A la meva família i

a la Júlia

“Qui lluita pot perdre,

qui no lluita ja ha perdut”

Bertolt Brecht

AGRAÏMENTS

En primer lloc voldria agrair als meus dos directors de tesi, els Dr. Carlos Semino i Dr. Salvador Borrós, el seu suport i les seves indicacions que m'han ajudat a dur a terme el treball aquí presentat. Gracias Carlos por haberme ayudado a crecer y fortalecerme como persona i gràcies Chicho per donar-me recolzament fins i tot quan no el mereixia.

També vull agrair l'ajuda i col·laboració impagables que he rebut de la Irene, la Mar, la Teresa, la Marta (ànims) i la Nerea. Sense la vostra implicació no hauria pogut fer bona part de la feina descrita en aquesta tesi.

Resto agraït a en Jerónimo Blanco i la Núria Rubio que tant m'han ajudat a millorar les meves relacions socials amb els ratolins. Gràcies per donar-me l'oportuniatat de treballar amb els simpàtics *nudes*. També agreeixo els consells que ens va donar el servei d'histologia del ICCC per tenyir i tallar les mostres de ratolins.

Voldria també agrair la col·laboració que vaig tenir al MIT amb els membres del CBE. Vull destacar la col·laboració del departament d'histologia del Whitehead Institute i a la Nikki Watson, que ens va ajudar a fer seccions de les nostres masses cel·lulars, així com la d'en David Shaywitz i el departament de biología cel·lular i molecular Harvard University, que ens va proporcionar primers de Brachyury.

Tothom és fruit del seu entorn, així que també són autors d'aquest treball tots els meus companys de laboratori, començant pel meu incansable company de batalletes i d'aventures, en Miki, que tot i lluir ja una certa *kipá* natural, té un esperit més jove que la majoria. Gràcies per tot i no canvïis mai. També ha estat clau en David, que tot i no poder resistir la temptació de fer safareig sempre que pot, ha estat un suport molt important i company d'habitació, amb el permís de l'Ana… Després, la Silvia, en Jordan i en Jordi, el trio calavera, han aportat molts moments dolços a la feina (brownies inclosos) i també el seu toc metal. Finalment també vull agrair a tota la gent amb qui he coincidit a materials: en Jordi, les dues Laures, la Laia, les dues Elenes, en SuperJose, les dues Núries, L'Anna, la Maria, en Toni i tots els altres TFCs i, per acabar, les happy Bertas, no perdeu el vostre somriure.

També voldria recordar els moments que he compartit amb la Dra. Carme Brosa durant el meu TFC, amb el Dr. Jordi Díaz com a auxiliar i amb en Carles Colominas tant en el GEMAT com també en l'assignatura d'algebra, que prous maldecaps ens ha portat.

Resto agraït als laboratoris de bioquímica i de cromatografia de l'IQS per la col·laboració que m'han brindat en matèria de biologia mol·lecular i cromatografia. La seva disponibilitat ha estat sempre modèl·lica, començant pels seu caps, els Dr. Planas i Dr. Broto, i seguint amb tots els altres integrants; la Cristina (gràcies per la paciència que has tingut amb mi), la

Magda, la Núria, la Neus, en Joan i la seva teoria de l'escalfament global i tota la resta de membres dels dos laboratoris.

Voldria destacar l'ajuda de Kelly Blackburn, Alice Chen, Roger Kamm, Alan Grodzinsky, Hayman Hartman, Hans Jörg Meisel, Jaime Rivera, Nicole Zur Nieden i Angelo Cardoso amb les seves opinions i contribucions al text d'aquesta tesi.

Les persones que més m'han ajudat a acabar aquest treball són la meva familia i la familia Lleal Rovira. El vostre suport ha estat molt important durant aquests quatre anys; m'heu sabut acompanyar en el difícil camí que ha estat la meva tesi.

Per acabar, gràcies a la Júlia senzillament per tot.

SUMMARY

Regenerative medicine is a discipline that has gained recognition in the last decades because many diseases are not treatable with traditional drugs. Many research groups and companies invest time and money in the production of new paradigms to cure conditions such as Parkinson's, arthrosis or spinal cord injuries. These approaches are based in the use of biomimetic tissues to replace damaged organs. In this work we present a new experimental model to study the formation of bone and cartilage and eventually to repair these tissues.

We have used Mouse Embryonic Fibroblasts (MEFs) combined with different biomimetic materials to study bone and cartilage formation *in vitro* and *in vivo*. MEFs have been cultured *in vitro* and *in vivo* in RAD16-I, a synthetic self-assembling peptide with structure similar to generic extracellular matrix milieu, to study the evolution of these cells in both conditions. Also, hydroxyapatite microparticles have been surface coated to produce biologically active bone-like inorganic charges for use in cartilage or bone substitutes. In order to improve the particles' coatings, we have developed a platform that allows us to perform combinatorial testing of growth factors and other biologically active compounds.

In vitro cultures of MEFs has shown that when primary mouse embryonic fibroblasts are cultured in a soft nanofiber scaffold, they establish a cellular network that causes an organized cell contraction, proliferation, and migration that ends in the formation of a symmetrically bilateral structure with a distinct central axis. A subset of mesodermal genes (brachyury, Sox9, Sox5, Sox6, Runx2) is upregulated during this morphogenetic process. The expression of brachyury was localized first at the central axis, extending then to both sides of the structure. Finally, the spontaneous formation of cartilage-like tissue mainly at the paraxial zone followed the expression of Sox9 and Runx2.

In vivo study of MEFs was facilitated by a non-invasive bioluminescence imaging (BLI) technique to detect luciferase-expressing cells, developed by Dr. Blanco's research group. These experiments showed that RAD16-I is a very permissive scaffold for cell survival and proliferation *in vivo*. Furthermore, it seems that the poor mechanical properties of RAD16-I are no disadvantage in terms of cell growth *in vivo*.

Finally, we have developed different types of coated and uncoated hydroxyapatite (HA) microparticles by plasma polymerization. The coatings permit to tune the properties of HA and produce particles that suit the needs of different medical applications in bone and cartilage repair. Moreover, we have developed a method to produce platforms based on 96-well plates that allow the combinatorial testing of biologically active compounds for various applications in regenerative medicine.

In conclusion, we have supplied new insights and tools that will enhance the finding of new regenerative tissues based on fibroblasts and biomimetic materials.

ABBREVIATIONS

AEC	Apical Epidermal Cap
Ala	Alanine
ALP	Acaline Phosphatase
AMMO	(3-aminopropyl)-trimethoxysilane
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BLI	Bioluminiscence Imaging
Brachy	Brachyury
BrdU	Bromodeoxyuridine
Coll	Collagen
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular Matrix
EGFP	Enhanced Green Fluorescent Protein
FACS	Fluorescent-Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FM	Fibroblast Culture Medium
GAG	Glycosaminoglycan
Gly	Glycine
HA	Hydroxyapatite
HPLC	High Performance Liquid Cromatography
HRP	Horse Radish Peroxidase
IGF	Insuline-like Growth Factor
IM	IntraMuscular
L-Gln	L-Glutamine
LP	Link Protein
MEF	Mouse Embryonic Fibroblast
MMP	Matrix Metalloproteinase
OPN	Osteopontin
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PECVD	Plasma Enhanced Chemical Vapor Deposition
PFA	Paraformaldehyde
PFM	PentaFluorophenylmethacrylate
PHC	Photon Counts
PLuc	Photinus pyralis luciferase
PVDF	Polyvinyldifluoride
qPCR	Quantitative Polymerase Chain Reaction
RF	Radio Frequency
RLU	Relative Light Unit
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SBF	Simulated Body Fluid
SC	SubCutaneous
SEM	Scanning Electron Microscope
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline and Tween 20
TGF	Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor
WB	Western Blot
XRD	X-Ray Diffraction

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1. Introduction

Humans are the only known living creatures that value the individual above all other things and medicine is the tool we have developed to fight against natural selection of the weakest thus providing every human being with help in case of disease. Our concern for every single human being over most things explains that medicine and medical care are major concerns in all societies throughout human history. Medicine began as a traditional knowledge that recognized useful properties of herbs, fruits and other natural products. It was not until the arrival of the organic chemistry in 1828, with Wöhler's synthesis of urea [1], that humans began developing tools to actively seek new means of tricking natural selection. The pharmaceutical industry was born with Aspirin in the late 19th century [2] and, henceforth, great efforts have been spent on finding new ways to overcome diseases. The twentieth century has given humanity a huge legacy of organic compounds that cure or withdraw symptoms of many diseases, but there are still many conditions for which simple compounds have had no effect.

The need for new ways to treat conditions such as Parkinson's, diabetes or cancer and the rapid development of biotechnology and bioengineering have led to the discovery of a new generation of biotechnology-based medicines in the last thirty years. Recombinant human insulin, first commercialized in 1982 by Eli Lilly and Genentech thanks to the work of Boyer and Cohen [3], is an example of such vital biotechnology-based medicines that have appeared since 1980, when the patenting of genetically modified microorganisms was allowed in the USA.

Since the 1980s, one of the fields of extensive research in biotechnology is the development antibodies for medical use. Therapeutic antibodies represent one of the largest classes of biological drugs today. To date, 22 monoclonal antibodies have been approved by the US FDA for a variety of indications, such as cancer, autoimmune diseases, infectious diseases, allergic asthma, and transplant rejection. Antibody-based drugs recorded US $26.3 billion in sales in 2007, and sales are projected to grow to US $49 billion by 2013 [4].

Nevertheless, all the advances achieved in biotechnology still leave black holes in medicine, conditions that are not convincingly curable yet. Good examples are the organ failures that require transplants. Donors are usually not enough to cover all the worldwide demand for healthy organs and, unfortunately, this feeds organizations dedicated to killing healthy young people to obtain their organs. Moreover, conditions such as Alzheimer's disease – a degenerative disease that affects the neural tissue in the brain – present a serious difficulty, which is that even if possible to stop the degenerative progress, full recovery will only happen when the lost neural tissue is replaced with new healthy one. Alzheimer's is predicted to affect 4 times the number of people it affected in 2006 [5]. This estimation forecasts that 1 of 85 people will suffer from Alzheimer's disease in 2050. Although this prediction might be inaccurate and organ transplants will continue to heal many patients, it is clear that the

development of regenerative treatments for these kinds of diseases is becoming crucial in medicine and many efforts are being focused in this direction.

There are other degenerating conditions with a high impact in society such as skeletal degeneration disorders. Bone and cartilage suffer a lot of mechanical stress so these are tissues that very frequently degenerate with age and lead to many types of prominent diseases within the elderly. Osteoarthritis is the most prominent condition affecting the cartilage and the subchondral bone. 27 million US citizens suffer of this disease, a number that has more than doubled in the last decade [6]. Osteoarthritis is a pain-intensive disease whose origin is the degeneration of articular cartilage and/or subchondral bone by a variety of potential forces –hereditary, developmental, metabolic, and mechanical–. Cartilage looses tissue mass and fails to accomplish its main function, load transfer and lubrication between bones. This dysfunction causes excessive friction between bones that result in chronic pain and motility problems [6].

In addition, the life expectancy found in many countries has grown significantly and degenerative diseases usually appear at late age, which explains why the problems derived from the natural degeneration of tissues have increased in the last decades and in the future. This presents degenerative diseases as a big market with the potential to grow even more. This juncture has encouraged big pharmaceutical companies to develop many strategies to ease, not yet cure, the symptoms of degenerative diseases. As said above, the reason cure is elusive is the fact that, even if possible to stop the degeneration of the tissue, recovery implies the reconstitution of the tissue.

So there is a growing need for new ways to regenerate damaged organs and this is exactly the main goal of regenerative medicine, which refers to the research into treatments that restore adult body parts. Thus, from all the areas within biotechnology, one of the today's most challenging is regenerative medicine. There are three strategies for future treatments in regenerative medicine: the injection of stem cells or progenitor cells; the induction of regeneration by introduced substances; and the transplantation of in vitro grown organs and tissues [7]. To fulfill its goal, regenerative medicine is very active in finding appropriate cells, materials and combinations of both to produce organ substitutes, which is also the core aim of tissue engineering [8]. In fact, regenerative medicine and tissue engineering are often used as interchangeable terms, which in my opinion is not strictly accurate.

Regenerative medicine has designed different bioinspired strategies to help the reconstitution of tissues that can be divided in 6 categories:

I) Use of biological materials (scaffolds of biological origin, biological compounds such as growth factors, cytokines and cells).

II) Use of biomimetic materials (synthetic scaffolds, substitutes of biological compounds such as synthetic small molecules, drugs, vitamins, etc.). Biomimetism refers to selecting ideas and inventive principles from nature and apply them to engineering products [9]. Nature is the best school for bioengineers and copying the best is one of the most efficient ways to innovate, Microsoft is a good example of that.

III) Use of cells of human or, less frequently, animal origin.

IV) Use of combinations of the previous three (i.e. cells and biological or biomimetic materials).

V) Use of engineered functional tissues produced artificially *in vitro* or *in vivo* (i.e. the development of artificial organs *in vitro* or the development of humanized organs in animals).

VI) Use of regenerative systems, tissues that copy naturally occurring regenerative models (i.e. blastema, blastema-like tissues, developmental tissues).

All these approaches are ultimately inspired in the nature. Millions of years of evolution have given rise to a humongous variety of solutions to different problems and it is the wish of regenerative medicine to take advantage of the knowledge stored in the nature to find solutions for the degenerative diseases.

Biological and biomimetic materials have been long used ranging from the previously mentioned hormones (insulin, growth hormone…) and therapeutic antibodies to biological scaffolds (hydroxyapatite, fibrin, collagen, hyaluronic acid…), biomimetic scaffolds, biocompatible implants, peptide motifs and more recently growth factors, siRNA, RNAi, micro RNA and even viral vectors or engineered substitutes. The mechanisms by which these compounds work depend on the targeted tissue and are subject to the particular molecular mechanisms involved in its degeneration or recovery. One example of a biomimetic material, developed in the past decade, is the self-assembling peptide RAD16-I, first described by Zhang et al in 1995 [10]. This peptide is the 16 amino acid chain AcN-$(RADA)_4$-$CONH_2$ that self-assembles in strong ionic or acidic environments to generate nanofibers that build a 3D scaffold with pore sizes ranging from 50 to 100 nm [10]. This soft scaffold has proved to be permissive for the growth of different cell types and non-toxic to rats [11-13].

Cell-based therapies have been used since the 1930s for some particular applications, for instance thyroid cells, blood transplants… But many of these treatments were not approved as drugs. Before the late 1990s only bone marrow transplants and organ transplantation were approved as medical treatments. One of the first developed cell-therapies available is the Autologous chondrocyte transplantation, an FDA approved (1997, Carticel™, Genzyme) cell therapy for articular cartilage repair [14]. This therapy consists on the injection of cultured chondrocytes in the damaged joints of the patients to increase the population of chondrocytes

within the cartilage. Other cell-based therapies are currently in clinical or preclinical trials including many types of adult stem cells –among others Bone Marrow Stem and Progenitor Cells [15-17], Adipose Derived Stem Cells [18-20], Neurospheres [21, 22] and recently Human Embryonic Stem Cells (Geron Inc.) [23]– targeting different diseases such as Spinal Cord Injury repair, Parkinson's, stroke, Perianal Fistula, Osteonecrosis, Myocardial infarction or muscular dystrophy. There's a lot of confidence that in the near future many brand new and revolutionary therapeutic treatments will come out from these trials. Nevertheless, cells implanted as suspensions are limited to applications where the importance of the tissue is not as much as the importance of the cells. When the tissue is key for regeneration, cells need to be seeded in materials that give the implant the proper biomechanical and biological properties.

Thus, the following step in regenerative medicine is the use of engineered mixtures of cells and biomimetic or biologic materials. To date, skin substitutes are the only FDA approved treatments using this tissue engineering approach. Integra (Integra Life Sciences) and Biobrane (Bertek Pharmaceuticals) were the first to be approved in 1996, followed by Transcyte® (Advanced Tissue Sciences) in 1997, Apligraf® (Organogenesis) in 1998, Dermagraft (Advanced Tissue Sciences) and Orcel (Ortec) in 2001 and Epicel® (Genzyme) in 2007. These skin substitutes have been developed to treat disorders such as diabetic or venous skin ulcers, burns and other skin disorders and consist of cells (fibroblasts and/or keratinocytes) embedded in a scaffold. But tissue engineering is a very young field in regenerative medicine and needs some time before new products for other conditions not involving the skin reach the bedside.

A more complex way of integrating all the different tools of regenerative medicine is the production *in vitro* of systems capable of regenerating; tissues that resemble developmental stages of human embryos. Adult humans have some inherent regenerative capacity in the fingertips, which fully regenerate after tip amputation, or in the liver, which can rebuild up to 2/3 of its normal size, but all other organs have lost this capacity. A future challenge in regenerative medicine is to produce types of tissues that behave similarly to developmental organs or pluripotential cell masses such as the blastema in regenerating limbs of amphibians –the blastema is a pluripotent cell mass that forms in the amputated limb and regenerates the whole limb again [24]–. This new approach would not only replace the loss of cells in damaged organs, but it would also retake the developmental program that cells follow during embryonic development of tissues. This is science fiction today, but it may come to pass in the future.

In this thesis we propose new insights in three different aspects of regenerative medicine with the common objective of developing new tools for the regeneration of bone and cartilaginous tissues, and more specifically, the regeneration of the interface between bone and articular cartilage. Natural unions of cartilage and bone are cartilaginous tissues with different

structures where the nearer the tissue is from bone, the more calcifications in the medium and the fewer amount of chondrocytes is present (**Figure 1**). Therefore, the composition of the extracellular matrix (ECM) is a gradual transition from cartilage ECM to bone ECM, with regions rich in collagen type II (near the core articular cartilage), in collagen X (in the transition part from cartilage to bone) and hydroxyapatite (at the outermost part of this bone-to-cartilage joint), which is a type of calcium phosphate present in the bone structure.

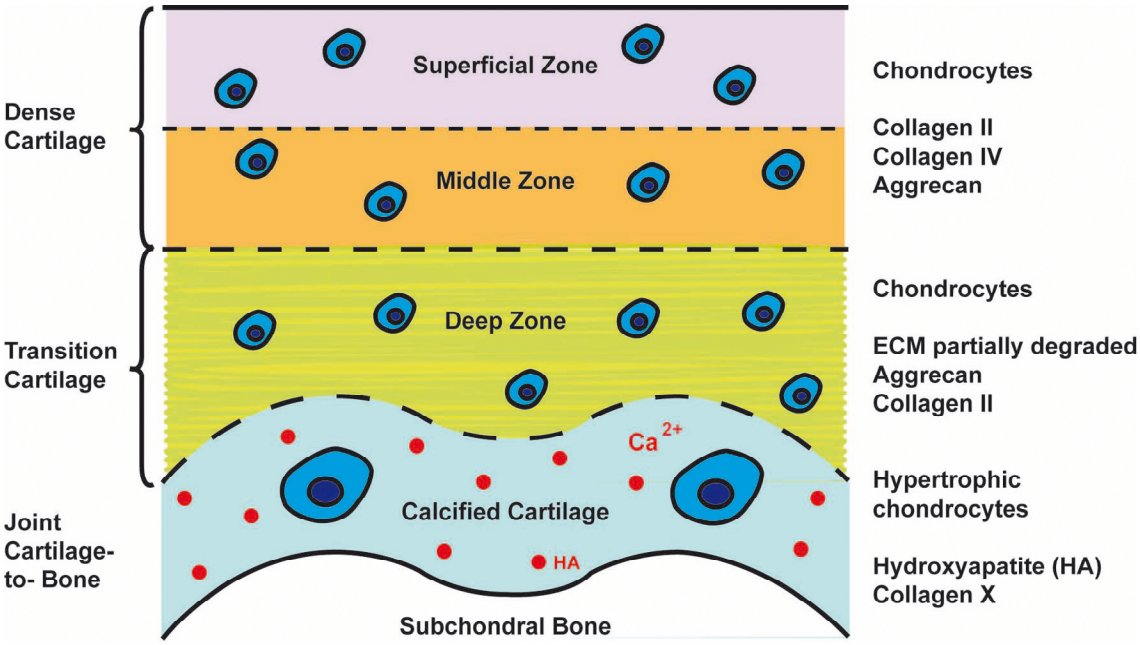

Figure 1: **Schematic of the articular cartilage's structure.**
From bottom to top, (1) Calcified cartilage is the first layer of cartilage lying just next to the subchondral bone. This layer is characterized by the presence of hypertrophic chondrocytes and high concentration of Ca^{2+} in the medium as well as hydroxyapatite precipitations and Collagen X in the ECM. (2) The second layer of the articular cartilage is the deep zone that contains mature chondrocytes and a ECM that combines intact and partially degraded Aggrecan and Collagen II. (3) The outermost parts of the cartilage are the middle and superficial zones, the densest part of the cartilage, which contains chondrocytes and high concentrations of Aggrecan and Collagen II. The ECM is strictly oriented horizontally in the superficial zone to decrease friction and increase loading capacity.

First we have developed a model based on the self-assembling peptide RAD16-I mixed with embryonic fibroblasts that could be a first step towards the creation of a regenerative system. This approach is inspired in the structure of the blastema, made of fibroblasts and dedifferentiated myoblasts in a very soft extracellular matrix. We have simplified the system by only using fibroblasts cultured in a 3 dimensional soft peptide scaffold made of self-assembled RAD16-I and in these conditions we have tried to recreate mesenchymal condensation *in vitro*. We have developed an assay with which embryonic fibroblasts, encapsulated in RAD16-I, proliferate and condense to form a dense cell mass with bilateral symmetry [25]. The volume reduction can be as high as 50 % but, more interestingly,

bilaterality is accompanied by chondrogenesis of the fibroblasts. This phenomenon has many similarities compared to mesenchymal condensation as well as limb bud regeneration and we expect that the model we present in this work might open a new path in the production of regenerating tissues for medicine.

Then, we have tested this mixture of fibroblasts + RAD16-I alone or combined with a structural elastomer in nude mice to evaluate cell survival and performance *in vivo* using bioluminescence as measurable response. This second part of the work has been possible thanks to the collaboration with Dr. Blanco's research group in the ICCC (Hospital de Sant Pau). In this study we have compared the cell response when loaded in suspension in a very soft 3D environment (RAD16-I) or in a soft 3D environment (RAD16-I) combined with a microporous elastomeric scaffold, which was used to reinforce the macroscopic loading capacity of RAD16-I hydrogels, as will be more extensively discussed below.

Finally, we have also developed modified materials to be used in the regeneration of bone-to-cartilage interfaces. As said, this tissue has a gradual composition that, among other things, has a gradual concentration of both Ca^{2+} and hydroxyapatite. We have followed previous work in the group [26-29] and evaluated the coating of hydroxyapatite (HA) as a way to improve its performance in this very particular environment. With the aim of improving the coatings of our particles, we have also developed a platform –a surface modified 96-well plate– for the combinatorial screening of biological and biochemical active compounds for use as coatings or in regenerative medicine and other biomedical applications.

2. Objectives

The main goal of this thesis is to develop a new model of tissue regeneration based on the use of the RAD16-I self-assembling peptide combined with fibroblasts. *In vitro* and *in vivo* studies will be addressed to evaluate the possibilities that fibroblasts in RAD16-I offer for the regeneration of bone, cartilage, the bone-to-cartilage joint and other tissues of mesenchymal origin.

In vitro studies will test the ability of Mouse Embryonic Fibroblasts to turn into different cell types when cultured in RAD16-I. The morphological changes of the construct associated to cell migration, cell-cell interaction and contraction as well as cell differentiation will be monitored and compared to the differentiation phenomena in development or limb regeneration.

In vivo experiments will enable to test the safety of this approach for tissue engineering as well as the performance of the combination of cells with RAD16-I alone or reinforced in terms of cell survival and migration.

The third aim of this thesis is to produce new materials for biomedical or research purposes. More specifically, we will develop new components for the use in cartilage joints and also platforms that permit to perform combinatorial biochemical or biological assays.