination of direct co-culture with a supportive feeder layer that has been an integral component of pluripotent stem cell culture. This removes the risk of contamination with xenogeneic pathogens and reduces variability in experimental outcomes due to feeder layer contribution. In addition, specialized biomaterials that include appropriate chemical and physical (topographic features) modifications, have contributed to successful differentiation of ESCs to multiple cell types. The use of a polymer-based substrates that can be synthesized with ‘off-the shelf’ constituents for ESC culture has several advantages, including: economic feasibility; reduction in the labor involved to maintain an additional cell line as feeders; elimination of the source of potential xenogeneic contamination; manipulation of chemical or physical and properties such as porosity, stiffness, and degradation to increase spatial complexity.

Locust Bean Gum as an alternative polymeric coating for embryonic stem cell culture

Biopolymers have received attention as tissue engineering substrates with several studies examining materials, such as alginate, chitosan, and gelatin as cell scaffolds for both 2D and 3D cell culture. Among biopolymers, LBG is a versatile polymer; it has been used in the past in numerous areas, such as food and cosmetic industry. Curiously, LBG has been used by ancient Egyptians to bind the wrapping of mummies. At present, the chemical stability and biocompatibility of natural products, such as LBG, in the body greatly accounts for their utilization. The adhesive properties of LBG make it an adequate polymer to use for example in drug delivery systems and in biopharmaceuticals applications (Dionísio and Grenha, 2012; Manjanna et al., 2013). The potential application of the LBG is vast but it had however not been fully explored. In the present thesis, we tested the potential use of the commercially available LBG polysaccharide as a novel coating to support pluripotent ESC in culture. Polymeric coatings of LBG were characterized by SEM and exhibited a rough topography, in opposition to the smooth gelatin coating. There is a general consensus that the topography of ECM can control cell spreading, which could determine whether the cell undergoes apoptosis or cell growth, maintenance of pluripotency or differentiation. In the present study, the topography of LBG coatings in combination with the chemical and biological properties of LBG, seem to be to be a great asset to support mouse ESC culture.
Although the natural polymers present high levels of biocompatibility, they should be used carefully due to the possible batch-to-batch and producer-to-producer variation. Furthermore, concerning the producer, batch or harvest time, the composition in galactomannan, protein and ash can vary, motivating several authors to perform purification of the polymer prior to use. Therefore, the study of the applicability of LBG as coating for stem cell culture was complemented with a comparative test performed using LBG from two manufacturers and using purified LBG. In general, when ESCs were cultured in LBG coatings, they were able to grow and retain their pluripotency regardless of the producer and purification step. In the present comparative study, one of the main aims was to test whether the ash and protein content affects ESC culture results or if the ESCs respond mainly to galactomannan, the major component of LBG. Taken together, the data presented here suggests that the possible protein and ash impurities did not cause

biological intolerance or benefit ESC culture system. Furthermore, ESC response among the different LBG coatings was equivalent, suggesting that maintenance of pluripotent ESCs is likely mainly due to the galactomannan.
In conclusion, the results suggest that LBG coating not only allows ESC survival in culture, but also promotes long-term ESC proliferation in the pluripotent state, while preserving their tri-lineage differentiation capacity. Therefore, LBG coating seems to be a suitable matrix to support pluripotent ESC cultures and an excelent alternative to the conventional gelatin-based ESC culture system.

Novel triblock copolymer nanofiber system as an alternative support for embryonic stem cells growth and pluripotency

Much of the knowledge of the biological mechanisms that underlie cellular functions, such as migration, differentiation and force-sensing has been garnered from studying cells cultured on glass or plastic surfaces (Freshney, 2005). However, more recently the cell biology field has come to appreciate the dissimilarity between these flat surfaces and the physical, chemical, mechanical cues of complex three-dimensional extracellular environments in which cells routinely operate *in vivo* (Baker and Chen, 2012). This has encouraged substantial efforts towards the development of *in vitro* biomimetic environments and has encouraged much cross-disciplinary work among biologists, material scientists and tissue engineers. Therapeutics is limited in part by *in vitro* cell expansion as well as materials issues that include the design of biocompatible scaffolds for further co-transplantation (Willerth et al., 2008). Recapitulating the various stem cell niches *ex vivo* is extremely challenging as it likely involves spatiotemporal regulation of biostimuli that extend to extracellular matrix architecture. Nonetheless, understanding the niche *in vitro* might help in translation to *in vivo*. The use of biomaterials properties to guide cell behavior is an attractive option for regenerative medicine, where controlling stem cell behavior is important for the establishment of a functioning cell population. A wide range of materials properties have been shown to influence many types of cells, inclusive the effects of topography on embryonic stem cells (Ji et al., 2012). It is recognized that topographical features such as ridges and grooves can dramatically influence cell phenotype (Park et al., 2007), which increase the need of developing substrates

with precisely biomimetic topography. Natural and synthetic polymers have emerged as a tool to develop and mimic stem cell niche. However, the use of synthetic polymers easily allows the control of modifications. The adhesion, growth and differentiation of stem cells are likely controlled by the surrounding microenvironment. Physical cues in the microenvironment, e.g. nanotopography, were shown to play important roles in stem cell fate decisions, mainly in maintaining pluripotency (Ji et al., 2012). Thus, controlling stem cell behavior by nanoscale topography has become an important issue in stem cell biology. Nanotechnology has emerged as a new exciting field and research from this field has greatly advanced. Nanotechnology allows the manipulation of sophisticated surfaces/scaffolds which theoretically can mimic the cellular environment and regulate cellular behaviors. As ESCs are adherent cells that respond to a wide range of substrate cues, including topography, the cell-substrate interface is therefore an important design parameter in regenerative medicine and tissue engineering applications. In an attempt to mimic the fibrous structure of *in vivo* ECM, we developed a new artificial support that is constituted of PEG-PTMSMA-PMAA-GRGDS nanofibers which main advantages are the elimination of animal-derived matrices and to make available a complex, synthetic and defined network of nanofibers in a scale similar to the native ECM. In the present work, we developed a new artificial support that can be used to grow pluripotent stem cells by converting an amphiphilic biocompatible peptide-copolymer into a nanofiber mesh, through a molecular self-assembly process. The developed nanofibers structure was shown to support mESC proliferation in an undifferentiated state after short- and long-term culture, indicating that this system is an alternative substrate and possible candidate to substitute gelatin coating in mESC culture. The nanofibers promoted self-renewal of mESCs without the requirement of a matrix coating with high levels of proteins and polymers, which not only provides a route for easier and economical stem cell culturing, but also promotes production of higher quality undifferentiated pluripotent stem cells with better control of cell proliferation and differentiation in a chemically defined matrix, especially when compared to gelatin coating.

It is common knowledge that within the stem cell niche, cell fate is controlled both spatially and temporally, as well as through cell-cell and cell-matrix interactions (Kim et al., 2012). The cell-substrate interface is therefore an important design parameter in tissue engineering applications, where substrate cues are used to

influence cell behavior. In the present work, the bioconjugation of nanofibers with GRGDS lead to a higher expression of *β1-Integrin* and *Collagen type I* suggesting that cell-matrix interactions were mediated via integrins. Furthermore, this results could indicate that PEG-PTMSMA-PMAA-GRGDS improve cell adhesion by inducing the expression of adhesion molecules that will allow the reorganization of the microenvironment and incorporation in a fiber-based matrix. Also, the interaction nanofiber-cells revealed to be beneficial for the maintenance of pluripotency in the culture system.

The culture of pluripotent stem cells on polymeric surfaces opens up avenues for the identification of synthetic microenvironments that can be easily synthesized and modified to form scaffolds to support the differentiation of ESCs into highly ordered structures. In ESC studies, biomaterials have been frequently used to direct differentiation to specific lineages in the presence of appropriate growth factors. However, maintenance of its undifferentiated state has proven to be a challenge. The use of natural and synthetic polymers in promoting ESC self-renewal is still in its nascent phase and has great potential. Much work remains to be done in the laboratory and in the clinic to understand how to use ESCs in stem cell-based therapies to treat disease. Furthermore, the use of appropriate matrices in cell culture is still an issue. The major challenge is to find a cheap, defined, user-friendly and feeder-free condition that properly mimics the ESC niche, in order to obtain high-quality undifferentiated ESCs. Many efforts have been made to closely mimic the real microenvironment of cells. It is expected that advances in biomaterial-based approaches will contribute immensely to the standardization of culture methodologies, leading to development of bioprocesses for ESC propagation and differentiation. Moving towards an animal-free matrix for culturing ESCs require the identification of polymeric substrates that support long-term proliferation and self-renewal. In the present thesis, the use of LBG coating and PEG-PTMSMA-PMAA-GRGDS nanofibers was validated as a support for proliferation, pluripotency and differentiation of mouse embryonic stem cell culture.

Characterization of Ccbe1 mutant MEFs. Ccbe1 and its effect in proliferation and migration

In vitro experiments using wild-type versus mutant MEFS is a suitable system to study the function of a gene thought to act in extracellular matrix remodeling and in migration because the function of fibroblasts is not only the maintenance of the structural integrity of connective tissues, but also the continuous secretion of the precursors of all the components of the extracellular matrix, primarily the ground substance and a variety of fibers.

The culture of primary mouse embryonic fibroblasts have been widely used by several researchers as a model to perform functional analyses (Chen et al., 2006; Kireeva et al., 1996; Kranc and Bamforth, 2003). The *Ccbe1* functional studies presented in this dissertation clearly suggest that *Ccbe1* plays a role in cell proliferation. *In vitro* analyses of survival growth curves showed a cell growth decreased in *Ccbe1*$^{-/-}$ MEFs. Furthermore, failure of proliferation in *Ccbe1*$^{-/-}$ MEFs was supported with cell cycle delay at G0/G1 and G2/M phase. Moreover, FBS starvation of *Ccbe1*$^{-/-}$ MEFs resulted in an increase of apoptotic cells suggesting that *Ccbe1* is important to protect cell viability. In addition, rescue of proliferation and viability was obtained by introduction of CCBE1 back to the culture system. The balance of cell proliferation and apoptosis is important for both development and normal tissue homeostasis. As fibroblasts have structural support and tissue remodeling functions, the low proliferation in *Ccbe1*$^{-/-}$ MEFs could maybe not be enough to maintain the integrity of tissues contributing for the development of extensive edema in mice. Furthermore, our preliminary results also show that EBs cultured on top of *Ccbe1*$^{-/-}$ MEFsi feeder layers were not able to properly differentiate namely into the cardiac lineage, as verified by the low expression of cardiac markers. This result suggests that, contrarily to LBG coating, PEG-PTMSMA-PMAA-GRGDS nanofibers and wild-type MEFs, *Ccbe1*$^{-/-}$ MEFsi have not the suitable properties to be used as support for mouse cardiac ESC differentiation. Moreover, the inability of *Ccbe1*$^{-/-}$ MEFsi to support differentiation is consistent with the requirement of *Ccbe1* for proper differentiation of ESCs towards cardiac lineages.

In cancer, it is known that senescent fibroblasts promote tumor growth and metastasis (Capparelli and Guido, 2012). Considering the fact that CCBE1 is downregulated in several cancer clinical cases and that *Ccbe1* mutant MEFs tends

to present the morphology of senescent cells, it is possible that the absence of *Ccbe1* in fibroblasts-like cells in tumors promote their senescence and consequently the growth and metastasis of tumors.

Bioinformatic analyses revealed that CCBE1 contains putative collagen and calcium binding EGF-like domains, and might have a function in extracellular matrix remodeling and migration (Barton et al., 2010). Indeed, *Ccbe1* mutant MEFs presented increased motility and seemed to exhibited weak adhesive bonds. In absence of *Ccbe1*, fibroblasts migrated faster and the expression of *uPA* and *Mmp2* was increased. Migration studies using human cell lines, also revealed an enhancement of cell migration upon downregulation of CCBE1 (Barton et al., 2010). Fibroblast migration was further increased in the presence of vitronectin and blocked in the presence of β-integrin blocking antibodies and even more evidently in the presence CCBE1. The collagen-like domain present in CCBE1 is a possible mediator of migration as collagen is an important regulator of ECM assembly and maintenance. Collagen has been shown to transduce signals via integrins or VEGFR3 to control cell spreading and migration (Alitalo, 2011). On the other hand, bioinformatic analyses performed in our laboratory has shown that CCBE1 has a conserved RGD domain which could allow further interactions with vitronectin or via RGD-recognizing integrins.

In the present work, we demonstrated that CCBE1 is involved in the following activities: (i) promotion of cell attachment and spreading; (ii) maintenance of cell viability; (iii) increase of proliferation rate and (iv) delay of cell migration. Taken together, these data indicate that *Ccbe1* is likely to function as an extracellular matrix signaling molecule and may regulate processes of cell proliferation, migration and adhesion during embryo development.

Future perspectives

Embryonic stem cells have a potential of application in drug discovery, developmental biology and disease studies due to their unique characteristics. Nevertheless, there are some concerns about conventional methods to maintain self-renewal of ESCs, since these methods include the use of feeder cells, as well as serum. Good manufacturing practice (GMP) quality, defined by both the European Medicines Agency and the Food and Drug Administration, is a

requirement for clinical-grade cells, offering optimal defined quality and safety in cell transplantation (Unger et al., 2008). Immune reactions against animal proteins in the cells and infection risk caused by animal microbes can be avoided using animal substance-free culture media, feeder cells or feeder-free matrix in derivation, passaging, expansion and cryopreservation procedures. In the present work, we replaced the animal-derived substrate by natural polymer from plant origin and by synthetic polymers, however ESCs were cultured in medium supplemented with FBS ESC screened. To support fetal growth and development, FBS contains mixed combinations of cell replication stimulators and cell differentiation inducers. Owing to the facts that serum is a biological complex mixture containing unknown compounds and that serum batches vary in their capability of maintaining ESCs at an undifferentiated stage, the replacement of serum with defined components would be optimal for GMP production of ESCs (Unger et al., 2008). Therefore, to overcome serum-related problems, several groups have optimized serum-free culture conditions for ESC lines using chemically defined KnockOut™ Serum Replacement (KSR), a defined, serum-free formulation optimized to grow and maintain undifferentiated ESCs in culture (Cheng et al., 2004), to replace FBS. In future work, as the next step towards optimization and standardization of ESC culture protocols and to obtain entirely xeno-free maintenance of ESCs, it would be interesting to combine the use of LBG or nanofibers in xeno-free defined culture medium, such as DMEM supplemented with serum alternatives (KSR) or in a chemically defined ESC medium containing differentiation inhibitors (2i medium). With this experiment we expect to elucidate if our system is also efficient in the maintenance of pluripotency even in culture conditions with reduced or zero animal-derived products.

The use of LBG and nanofibers coatings for mouse ESC culture was validated in the present thesis, however, it would be interesting to validate the use of these supports for culture and differentiation of human ESC and iPS. In the possibility that these coatings are able to support not only mESC, but also hESC and iPS, LBG and nanofiber based-coatings can be used, for instance, to culture hESC for further drug testing and disease studies. Furthermore, the coatings used in this work, LBG and nanofibers, were characterized by SEM and TEM. Nevertheless, it may be also important to study the stability of the nanofibers and LBG solutions along the time

and at different temperatures (i.e. room temperature, 37 and 4ºC) by SEM or TEM to verify if their morphological structure is preserved.

Surface charge, apart from other factors, is a crucial parameter for cell adhesion (Lee et al., 1994; Vancha et al., 2004). According to published data, several cell lines and primary cultures benefit from the use of positively charged extracellular matrix proteins or polymers that enhance their ability to attach to culture plates (Berchtold et al., 2005; B. Liu et al., 2008). LBG is a non-ionic or neutral polysaccharide. One of the future tasks could be to test cell growth and pluripotency of ESCs cultured in aminated LBG (positively charged), sulfated LBG (negatively charged) or in blending of both LBG derivatives.

Ccbe1 seems to be involved in both proliferation and apoptosis process. The proliferation experiments and the studies concerning the role of *Ccbe1* in preventing cell apoptosis will be complemented to include more biologial replicates in the experiments of cell proliferation dye assay (eFluor®670) and expression of genes related to apoptosis process, such as Caspase3.

Since the analysis of the Affymetrix array data generated in Ieda *et al* revealed that *Ccbe1* is highly expressed in mouse cardiac fibroblasts (embryonic and adult) (Ieda et al., 2009) and that co-culture of differentiating ESCs with $Ccbe1^{-/-}$ MEFsi feeder layers lead to inhibition of cardiac differentiation, we would test the effect of $Ccbe1^{-/-}$ and $Ccbe1^{+/+}$ embryonic cardiac fibroblasts in proliferation and gene expression profile of cardiomyocytes. In addition, we would test endoderm and ectoderm differentiation markers to verify whether the defect in differentiation is related only with the mesoderm and cardiac differentiation or related to a general defect in differentiation.

Ccbe1 is a secreted protein with two putative distinct functional domains: an EGF-like domain, and a collagen-like domain. EGF-containing molecules signal via EGF-receptors but also through other receptors, such as integrins. EGF signaling is involved in the morphogenesis and homeostasis of several tissues, controlling cell migration, proliferation, survival or differentiation. Collagens are important regulators of ECM assembly and maintenance, and have been shown to signal via integrins and more recently through VEGFR3 to control cell spreading and migration (Alitalo, 2011). Furthermore, *Ccbe1* has been shown to interact with vitronectin, collagen type I and V (Bos et al., 2011). In addition, our results showed

that *Ccbe1* may modulate cell migration. Therefore, concerning the possibility that *Ccbe1* may have a role as a modulator migration through integrin signaling or the regulation of ECM-integrin signaling, *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs could be tested in a Mouse Extracellular Matrix & Adhesion Molecules RT profiler PCR Array (Quiagen) to assess the expression of cell adhesion molecules such as transmembrane molecules, cell-cell and cell-matrix adhesion molecules, and ECM proteins like basement membrane constituents, collagens and ECM structural constituents, ECM proteases and ECM protease inhibitors. The use of qPCR would allow an easily and reliably analysis of the expression of a focused panel of genes related to cell adhesion and the effect of *Ccbe1* in their expression, thus giving clues about possible pathways or mechanisms of action and about potential partners of CCBE1. In addition, in an effort to find the mechanism of action of Ccbe1, a RT profiler PCR Array (Quiagen) could be performed using *Ccbe1*$^{+/+}$ and versus *Ccbe1*$^{-/-}$ MEFs to assess the expression of target genes that make part of extracellular signal-regulated kinase (ERK) signaling, phosphatidylinositde-3-Kinase (PI3K) signaling, signaling through focal adhesion kinase (FAK) or expression of target genes of integrin pathway.

To characterize which is the region of *Ccbe1* responsible for the binding to ECM proteins, ECM produced by *Ccbe1*$^{+/+}$ MEFs would be incubated separately with each CCBE1 protein: the full-length and putative N-terminal (EFG-like domain containing region) and C-terminal (collagen domain containing region) fragments and immunofluorescence analysis would be performed using antibodies to detect *Ccbe1* and ECM components. This experiment would be elucidative about the localization of *Ccbe1* in the ECM, and would allow identification of which ECM components interact with each portion of CCBE1 protein.

The wound-healing assay used to explore the interaction between β-integrins and CCBE1 may be re-designed to increase the resolution of the experiment. To do so, both *Ccbe1*$^{+/+}$ and *Ccbe1*$^{-/-}$ MEFs can be incubated with antibodies against β1-integrin (collagen receptor), β3-integrin (vitronectin receptor) and β4-integrin (laminin receptor). The results obtained for *Ccbe1*$^{+/+}$ MEFs incubated with anti-β-integrin antibodies would be used to establish a baseline of the ability of each antibody to block the corresponding receptor. The possible β-integrin-dependent pathway by which CCBE1 interacts with ECM in migration events should be identified by considering the antibody that gives rise to a higher block of migration of *Ccbe1*$^{-/-}$ MEFs after normalization to the blockade observed in the *Ccbe1*$^{+/+}$

MEFs by the same antibody. The β3-integrin, in particular αvβ3 integrin, is a potential candidate to interact with CCBE1 since CCBE1 has an RGD domain and αvβ3-integrin usually the receptor of proteins with exposed RGD peptide, including vitronectin, fibronectin, and collagen (Srichai and Zent, 2010). On the other hand, the most evident differences in migration between *Ccbe1*$^{-/-}$ and *Ccbe1*$^{+/+}$ MEFs were obtained in vitronectin coating and it is known that vitronectin, a substrate for αvβ3 integrin receptor, promotes cellular spreading and migration following αvβ3 integrin ligation to the RGD sequence found in the connecting sequence. Also, a shift in the expression of *uPa*, which is involved in proteolytic activity on the cell membrane, or in the expression of αvβ3 can result in aggressive migration, as seen in cancer invasion, which is consistent with the increased expression of *uPa* in *Ccbe1*$^{-/-}$ MEFs. In fact, it is known that the effects of vitronectin and *uPa* on cell migration are additive, both induce cytoskeleton and microtubule reorganization and focal contact redistribution (Degryse et al., 2001). Furthermore, vitronectin can connect the uPa and the integrin systems as it can bind to both *uPa* receptor and the integrins (Degryse et al., 2001). Therefore, it may be interesting to analyze the role of Ccbe1 in the regulation of Integrin signaling.

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